ISSN Print: 2518-4261
ISSN Online: 2518-427X
Vol. 60(3), September 2023

PROCEEDINGS OF THE PAKISTAN ACADEMY OF SCIENCES: B. Life and Environmental Sciences

PAKISTAN ACADEMY OF SCIENCES
ISLAMABAD, PAKISTAN

Proceedings of the Pakistan Academy of Sciences: Part B Life and Environmental Sciences

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Annual Subscription: **Pakistan:** Institutions, Rupees 4000/- ; Individuals, Rupees 2000/- (Delivery Charges: Rupees 150/-)
Other Countries: US$ 200.00 (includes air-lifted overseas delivery)

HEC Recognized; Scopus Indexed

Published by **Pakistan Academy of Sciences**, 3 Constitution Avenue, G-5/2, Islamabad, Pakistan
Email: editor@paspk.org; **Tel:** 92-51-9207140; 92-51-920 6770; **Websites:** www.paspk.org/proceedings/; www.ppaspk.org

Printed at **Graphics Point.,** Office 3-A, Wasal Plaza, Fazal-e-Haq Road, Blue Area, Islamabad
Ph: 051-2806257; E-mail: graphicspoint16@gmail.com

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PAKISTAN ACADEMY OF SCIENCES: PART B
Life and Environmental Sciences

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Proceedings of the Pakistan Academy of Sciences: B
Life and Environmental Sciences 60(3): 319-325 (2023)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(60-3)701

Pakistan Academy of Sciences

Review Article

Vertical Farming of Horticulture Crops: A Recent Trend

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Abstract: Vertical farming now a day is gaining status as complementary to traditional farming practices, allowing for more sustainable food production for the world's growing population. While early studies on vertical farming systems focused primarily on the advancement of technology through innovative designs, hydroponic cultivation automation monitoring, and the use of advanced LED lighting systems, more recent studies have focused on the resilience and circularity of vertical farming. Over the last few decades, there have been elevated concerns about ethical cultivation practices and environmental issues, reliance on non-renewable resources, commitment to biodiversity conservation, ration scarcity, and its leverage to hunger. Environmental issues, such as economic justice issues, biodiversity commitments, and a focus on food security issues, have gained traction in social work. Vertical farming is one of the solutions to many problems including food and nutritional security, environment safety, resource utilization, land fragmentation, climate resilience agriculture, etc.

Keywords: Vertical farming, Horticulture, Fruits and vegetables.

1. INTRODUCTION

Vertical farming is a way of farming utilizing available vertical surfaces instead of traditional farming practices. Cultivation is taken up in vertically stacked layers allowing the farmers to utilize the space and produce more food on the same area of land. Often these layers are integrated into buildings such as skyscrapers, housed in warehouses or shipping containers, greenhouses or otherwise placed in spaces that would otherwise be unfit for farming. Gilbert Ellis Bailey coined the term vertical farming, but he gave it a completely different explanation or suggested that farmers use explosives to reach the depths of root growth. Vertical farming has a variety of definitions based on its size, layout, type of building, density, level of control, and location [1].

Today, the framework of vertical farming has utterly changed, and it is at present limited to the aim of utilizing every available inch of land or free space, whether in the city or a village, in sort to grow as much food as possible for the world's hungry population. It has now become a popular farming method all over the world and is gaining attraction in India as well. Many entrepreneurs are interested in vertical farming because of the high net returns. Buildings, warehouses, rooftops, and balconies can all be used for vertical farming [2]. The world's population is estimated to grow by another 2 billion people by 2050, posing a greater challenge than with limited land resources feeding more people. The use of a vertical farming system is the answer to this problem [3-6] as shown in Figure 1. Today we are facing a shortage in food supplies coupled with rising market prices which has impacted the poor and developing countries and specifically the urban households [7].

Vertical farming is regarded as one of the most cutting-edge agricultural technologies for reducing the amount of land used and is also known as 'building upwards' [8]. Vertical gardening is gardening in which plants are grown on vertically inclined or vertically stacked layers [9]. This system helps to grow more crops within the same growing area in the field [10]. Leafy greens (57 %) are the most commonly planted crop in the United States and Canada, followed by tomatoes, flowers, and

Received: July 2022; Revised: March 2023; Accepted: June 2023
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microgreens (Table 1) [11]. The area of cultivation can be increased by 3 to 4 times, by the use of a vertical farming system, and high quantities of nutritious and high-quality fresh food can be produced throughout the year [6, 12]. Cultivation distance is determined by the availability of resources to be utilized by the crop, such as water, nutrients, and lighting, which is reflected in the growth and yield of the resulting plants [12]. Countries facing population pressure like India and China are dealing with shrinking cultivable land and sustainability of production, so there is a need to innovate new possibilities for the cultivation of food items. Developed countries have already adopted the concept of vertical farming popularly known by the term Sky Farming [13, 14]. Thus, this approach has greater potential to ensure food and nutritional security in developing countries.

Many start-ups recognized as greenhouse industry companies, and even companies (Table 1 & 2) which were previously unknown to horticulture are now entering the vertical farming space. Furthermore, growing awareness of vertical farming has boosted research into controlled environments, which has a positive impact on the horticulture industry [1, 15]. This review paper investigates the implementation of a sustainable urban agriculture project, also known as vertical farming, and it suggested that it holds assurance for addressing persistent food security issues in urban areas. Finally, it implies that there is a unique set of skills in social work and fundamental values, such as its contribution to social justice and human rights, as well as its ability to advocate for policies and society exercise, making it a valuable partner in the development of sustainable agriculture programs in urban areas [16].

2. REASONS TO SHIFT FROM CONVENTIONAL TO VERTICAL FARMING

2.1. Exponential Increase in Population

India's population is rapidly increasing, and it is predicted that it will soon surpass that of China. With natural resources such as water and arable land becoming scarce, these figures pose an even greater challenge. Vertical farming is, in fact, the most advantageous solution for this [17].

2.2. Scope of Quality Food Production

When compared to the refrigerated produce typically available at supermarkets, a vertical farm allows farming within the confines of a city, and the produce is quickly delivered and always fresh, when the farms are nearby [3, 14, 17].

2.3. Negligible Wastage of Water

The agricultural industry is one of the most polluting industries on the planet, using up to 90 % of the world's water. However, vertical farming has the potential to change that. Vertical farms use 95 % less water than conventional farms due to the regular circulation of water [3, 17].

2.4. Optimum use of Energy

Solar energy is used in vertical farms, which is renewable energy so saves energy [17, 18].

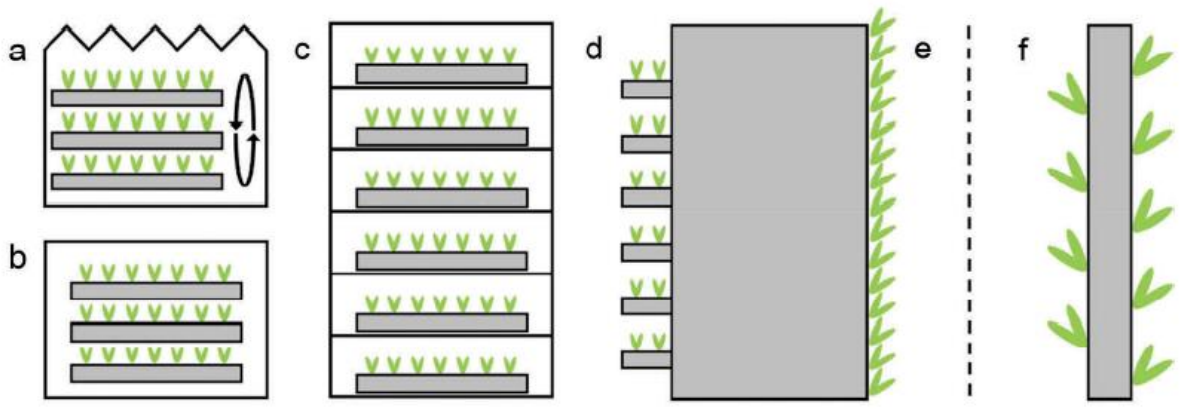

Fig. 1. Representation of vertical farming system (Source: [5])

Table 1. Exploitation of vertical farming in the world

S. No.	Name	Location	No. of Storey	Products	Area (sq. ft.)	Technology	Referance
1	Green Sense Farms	First farm in Portage, Indiana -Shenzhen, China		Micro Greens, Herbs and Lettuces are major crops	20,000	Automated computer controls system used, which provides the exact amount of water, nutrients, temperature, light and humidity for growth of the plant. Minimize the waste and recycle water technique	[19]
2	The Plant Vertical Farm	Chicago, IL	3	Mushroom and Tilapia	100,000	Aquaponics and hydroponic systems, Waste is recycled into energy, Use of biogas from an anaerobic digester, Sunlight is used as natural energy.	[20]
3	Plant lab VF	Den Bosch, Holland	3	Tomatoes, cucumbers in vegetables and strawberries in fruit crops		LEDs are used as an energy source. Hydroponic and Aeroponic system	[21]
4	Vertical Harvest plants	Jackson Wyoming, USA	3	Tomatoes, lettuce, micro greens and strawberry	18,000	Recirculating hydroponic methods LEDs are used as an energy source.	[22]

3. OPPORTUNITIES

3.1. Agricultural Productivity

Agricultural cultivation has its limits viz. the cultivable area is limited and with the increasing population, it may not be apt for meeting the food requirements of future generations. In such a scenario, vertical farming can increase the yield up to more than ten times by utilizing the space component [17]. Currently, financial competency is analyzed for a period from 2019 to 2026 foreseeing the market analysis for a stronger performance of vertical farming [3, 15, 18].

Table 2. Indian companies involved in vertical farming

Company	Location	Remarks
Urban Kissan	Hyderabad and Bangalore	It was found that 30 times more production is taken by the vertical farming system than traditional farming with the use of 95 % less water.
Urban Green Fate	Mumbai	Started in 2012. Main focus on converting the free spaces such as empty plots, areas between buildings and homes or on walls, and restaurants into thriving hydroponic micro-farms.
Triton Food Works	Northern India	Here many crops are cultivated in vertical units such as strawberries, tomatoes, coriander, broccoli, cherry tomatoes, bell peppers, cucumbers, and oregano.
365D Farms	Pune	It is the first movable hydroponic vertical farming unit in India. It was built in a shipping container and mainly used for the production of lettuce overall the year under high-tech conditions.

3.2. Weather Resistance

Agriculture is traditionally taken up as an outdoor activity which is highly prone to environmental conditions. Many times, these factors play a spoilsport and cause a lot of damage to the crops. Such concerns can be minimized by relying on vertical farming practices [17, 23].

3.3. Constant Demand

Profitability usually necessitates continuous operation, so the crop(s) grown must have sufficient year-round market demand. Horticulture makes it much easier to grow the same crop year after year, and it allows growing systems to be engineered and optimized specifically for that crop [3, 17].

3.4. Limited Labour

Vertical farmers often report that one of the most common costs mentioned by vertical farmers is labour. Vertical farming lends itself to crops that can be "sown and grown" with little effort. Automation reduces labour inputs, but it usually comes at a high cost in terms of design, purchase, and installation [17,23].

3.5. High Harvestable Yield

The portion of the crop that can be harvested and sold is referred to as this. In the case of lettuce, almost the entire plant can be sold, resulting in a high harvestable yield [3, 6, 14, 17, 24]. Throughout the year, produces large quantities of nutritious and high-quality fresh food [4, 24].

4. AUTOMATION IN VERTICAL FARMING

In vertical farming, the monitoring process is imperative because it helps humans to keep track of how much nutrients they are giving to the plants. However, the costs of implementing advanced technologies for vertical farming, such as the buildings or greenhouses for the crops, as well as the lighting and automated monitoring systems, are prohibitively expensive [3, 10, 25]. The use of robotics in agriculture, particularly in vertical farming, is becoming more relevant and practical. This allows for greater precision in operating, controlling, and guiding machines to more efficiently perform agricultural tasks.

The main purpose of this project is to create a system for vertical farming which is automated that can observe or monitor the state of the nutrient solution while using a lesser amount of water and electricity for producing vegetables [10]. The time limitation is critical in this system because plants take a much time to grow and people must conduct daily observations. Due to a lack of daily observation, it resulted that growth process of plants was slowed [10].

For improving propagation efficiency and runner quality in strawberry high light intensity and long photoperiod is useful [26]. A justifiable estimate for evaluating the potential for growing plants canbe PAR values which are critical for the cultivation of leafy vegetables [27]. In a vertical hydroponic system, it was observed that half-dose fertilizer produced more fresh and dried leaf weight than full-dose fertilizer. Furthermore, using half a dose of fertilizer on lettuce may be more cost-effective [28].

When compared to plants grown with standard white LEDs and combined Red/Blue LEDs, white LEDs with a shorter blue peak wavelength of 437 nm aid in qualitative parameters of lettuce plants, according to a study [29]. LED lighting technologies and rising consumer demand for fresh, healthy, and locally grown produce with minimal inputs are driving this trend. [9]. Reported findings suggest that the function of space (volume) can be exploited to improve the efficiency of vertical agriculture systems so that our production systems can fully exploit the inputs [30].

5. PRODUCTIVITY AND ECONOMICS OF VERTICAL FARMING

Vertical cultivation allows for a multiple-fold for increasing the number of plants per sqm, depending on the diameter of the pipe used, which has a positive impact on the square meter's productivity because by using a vertical farming system area of cultivation can be increased by 3 to 4 times [3, 12]. With a production of 1,366,850 tonnes, the USA leads in the production of strawberries through vertical farming among countries, accounting for

more than a quarter of global production [12]. Vertical farming is the system that is completing the demand of consumer for local production of high-quality fresh vegetables and fruits that is healthy, safe, tasty, and produced sustainably in or near cities is growing and it is rapidly expanding in the cities [24].

Land, irrigation water, fertilizers, and other resources are in short supply in modern agriculture. Furthermore, in wild areas or open field systems farmers prefer to grow crops and vegetables by the soil-based traditional method, and in most regions, seasonality, environmental extremes, and soil-borne diseases make it unfeasible to grow most crops consistently continual with high quality. As a result, current agricultural systems are unsustainable, and market prices for vegetables are volatile. For growing vegetables vertical farming systems inherit advanced greenhouse technologies as a recent development and good quality plants in a completely controlled environment and multiple stack layers help in increasing crop yield per unit area. These types of systems allow for the constant production of high-quality plants throughout the year while using fewer resources [15, 30].

Different cropping systems or cropping sequences influence the gross return, net return, and benefit-cost ratio, according to an economic analysis of different cropping systems in horticultural crops [18, 31]. When intercropped with Ziziphus mauritiana and then Emblica officinalis all of the cropping sequences yielded a higher economic return. Fenugreek-Okra cropping sequence with Z. mauritiana recorded the highest gross return (982275), net return (809215), and BCR (4.68) which was followed by crop sequence of nigella-cowpea with Z. mauritiana, registering a gross return of Rs. 873750/ha and net return was Rs. 705450/- ha, respectively [31].

6. CONCLUSION

Vertical farming of horticulture crops is a promising trend offering advantages like optimized land use, year-round production, and better crop quality. Controlled environments enhance yields, but research is needed on efficiency, energy consumption and sustainability aspects. It holds potential for urban agriculture and addressing global food demands. Vertical farming holds the key to transforming the future of horticulture cultivation and satisfying the rising global food demand with continued technological innovations.

7. LIMITATIONS

Vertical farming is unquestionably a solution to critical issues in Indian agriculture, such as a lack of or excess supply of farm produce, excessive pesticide use, excessive fertilizer use, and deteriorating soils. However, there are obstacles, such as the Indian farming community's acceptance of vertical farming. Availability of electricity throughout the day, assurance of minimum support prices, public awareness, farming community inclusion, technical know-how Expenses associated with managing and maintaining vertical farm systems [2].

Vertical farming is capital intensive, especially considering the initial cost of establishment. Cost-incurring aspects include the erection of structures and automation, computerized monitoring systems, cost of software, racking and staking facilities, climate control systems, LED lighting, and so on. Plant grown entirely under artificial lighting requires high energy costs.

In vertical farming excess nutrients used may interfere with contamination in the main water channel if not properly managed. Although light emission from LED is on the lower side still is a source of heat but especially during the summer months, they can cause problems with temperature control and overload air conditioning systems which results in higher energy costs. It will need to be properly disposed off of plant residues and other waste which is present around the buildings in a vertical farming system. Vertical farming system needs skilled labour because of a lack of knowledge of technologies they will be unavailable to handle at first and requires training [2].

8. FUTURE PROSPECTS

India is a major producer of vegetables, fruits, and a variety of other crops or products. Vertical farming has been now introduced in India for the production of various crops. The concept of vertical farming is developed by experts from ICAR in soil-free conditions. In metros cities such as Mumbai, New

Delhi, Chennai, and Kolkata in soilless media or without the use of pesticides or chemicals, evenly crops can be grown on multi-story buildings [10]. Options are too many for exploring the cultivation strategies in vertical farming like leafy vegetable crops, strawberries, eggplant, and herbs are the crops which can be successfully grown up to two-three upper levels of tower and onion, leek, collards, and some Cole crops can be suitable for lower levels [32]. A successful hydroponically vertical farming on a small scale was started by scientists at Nadia's Bidhan Chandra Krishi Viswavidyalaya. Vertical farming adaptations on a small scale have been seen in Nadia, West Bengal, and Punjab. Brinjal and Tomato were successfully grown in Nadia's Bidhan Chandra Krishi Vishwavidyalaya. In vertical farming, Punjab has also succeeded to produce potato tubers. Vertical farming system is preferred because organic food is grown with high quality, and predictable supply, according to Idea Farms, an Indian design-in-tech company. Greenopia, a Bengaluru-based start-up, sells kits that include smart self-watering pots, enriched soil, and the appropriate seeds. The sensor-embedded pots replenish moisture in the soil as needed and alert you when it's time to refill water from the outside. U-Farm Technologies, a Mumbai-based start-up, is customizing modular farms for individual apartment complexes or supermarkets using hydroponic gardening techniques. In India, a growing number of vertical farming start-ups are emerging [2]. Farmers give first preference to traditional farming because of caring for plants under human supervision but still,in many places, the vertical farming system was commonly used. But automated monitoring system in a vertical system helps people to monitor the nutrient condition without manually measuring the nutrient condition which provides nutrients at the time and this controlled system also helps in reducing the time taken needed during changing the nutrient solution [10]. Vertical farming is also capital-intensive and necessitates technical knowledge in order to utilize the new techniques and equipment available [33].

9. CONFLICT OF INTEREST

There is no conflict of interest among the authors.

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Proceedings of the Pakistan Academy of Sciences: B
Life and Environmental Sciences 60(3): 327-336 (2023)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(60-3)847

Pakistan Academy of Sciences

Review Article

Significant Role of Medicinal Botanicals Hostile to Cancer

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Abstract: In medical systems, medicinal plants have always held an important place. Plant phytochemicals with known biological activity, such as antiviral, antibacterial, and anticancer properties, are crucial in treatment. It has demonstrated little to no negative effects in recent years and is regarded as safe to use. The use of medicinal plants is essential for preventing illnesses, especially cancer, which is the second leading cause of death worldwide. It was discovered that scientists had been effective in finding anti-cancer compounds up to this point such as eugenol, allicin, catechins, curcumin ursolic acid, anethol, lycopene, resveratrol, 6-gingerol from ginger, tomato, garlic, turmeric, blueberries, milk thistle, cranberries, walnuts which can assist in blocking or activating cancer cell activation signalling pathways cycloxigenase, matrix metalloproteinases MMP, COX-2, topoisomerase enzyme, Bax, Bak proteins and accelerating enzymes (antioxidant potential) that protect the body. Cancer is now treated with a few plant-based products and their phytoconstituents. Even though the management and control of cancer progression have advanced significantly, there are still many gaps and untapped prospects. As a result, this review article emphasizes the value of medicinal plants in maintaining human health as well as lists the phytochemicals from medicinal plants that can be used to cure cancer.

Keywords: Medicinal plants, Cancer, Phytochemicals, Secondary metabolites, Therapeutics.

1. INTRODUCTION

Ancient therapeutic knowledge has been pioneered ever since the beginning of the historic era, which has been saved as well as transferred to every single corner of the globe. All the ancient discoveries regarding drug detection come in nature along with the herbal side. Countless crucial drugs are obtained through organic products or natural means. Plants are one of the extremely significant means of novel pharmacologically effective elements which enter the medicine production sector for the formation of drugs, having a sustained record in the therapy of numerous disorders. To date, there are plant species (35,000-70,000) that have already been analyzed for medicinal purposes [1]. Countless individuals have relied on medicinal plants for basic healthcare since prehistoric times. The traditional knowledge of local communities and indigenous peoples is closely related to this type of traditional medicine. Due to the fact that many traditional herbal practices have contributed to the discovery of medications, interest in traditional knowledge has increased. Ethnobotany is the name of the scientific field that investigates how local groups and indigenous peoples use plants. In reality, there are various approaches that can be used to choose plants for pharmacological studies, and the ethnobotanical paradigm is just one of them. Drugs found via ethnobotanical leads include aspirin, codeine, ipecac, reserpine, and others. In fact, it gives the scientist quick hints for choosing the candidate species for more research [2, 3]. Paul Alan Cox and Michael L. J. Balick deserve credit for exploring and emphasizing the role that conventional knowledge plays in contemporary drug development in the early 1990s [3]. The discovery of quinine is regarded as the greatest medical advance of the seventeenth century. Since the 1600s, quinine, a substance found in the bark of the cinchona (*Cinchona pubescens*) tree, has been used to alleviate malaria. Indigenous consumption is also the source of the discovery of artemisinin, another antimalarial medication. Since the

Received: January 2023; Revised: May 2023; Accepted: June 2023
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discovery of artemisinin, the Artemisia annua plant has been used in China to cure fevers for at least two millennia. The value of documenting conventional understanding scientifically is clearly demonstrated by these examples. Despite the development of new chemical, biological, and screening methods, conventional knowledge persists to be extremely applicable as natural product research progresses [3]. Plants possessing ethnopharmacological ability are principal resources of medication intended for initial drug detection concerning their novel ethnopharmacological uses. In the current era, the exploration of drugs originates from plants depending on their bioactivity. About 1/3 (39.1 %) of each of the authorized drugs by the FDA are of natural source. Natural compounds are considered one of the most valuable bases in the procedure of drug detection. In the practice of drug finding, the incidence of more than 200,000 natural metabolites represents the diverse bioactive properties giving immense importance to natural products. However, therapeutic (medicinal) plants happen to be the most prominent sources of natural products [1]. Between 350,000 and almost half a million species of vascular plants, or 10 % of all plants, are thought to be utilized as medicines. Initially, the trial-and-error approach was employed to treat illnesses or even just to feel better, and in this way, valuable plants with positive properties were identified [4]. The major goal of this review is to highlight the usefulness and significance of medicinal plants for maintaining human health, as well as their phytochemicals for oncogenic treatment, which represents a novel strategy that may contribute to the advancement of the pharmaceutical industry.

2. MEDICINAL PLANTS

Medicinal plants can be described as plants that hold healing attributes otherwise, they employ positive pharmacologic outcomes over an animal or human health [5]. Medicinal Plants are currently in need, also their acknowledgment is gradually rising. Unquestionably, plants possess a significant position in offering important services in bio-networks. However, devoid of plants individuals as well as other living organisms are unable to thrive in the manner living beings would. Nonetheless, herbals, particularly medicinal herbs have continuously operated as a general indicator of the health of bionetwork. Also, medicinal plants have undeniably been reflected by individuals ever since the prehistoric era. It can be supposed that previously as well as early human beings acknowledged as well as manipulated plants surrounding them for shelter, clothes nutrition, along with fuel because they turn out to have awareness of their properties. Medicinal plants have been transmuted in some of the earliest disciplines in nations like Greece, Egypt, India as well as in China. Plants were frequently utilized as medicine as well as a disinfectant in ancient Persia (currently named Iran). Meanwhile, above a tenth of the plant varieties (greater than 50,000 species) are employed in cosmetics along with therapeutic products. Though the distribution of medicinal plants is not the same across the globe, in addition, those medicinal herbs are assembled largely through the natural world. Certainly, the need for natural resources has been boosted by dint of 8 % to 15 % in Asia, Europe along with North America annually in current years. The phrase medicinal plant describes a range of vegetation that holds therapeutic features. Such plants are a valuable resource of components that can be utilized for drug-making (Figure 1). The portion of medicinal plants which might be consumed is distinct kinds of skin, seeds, flowers, fruit, leaf, root, or even entire plant. Dynamic components inside major portions of such plants pose explicit or else implicit healing results thus being employed as medicinal agents. The parts of such plants carry a variety of substances that are being formed and then accumulated which are described as active substances, exerting physiological impacts on living organisms. Human beings are dependent upon natural plant substances to be acquainted with therapeutic requirements for sustaining health as well as disease treatment. Medicinal plants are utilized for medication since they have specific properties, which include interactive activities. The components of plants might act together then such an interface would be advantageous or unfavorable to any of them or else reduce the toxic consequences of both [6].

The most primitive data being acknowledged is the utilization of 1000 plants which includes *Cedrus duham* species, *Papaver somniferum* L, *Cupressus sempervirens* L*, Glycyrrhiza glabra* L, along with *Commiphora myrrha* Engl. Such drugs (Table 1) were originally applied in raw types involving teas, powders, dressings, and tinctures [7].

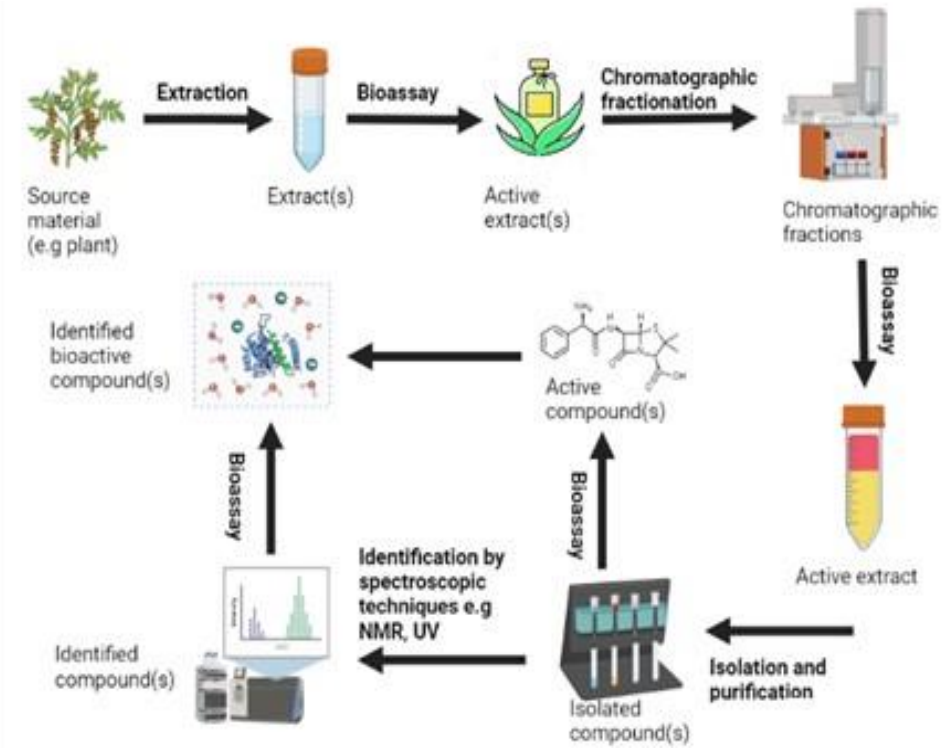

Fig. 1. Timeline for discovering natural drugs.

Table 1. Drug compounds isolated from medicinal plants [6].

Drug	Plant	Activity
Taxol	*Taxus brevifollis*	Anti-cancerous
Morphine	*Papaver somniferum*	Narcotic and powerful pain reliever
Serpentine	*Rauwolfia serpentia*	Hypertension
Quinine	*Cinchona* sp.	Antimalarial
Vincristine	*Catharanthus rosesus*	Anti-cancerous

2.1. Secondary Metabolites of Plants

The therapeutic power of plant components is predominantly because of the blend of components designated as the secondary metabolites of plants (SMoPs). SMoPs are a distinct biochemical cluster of elements formed through plant cells via secondary metabolic paths (derivatives of the primary metabolic paths). Contrary to the primary metabolites being tangled in the key metabolic pathways which are fundamental for subsistence secondary metabolites of plants are not needed for life as well as development, however, they portray significant tasks in interspecies defense besides antagonism. Currently, there are two hundred thousand diverse secondary metabolites of plants being isolated as well as recognized. SMoPs are categorized as shown in (Figure 2) for their chemical

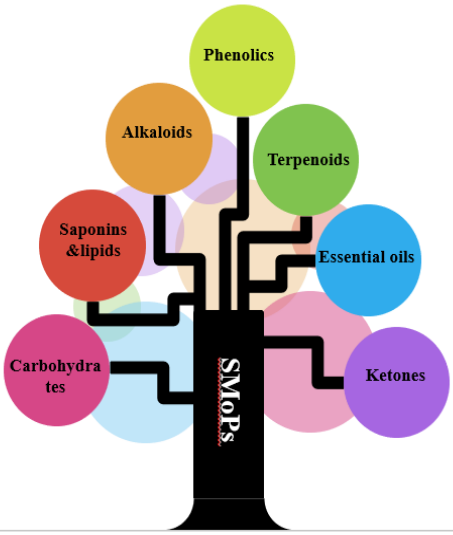

Fig. 2. Categorization of secondary metabolites of plants.

arrangements otherwise biosynthesis paths [8].

2.2. Antioxidants

Antioxidants have been reported to stop oxidative damage instigated by free radicals. All organisms are protected from the attack of free radicals by the defense processes which search as well as stabilize the free radicals nonetheless while the formation speed of free radicals surpasses then oxidative stress is created. It is a detrimental process that can harm cell structures involving lipids, DNA, as well as proteins. So, there is an upsurge in the application of naturally occurring antioxidants and their use in cosmetics, medicine, foods, or raw materials instead of man-made materials [8]. The antioxidant depicts a defense approach to which the body of a human is safe from free radicals which create oxidative harm. However, free radicals' abundance advances toward cellular stress which ultimately harms cellular structures as well as their functions, DNA, and proteins. Reactive oxygen species commonly known as ROS are a class of reactive compounds, free radicals, as well as ions produced through oxygen. Medicinal plants have emerged as possible natural antioxidant sources, producing a variety of anti-oxidative chemicals with medicinal and therapeutic characteristics. Antioxidant-containing drug formulations are commonly used to treat and prevent a wide range of infectious and complicated disorders. Antioxidants are said to protect against oxidative stress stimulated via reactive oxygen species as well as free radicals. Natural antioxidants come in a wide range of compositions, physical and chemical characteristics, processes, and action sites. Antioxidant activity has been documented in medicinal plants that are high in flavonoids, vitamins, polyphenols, and anthocyanins [9].

3. CONSEQUENCES OF SYNTHETICAL MEDICATIONS

Chemical or synthetical pharmaceuticals may have stronger or faster effects than herbal remedies, but they also come with a slew of consequences and hazards. Herbal medications are thought to be free of such side impacts for the reason that they have been used by millions of people around the world for thousands of years to treat a variety of ailments [10].

3.1. Expensiveness of Synthetic Drugs

In comparison with synthetic medicines, herbal remedies are less expensive. Medicinal plants continue to make a meaningful contribution to current prescription pharmaceuticals by providing key ingredients which can be used to make new ones [11].

4. IMPORTANCE OF MEDICINAL PLANTS IN LONG-TERM HUMAN HEALTH

For thousands of years, civilization has relied on therapeutic plants as a resource of medications. Early human beings were completely reliant on plants intended for all their medical requirements, including restraint to diseases, therapy, along with various forms of medication, and have been using plants as pharmaceuticals for millennia. The usage of medicinal plants has had a spiritual impact during the course of the evolution of individual society, as well as varied perspectives over conceptions of wellbeing coupled with sickness that each culture's existence embraces. For almost 3,000 years, a huge variety of vegetation has been employed in the medical field techniques for instance traditional medicine in India, Africa as well as China with the majority of them containing medicinal properties according to Western (Figure 3). Moreover, some more herbs were being employed by various civilizations for thousands of years while being more unlikely to be validated by dint of Western criteria. The importance of therapeutic (medicinal) vegetation on the well-being of humans is undeniable. The World Health Organization identifies 252 medications as fundamental in addition to essential, 11 % of the total are exclusive of plant derivation and a high portion of it is synthesized via natural precursors. Digoxin, quinine, and quinidine derived through *Cinchona spp*., vinblastine along with Vincristine, isolated *viz Catharanthus roseus*, atropine as of *Atropa belladonna*, as well as *Papaver omniferum* provide morphine along with codeine are some of the medications derived from natural sources. Natural origin medications account for 60 % of anti-cancer along with medications (anti-infectious) now in the marketplace otherwise appearing in experimental trials. These plants offer chemicals for the formation of innovative medications, and biomimetic synthesis, along with the detection

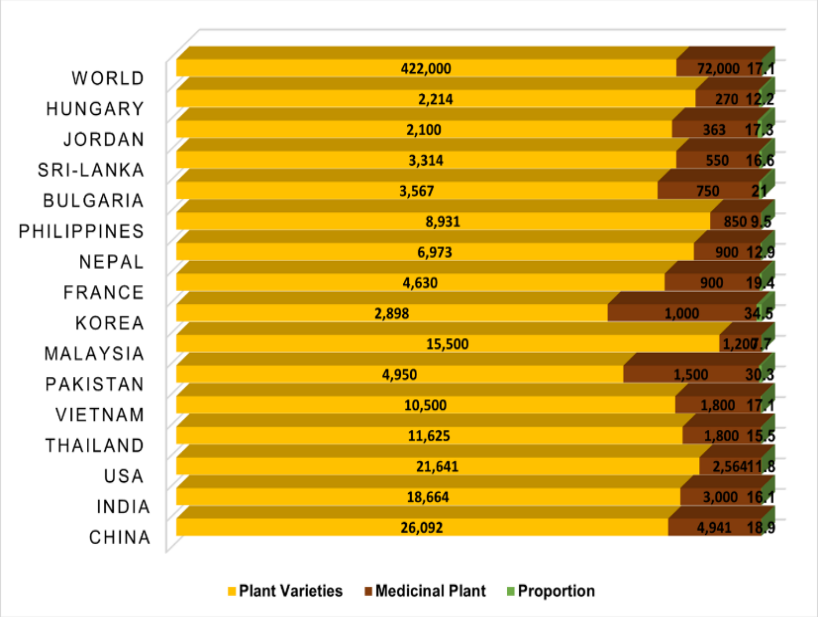

Fig. 3. Worldwide usage and proportion of medicinal plants [5].

of new therapeutic characteristics which are not previously associated with identified molecules [11].

4.1. Effectiveness of Herbal Medicines

The fact is that plant remedies can be utilized in the treatment of some ailments when traditional medicine fails and that medicinal plants are effectual, mild, as well as much of the time specialized in their role over the organs of humans [10].

5. CANCER

Cancer is a life-threatening disease caused by aberrant cell proliferation in the human body. The normal function of the damaged organ is significantly interrupted as a result of the abnormal proliferation, which may end in the patient's mortality. Mutations in two types of genes are commonly responsible for cancer: cancer-inducing oncogene and tumor-suppressing genes are genes that play a significant impact in tumor suppression. To metastasis, cancer cells normally spread throughout the body via blood arteries and lymphatic systems. Traditional therapies have included botanicals (medicinal) from the dawn of time on this planet. Because medicinal plants have therapeutic benefits, are less poisonous, and are less expensive than conventional ailments, 80 percent of the world's population, particularly those in rural areas, are directly dependent on them. Many natural metabolites or subtly modified metabolites generated from plants have anticancer properties, indicating that these plants can be used to treat cancer [13]. Natural compounds or derivatives account for 48.6 % of all cancer medicines that have been registered since the 1940s [1].

Previously, 24.6 million cancer survivors, 10.9 million new cancer cases, as well as 6.7 million fatalities due to cancer were reported each year throughout the globe. According to statistics from the World Health Organization, globally, 14.1 million new cancer cases and 8.2 million deaths have been reported in 2012, with an estimated 70 % surge in new cancer cases over the following two decades [13]. The coronavirus disease 2019 (COVID-19) pandemic, on the other hand, in 2020, had a detrimental impact on cancer detection and treatment. Healthcare facility closures have reduced access to care and fears of exposure to COVID-19 caused delays in treatment and diagnosis, which could cause a brief drop in cancer incidence followed by an increase in advanced-stage cancers along with, a higher death rate. However, owing to the halt in the dissemination of population-centered surveillance data, it would take several years to determine these and other pandemic-related secondary effects on the population. For instance, cancer mortality and incidence data are available for 2018 and 2019, correspondingly [14].

5.1. Medicinal Plants possessing Chemotherapeutic Properties

After significant research on medicinal plants with chemotherapeutic potential over many years, (*Alhagi pseudalhagi, Aphanamixis polystachya*, *Calamus rotang, Cirsium rhinoceros, Aphanamixis polystachya, Annona squamosa, Terminalia arjuna, Polygonum cuspidatum, Euphorbia jolkinin, Centella asiatica, Bupleurum kaoi, Stephania tetrandra, Ochrosia elliptica, Labill Ophiorrhiza mungos, Ornithogalum umbellatum* (*Ornithogalum*). *Taxus brevifolia* is a species of *Taxus Tabernaemontana divaricata, Scandinavian, Paederia, Elephantopus scaber, Impatiens balsamina, Coix lachryma, Rhei Rhizoma, Taxus wallichiana, Moringa oleifera, Vitex negundo*, and a variety of other plants are among them). Scientists were successful in discovering anti-cancer phytochemicals (Table 2) [15] such as eugenol, silymarin, allicin, catechins, curcumin ursolic acid, anethol, ellagic acid, lycopene, resveratrol, 6-gingerol, S-allyl cysteine, capsaicin, along with others. [16].

5.2 Phytochemicals as cancer treatment: a unique approach

Phytochemicals are compounds found in therapeutic (medicinal) plants that hinder cancer development and development. According to studies, the plant kingdom contains over two hundred fifty thousand different plant varieties, but 10 % of them have been explored and intended for the cure of numerous ailments. Phytochemicals along with their counterparts can be discovered in a variety of plant components, including the flower stigmas, pericarp, sprouts, fruits, seeds, roots, rhizomes, stems, leaves, embryos, and bark are all examples of plant parts, and a variety of pharmacological effects. Saponins, gums, minerals, taxanes, flavonoids, lignans, vitamins, glycosides, alkaloids, oils, terpenes, biomolecules, and other primary and secondary metabolites all play a part in the process of either blocking or activating cancerous cell activation proteins, signaling pathways (Figure 4) [17] (cycloxigenase, CDK4 kinases and enzymes (matrix metalloproteinases) MMP, Cdc2, COX-2 (Cycloxigenase), CDK2), topoisomerase enzyme, B cell lymphoma 2 (Bcl-2), cytokines, automates target of rapamycin (mTOR), MAPK/ERK, PI3K, Akt, (tenecteplase) TNK via inducing DNA repair (p21, p27, p51, and p53 genes) as well as their products (protein), Bax, Bak, Bid proteins, accelerating enzymes that defend the body (Caspase-3, 7, 8, 9, 10, 12) forming antioxidant enzymes (antioxidant activity) e.g. glutathione peroxidase (GPxn), glutathione (GSH), as well as glutathione S-transferases (GST). As a result, they have a high anti-cancerous impact in the context of effectiveness [18]. Recent studies revealed that medicinal plants and the bioactive substances they contain had been thought to be important agents in treating breast cancer through various processes. Asthma, scrofula, and other respiratory conditions are commonly treated with *Citrullus colocynthis*. Additionally, it is used to treat cancer, leukoderma, splenomegaly, blockage, dyspepsia, urine incontinence, and weakness. *C. colocynthis*, which has cytotoxic activity and is thought to be helpful in the treatment of cancer, contains cucurbitacins as well. Rameshbabu et al. looked at the antiproliferative effects of methanolic and aqueous

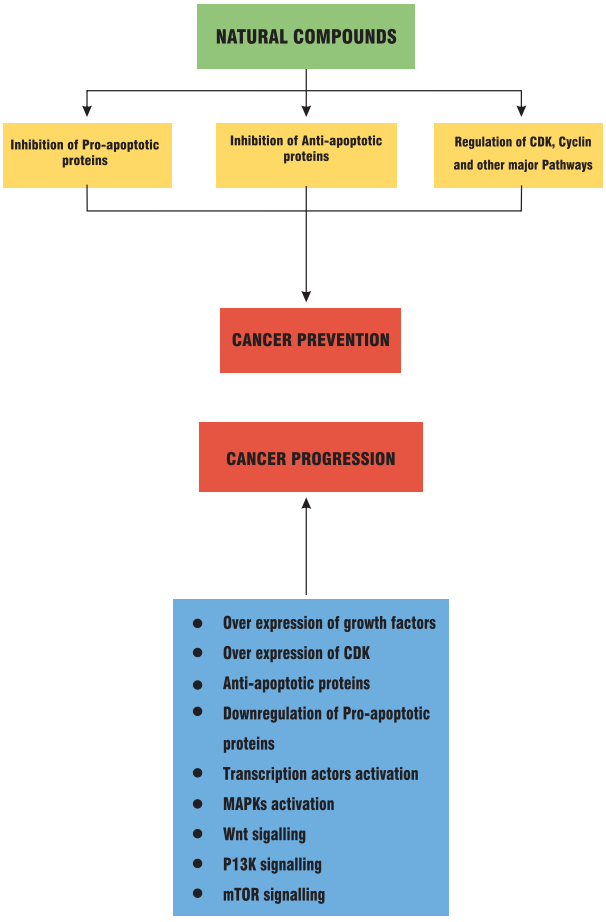

Fig. 4. Utilization of natural compounds for cancer prevention [17].

Table 2. List of medicinal plants with anti-cancer properties [15].

Anticancer chemical compounds	Molecular process of anti-cancerous activity	Medicinal plant
Apigenin	Impacts leptin or leptin receptor path, as well as stimulates cellular apoptosis via initiating (p38) mitogen-stimulated protein kinases (MAPK) path.	*Petroselinum crispum, Moringa peregrine*
Betulinic acid	Provokes mitochondrial apoptosis path.	*Betula alba, Ziziphus mauritiana* Lam.
Genistein	Hinders tyrosine kinase enzymes via inhibition of DNA topoisomerase (II), as well as engages in c-Jun N-terminal kinases (JNK) path for the promotion of activity of AP-1.	*Glycine max, Lupinus albus, Flemingia vestita*
Crocetin	Inhibition of nucleic acid formation generates apoptosis as well as delays developmental factor signifying paths, and enhancement of the anti-oxidation process.	*Crocus sativus L., Saffron crocus*
Diindolylmethane or Indole 3-carbinol	Epigenetic performance of cancerous cells via modulation of the receptor tyrosine kinase, PI3K, or Akt signaling paths as well as modification of invasion and metastatic angiogenesis.	Cabbage as well as mustard family.
Phenoxodiol	Boosts the apoptosis process, and inhibits anti-apoptotic elements quantity.	*Glycine max* (L) Merr.
Protopanaxadiol	Halts the cell in the G0 or G1 stage of the cell cycle phase.	*Panax ginseng* CA. Mey.
Curcumin	Intervenes with NF-κB to regulate tumor cell development via management of multiple cells signaling paths involving cFLIP, c-IAP1, cyclin D1, c-myc, XIAP, Bcl-2, and Bcl-x.	*Curcuma longa.*
Camptothecin alkaloids byproducts (irinotecan along with topotecan)	Apoptosis induction through telomerase complexes suppression.	*Saussurea lappa, Camptotheca acuminate* Decne
Indigo (meisoindigo along with indirubin)	Promotion of cell differentiation apoptosis, as well as tumor growth inhibition.	*Indigo naturalis*
Flavopiridol	Cell cycle progression inhibition in malignancy.	*Dysoxylum binectariferum, Amoora rohituka*
Homoharringtonine and Harringtonine	Inhibition of protein synthesis in melanoma.	*Cephalotaxus* species
Salvicine as well as saprorthoquinone	Inhibition of DNA topoisomerase (II).	*Salvia prionitis*
Roscovitine	Hinders histone deacetylates action in cancerous cells in addition to that activates SIRT1.	*Vitis vinifera, Raphanus sativus*
Podophyllotoxin (etoposide plus teniposide)	Topoisomerase (II) enzymes inhibition.	*Podophyllum emodi, Podophyllum peltatum*
Vincristine combined with vinblastine	Blockage of cell development employing preventing microtubule formation.	*Vinca rosea* Linn., *Catharanthus rosea* (L.) G. Don

extracts from several *Anastatica hierochuntica* (L.) components, including the seeds, stems, as well as leaves. The outcomes demonstrated that following treatment with both extracts, procaspase-3 expression and MCF-7 cell viability both dropped. Additionally, both extracts increased the expression of genes associated with apoptosis and the cell cycle, including Bax, TP53, and CDKN1A [19].

According to investigations, flavonoids can prevent the growth of tumor cells by preventing the production of ROS and suppressing the activity of the enzyme's xanthine oxidase, cyclooxygenase-2, and 5-lipoxygenase, all of which are crucial for the growth and progression of tumors. Kaempferol demonstrated antiproliferative and apoptotic efficacy against human osteosarcoma, stomach (SGC-7901), and lung (A549) carcinoma cells in a different investigation. Hesperidin also exhibits hepatoprotective and anticancer properties against the growth of hepatocellular carcinoma. Human epithelial colorectal adenocarcinoma cells treated with cyanidin demonstrated both an inhibition of proliferation and an induction of apoptosis [17]. Triterpenoid saponin tubeimoside-V, a substance from the plant *Bolbostemma paniculatum*, was studied following the plant's extraction and fractionation, which enabled isolation and characterization. It demonstrated the clearing of glioblastoma cells through apoptosis and acted as an antitumor chemotherapeutic [20]. Paclitaxel (PTX), a well-known first-line chemotherapy treatment/ therapy for cancer diseases like ovarian and breast cancer, is the most frequent herbal medicinal ingredient isolated from (Rehd) *T. chinensis*. The compounds vindesine, vincristine, vinorelbine, and vinblastine are found in *Vinca rosea*. The Food and Drug Administration (FDA) has approved and granted licenses to all of these vinca alkaloids, which were the first all-natural substances to enter clinical trials across a number of tumors. It is known that utilizing these alkaloids in low concentrations disrupts microtubular function, while administering them in high dosages results in cell cycle arrest and apoptosis. Currently, these alkaloids are used to treat a number of tumors [21]. Anthocyanins and phenolic acids, the primary active components of mulberries, have been demonstrated to inhibit cell proliferation in a variety of cell lines, including MCF7 cells. *M. nigra* reduces mutant p53 levels in HT-29 cells, which results in cell death through a caspase-3-independent mechanism [22]. In lung cancer cells like H1650 and A549, *Polygonum cuspidatum* extracts block free radical molecules like DPPH (2,2-diphenyl-1-picrylhydrazyl) and hydroxyl. *Toona sinensis* leaf extract inhibits the production of ROS and causes apoptosis in lung adenocarcinoma cells, particularly H441 [23].

6. CONCLUSION

It is challenging to standardize a specific medication treatment for a patient because tumorigenesis depends on a variety of pathways and diverse sets of mutations. Chemotherapy addresses important problems such as medication resistance, side effects, and the return of the disease. Combinatorial therapies shed some light on the fact that monotherapies don't always work for cancer patients and have the potential to cause several negative effects. It has been observed that even after successfully treating those cancers, some of the cells stay excluded and trigger tumor recurrence because the tumor is made up of different types of cells. Long-term usage of medicines or radiation therapy renders cancers resistant to these treatments, which lowers their effectiveness. In order to lessen side effects and ultimately regulate the resistance developed by the tumors, an alternate therapy would therefore involve combining the aforementioned therapeutic modalities. These include any chemotherapeutic drug combinations as well as any combination of two therapies, such as radioimmunotherapy or radiochemotherapy. Its primary goal is to interfere with the homeostasis of tumor cells by focusing on aspects that can increase therapeutic effectiveness and inhibit or postpone the development of acquired resistance. Natural compounds would be an excellent choice and could possibly inspire the development of new drug candidates since they might be employed as new discoveries in the drug discovery process. The main task at hand is to refine these natural compounds' pharmacokinetic qualities such that in vivo tests on humans might rely on their safety and effectiveness. It is imperative to research the role of natural substances as cancer cures due to the different negative effects of conventional cancer therapy alternatives. Since they have fewer negative side effects, the use of these compounds for treating carcinogens has become more significant in drug delivery. Future research on these phytochemicals appears to be both promising and active. Compared to manufactured medications, these are less harmful and more helpful. In this review paper, we attempted to condense the list of some of the plants and naturally occurring substances produced from plants that have anticancer qualities for different malignancies, which would eventually lead to some

novel compounds and be approved for use in the treatment of cancer.

7. CONFLICT OF INTEREST

All authors declare no conflict of interest.

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Abbreviations:

FDA: Food and Drug Administration
M.Ps: Medicinal Plants
SMoPs: Secondary Metabolites of Plants
ROS: Reactive Oxygen Species.

Proceedings of the Pakistan Academy of Sciences: B
Life and Environmental Sciences 60(3): 337-365 (2023)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(60-3)857

Pakistan Academy of Sciences

Review Article

A Wonder Plant *Aloe vera* L. (Liliaceae): An Overview of its Folk Traditional Uses, Phytoconstituents, Biological Activities, and Cosmaceutical Applications

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Abstract: *Aloe vera* L. (Lililaceae) bears various medicinal applications that likely date back more than a thousand years ago. The current review provides an overview of the folk traditional uses, phytochemistry, biological activities, and cosmaceutical applications of the *A. vera* plant to date. The data have been retrieved from different scientific databases, including PubMed-Medline, Researchgate, Google Scholar, Science Direct, Scopus, SciELO, Taylor & Francis, Web of Science, books, conference papers, Masters and Ph.D. dissertations. As per the collected data of this review, almost 40 active phytoconstituents in *A. vera* have been reported so far with varying concentrations. Ethnobotanical data displayed that *A. vera* is still used as traditional medicine among communities against more than 20 different health-related problems. The DPPH, FRAP, TAC, and ABTS assays were commonly employed where *A. vera* extracts showed varying antioxidant activities against reactive oxygen species (ROS). Data on the biological activities showed *A. vera* plant extracts with remarkable anti-inflammatory activities through the inhibition of TNF-α and prostaglandin E2 factors and also exerts anti-diabetic activity against type 1 and type 2 diabetes. As per the collected data of this review, *A. vera* extracts have been reported with anti-microbial activities against more than 12 bacterial and 7 fungal strains and also obstruct the uncontrollable proliferation of specific types of cancer cells like HCT-116, HepG2, HeLa, A549, and MCF-7. Conclusively, *A. vera* possesses wide-ranging applications in the treatment of various diseases. However, more controlled investigations and clinical trials with the elucidation of the mechanism of action activities are prerequisites in the future to substantiate the outcomes and efficacies of *A. vera* under different circumstances. Any toxic effects of *A. vera* if associated with specific extracts or compounds should be addressed for safer consumption of *Aloe-based* food and cosmetic products.

Keywords: *Aloe vera*, Biological activities, Cosmeceutical uses, Folk traditional uses, Lililaceae, Phytoconstituents

1. INTRODUCTION

Plants have a wide background in the pharmaceutical and cosmetic industries along with food fields and are very significant in the development of human civilizations [1-4]. Some plants are used pliantly as folk medicine or herbal drugs since the time of the bible. Different classes of plants including bryophytes, tracheophytes, and their subclasses have been used to treat certain human diseases. It is well explained in the ethnobotanical field studies from different regions [5, 6]. The plants mostly used in traditional medicine belong to the tracheophyte's subclass angiosperm (94.6 %), followed by the pteridophytes (3.3 %), and gymnosperm (2 %) [7]. Mosses with ~29 species are the only group with the most uses, whereas liverworts only contributed 3 species and with no available data on traditional uses of hornworts [8].

The phytochemistry and biological activities of different plants and percentage of plant parts used

Received: February 2023; Revised: May 2023; Accepted: June 2023
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for therapeutic purposes globally were described by numerous authors [9-18].

Ali *et al*. [7] reported the percentages of parts of plants for ethnomedicinal use including leaves (44 %), stem (12 %), roos (10.66 %), fruit bark (5.33 %), rhizome (5.33 %), stem bark (2 %), blub (3 %), shoot (1.33 %), resin (1.33 %), and come pedicle, the capsule was 0.66 %. The contribution of trees was 16 %, shrubs 11 %, and herbs 73 % (Figure 1a-b).

Among all the higher plants, perennial succulent plants of the genus *Aloe* of the Liliaceae family are found in moderate and subtropical areas of the world. The word *Aloe*, which means a bitter, shining material, comes from the Arabic "Alloeh" or the Hebrew "Halal." Africa is where this plant genus first appeared [19]. There are almost 200 species in the genus some of them are grown for the sticky latex that their large, meaty leaves. *Aloe* plants have been used as purgatives and cure for skin conditions since the time of the Bible [20]. *A. vera* is also called elephant's gall, burn plant, "lily of the desert" and *Aloe*. This plant is sometimes known as *A. brobadensis* having green, stiletto-shaped, marginated, tapering, fleshy, spiky leaves that contain a clear viscid gel [21, 22].

A. vera leaves discharged two different forms of exudates on cutting, one is a sour reddish-yellow juice found in pericyclic cells under the leaves' heavily cutinized epidermis. This "juice" has often been used in dry form as a laxative [23]. Aloin, aloe-emodin, and other similar chemicals give it a bitter flavor. The thin-walled, cylindrical cells in the inner center region (parenchyma) of the foliage generate the other exudate, a translucent, slick mucilage or gel [20, 24].

The chemical makeup of *Aloe* varies by species, climate, and growth conditions and some important phytochemicals reported in *Aloe* include alkaloids, flavonoids, saponins, terpenoids, glycosides, tannins, phenolic compounds, carbohydrates, protein, sterols, protein, tri-terpenoids, glucose, and galactose, etc. and some derivatives [17, 25-27]. Vitamins, enzymes, micro and macro minerals, sugars, anthraquinones, campesterol, sitosterol and lupeol, salicylic acid, and amino acids were also reported [28].

The pharmaceutical and cosmaceutical industry most often uses the plant's latex and gel as it contains a range of organic components believed to contribute to the gel's alleged emollient, moisturizing, and are few skin conditions where *A. vera* is used to treat these complications. *A. vera* sap has healing potential [20] for cuts, burns, and eczema supposedly decreasing inflammation and relieving discomfort. However, there are still some disagreements on the benefits of *A. vera* sap on the healing process [1, 29-31]. Some important reported *A. vera-based* food, pharmaceutical, cosmaceutical, and other products developed are shown in Figure 2.

Considering the potentials of *A. vera*, the present review provides a brief account of ethnobotanical uses with the range of chemical

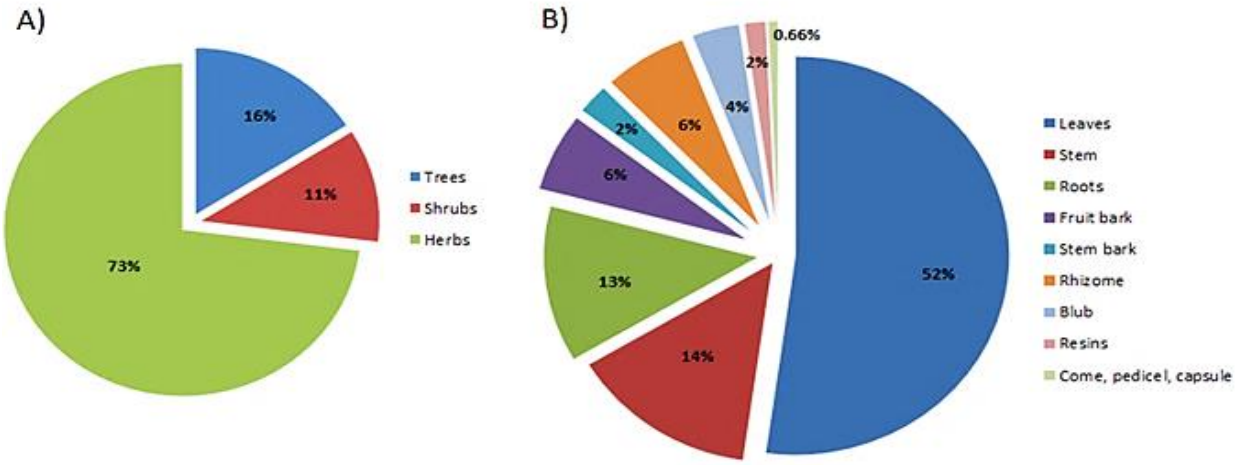


Fig. 1. A) Contribution of trees, herbs, and shrubs used as folk traditional medicine, B) Contribution of parts of plants used for ethnomedicinal purposes. Source: Ali *et al*. [7].

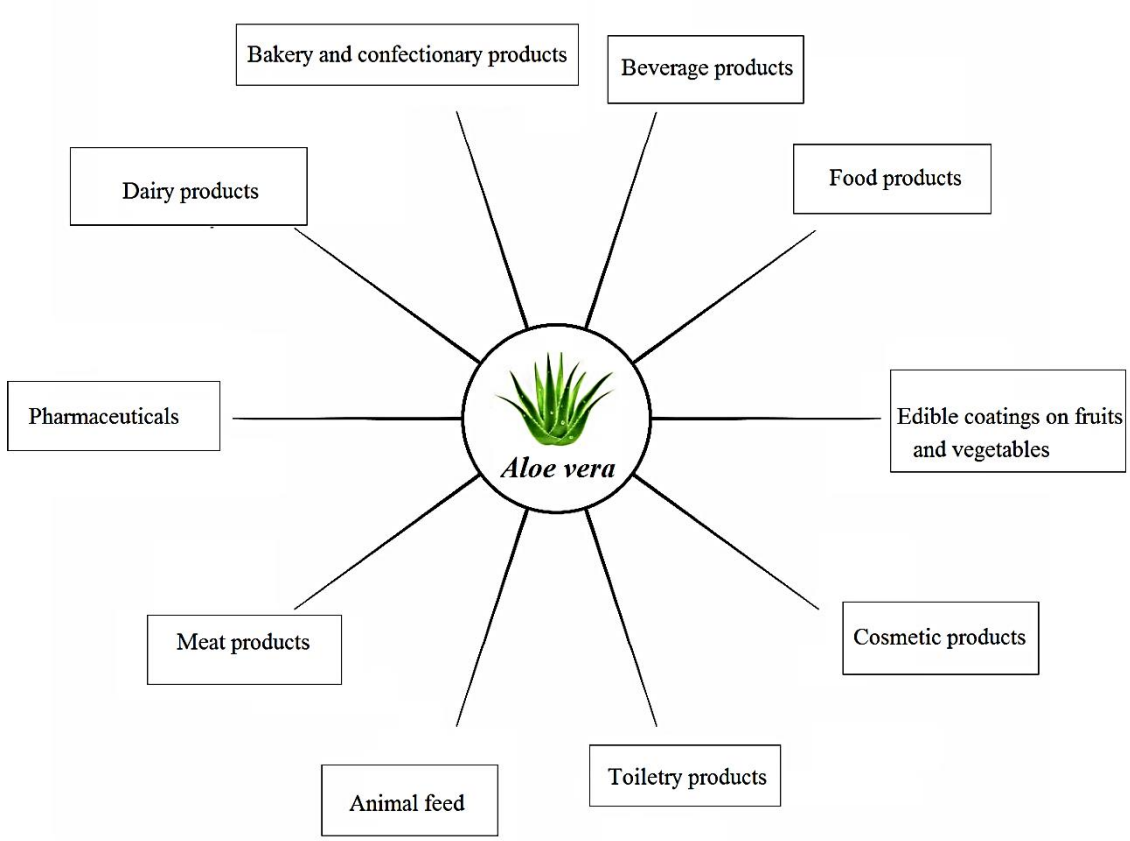


Fig. 2. Different *A. vera-based* food, pharmaceutical, cosmaceutical, and other products developed in the industries

compounds that make *Aloe* plants capable of providing antimicrobial, antioxidant, antidiabetic, anticancer, and anti-inflammatory activities. The review further emphasizes on *Aloe* based food and cosmetic products with improved nutritional and therapeutic effects.

2. METHODOLOGY

1.1. Data Search Approach

The data have been compiled after going through a detailed study on morphology, phytochemistry, folk traditional uses, biological activities, and cosmaceutical and food products of *A. vera* from different databases like Pubmed-Medline, Researchgate, Google Scholar, Science Direct, government reports, SciELO, Web of Science, Scopus, Springer Link, and Taylor & Francis, and from Masters and Ph.D. dissertations. While searching the data, specific keywords were used including ethnobotany of *Aloe vera*, *Aloe vera* morphology, *Aloe vera* phytochemistry, *Aloe vera* medicinal uses, *Aloe vera* biological activities, *Aloe vera* antioxidant activity, *Aloe vera* antimicrobial activity, *Aloe vera* antidiabetic, *Aloe vera* anti-inflammatory, *Aloe vera* cytotoxicity, and *Aloe vera* based cosmaceutical and food products, etc. The data for this review was collected from the articles published up to 2023 in the English language.

3. RESULTS

Overall, 132 articles were studied and the required data have been retrieved and compiled, and consequently presented. The detailed review of the selected articles showed that *A. vera* has been long used as traditional medicine in almost 12 countries against more than 20 different disease categories (Table 1). Almost 40 different types of active phytochemicals have been reported in the extracts of *A. vera* so for (Table 2) including phenols, flavonoids, alkaloids, tannins, saponins, etc. The total flavonoid content was estimated by the colorimetric method and the total phenol content was estimated by the folin-ciocalteu method.

Data showed that the total tannin content was mostly quantified by the vanillin-HCl method, and alkaloid content was determined with the bromocresol green method. For the antioxidant activities of *A. vera* extracts against reactive

oxygen species, DPPH, FRAP, ABTS, and TAEC were the most widely used techniques (Table 3). It was confirmed that the phytochemicals of *A. vera* extract have anti-inflammatory activities by targeting/inhibiting the TNF and MMP-9 factors (Table 4).

Aloe extracts also possess anticancer activity by preventing the uncontrollable division of specific types of cancer cells like HCT-116, HepG2, HeLa, A549, and MCF-7, hence reducing the chances of blood cancer/leukaemia, liver cancer, breast and lung cancer (Table 5). The restoration of insulin level was evident for the extracts of *A. vera* that authenticates its potential antidiabetic activity (Table 6). Antimicrobial activity data showed that the extracts of this plant were active against 12 bacterial and 7 fungal strains (Table 7). The current review also manifested that there are a lot of *Aloe-based* food and cosmetic products that have been commercialized with nutritional and cosmaceutical applications (Table 8).

3.1. Morphology of *A. vera*

The *Aloe* plant is comprised of leaves, short stems, roots, and flowers (Figure 3), and its morphology has been extensively studied by various authors [22-24, 32]. *A. vera* live for more than two years and has sukers at the base. The green, glabrous, glaucous, margin sparsely dentate leaves are sessile, erect, and linear-lanceolate with a length range from 15 – 35 cm and a width range from 4 – 8 cm [23, 24].

Malik *et al.* [22] explained some physical characteristics of the fresh and dried *Aloe* leaves such as length, weight, width, etc. In their study, the reported lengths of the fresh and dried leaves were 43.3 and 38.5 cm, weight was 5.4 g and 5.2 g, and width was 8.2 cm, and 6.1 cm respectively. The reduction in these physical parameters of fresh and dry weight, length, and width of the leaves is due to dehydration causing the reduction of gel weight [22].

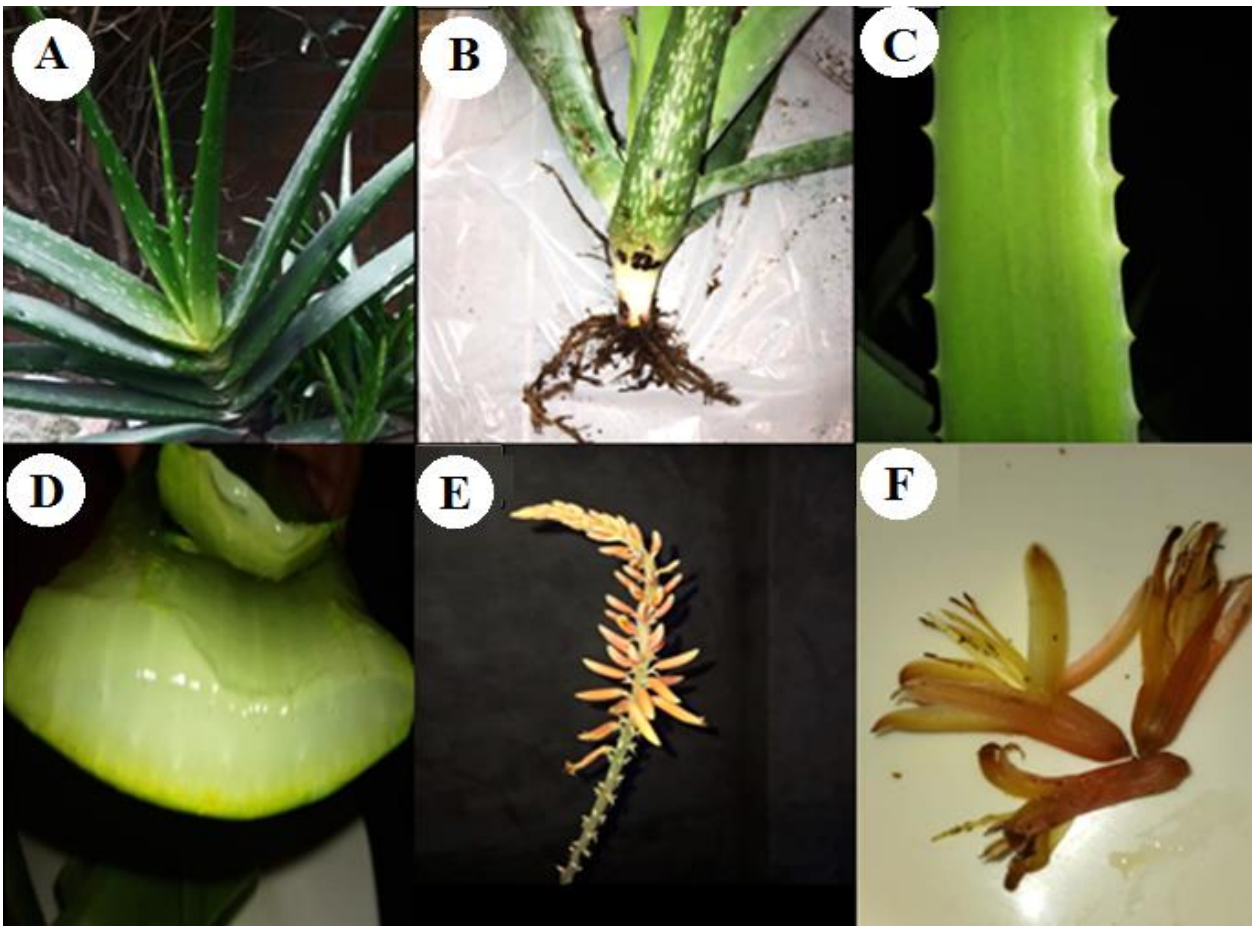


Fig. 3. Morphology of *A. vera* plant. **A)** Habitat**, B)** Roots, **C))** Abaxial surface of leaf and thrones, **D)** Leaf gel**, E)** Synflouresence, F) Flowers. Original photographs by Maira Batool.

The short stem had 1 – 2 branches on which erect pedunculate raceme inflorescence occurs which may be 60-100 cm in length. It has ovate-lanceolate and persistent bracts; 9-12 x 5-6 mm. The flower of *A. vera* formed on the short pedicel and the perianth is dull reddish, lobes 6, almost equalling the tube, and 2.5-3 cm long. The color of the flower ranges from reddish to pale yellow capsule of 1.5 cm [22, 33]. The roots are fibrous that absorb water and nutrients required for proper growth and development. Some physical parameters of *A. vera* roots studied [23] showed varying color, length, weight, and width. The color of the fresh roots is brown and became dark on drying. The fresh and dried root lengths reported were 35 cm, and 34 cm long, 5.4, and 5.2 cm wide with 0.5 g, and 0.4 g weight [22].

3.2. Folk Traditional Uses of *A. vera*

Plants have various beneficial traditional uses and different plant species are used as medicine by people of different regions according to the information they know about that particular plant [34-36]. The traditional uses depend on the cultural and religious knowledge of the plant. The ethnobotany of

Table 1. Folk traditional uses of *A. vera* reported from different regions of the world

Region	*Aloe* spp.	Part used	Folk medicinal use	Reference
Machakos	*A. vera*	Leaves	Hypoglycaemic effect	[46]
India	*Aloe barbadensis,* and *Aloe arborescens*	Gel and juice	Used against mild fever, diabetes, wound and burns, AIDS, liver infection, improve fertility and gastrointestinal disorder	[39]
Singapore		Leaves	Used to treat respiratory disorders, boost immunity, skin and hair care, mouth ulcer, control bleeding, itching and reduce muscle pain	[38]
Ghana		Leaves	Used against diabetes mellitus	[40]
Cameroon		Gel	Used for hair care, visage, sunblock, analgesic and anti-inflammatory agent	[41]
Tanzania	*A. vera*		Used against constipation, toothache, and skin complaints. Used to assist labor and induce abortion. Used to treat arthritis, pneumonia, gonorrhea, sleeping sickness, alleviate pain, inflammation, retarded growth of tongue, pneumonia, aid wound healing, syphilis, diseases in poultry and goats, testicular and scrotum cancer, chest pain, malaria, colds, reduce labor pain, cough, typhoid, ulcers, vomiting, swollen diaphragm, nosebleed, ringworm, skin diseases, diarrhea, anemia, backache, stomach ache, burns, gonorrhea	[42]
Yemen	*Aloe lavranosii*	Leaves	Used for wound healing, to treat malaria, abdominal pain, fever, gynaecological pain after childbirth intestinal infection, burns, intestinal colic, obesity, newborn infection, and intestinal infection,	[46]
	Aloe rubroviolacea			
	Aloe vacillans		Used against constipation, scabies, intestinal worm, low immunity by infant malaria, fever, low immunity, abdominal pain, body pain, and hair fall	
India			Used against dental problems	[44]
North Africa	*A. vera*		Used as a wound healer and taken against skin ulcers, mouth ulcers, herpes simplex, psoriasis, gastric ulcers, and age-related problems	[48]
Nigeria			Used against malaria	[43]
Kenya			Used to treat malaria, peptic ulcers, cough, wounds, and swelling of the diaphragm	[47]
Tanzania	*A. latcritia, A. secvndiflora,* and *A. duckcri*		Used to treat malaria, general stomach in humans, new castle disease in chicken, wounds, hernia, and typhoid	[45]

A. vera (Table 1) has been extensively studied among communities of different regions where it has been used as a traditional medicine against many diseases [37-45].

In one study, *A. vera* leaves gel was used to treat the hypoglycaemic effect (condition of blood when the glucose level is lower than the normal 70 mg/dL) because the gel controls diabetes [48]. The study by Siew *et al.* [38] in Singapore described local uses of *A. vera* against respiratory diseases and cancer by using its leaves as decoctions, eaten raw or in the form of juice. As per their data, it improves immunity and blood circulation and is a better remedy against acne, mouth ulcer, itching, and arthritic pain.

A study in India described two species of *A. vera* i.e, *Aloe barbadensis* miller and *Aloe arborescens,* whose gels and juices were used to treat mild fever, gastrointestinal disorders, AIDS, liver infection, muscle pain, and cancer [39]. Asase and Yohonu [40] proclaimed that in Ghana, *A. vera* leaves were used to treat diabetes mellitus when added 15 % of plants in food or taken in the form of decoctions. Fongnzossie *et al.* [41] reported that *A. vera* from the east of Cameroon was used to treat hair and skin problems. Moreover, *A. vera* was included in the top 10 cosmetic ingredients, and different phytochemical constituents present were used to treat inflammation, block UV rays for skin, and regenerate tissues.

From Tanzania, Amir *et al.* [42] found that malaria is frequently treated with *Aloe* leaves. Besides that, 11 species of *Aloe* were used as traditional medicine in Tanzania to treat diseases such as constipation, induction of abortion, toothache, skin complaints, assist labor, arthritis, pneumonia, gonorrhea, alleviation of pain, inflammation, retarded growth of tongue, pneumonia, wound healing, syphilis, diseases in poultry and goats, testicular and scrotum cancer, malaria, colds, ulcers, vomiting, swollen diaphragm, nosebleed, ringworm, skin diseases, reduce labor pain, anemia, backache, stomach ache, burns, gonorrhea, wounds, fever, pneumonia and skin diseases, edema, headache, chest pain, pneumonia, conjunctivitis were found to be treated with *Aloe* species.

Another study reported the ethnobotanical uses of *Aloe lavranosii, Aloe rubroviolacea, Aloe sabaea,* and *Aloe vacillans* against wounds, malaria, intestinal infection, fever, intestinal colic, obesity, gynaecological pain after childbirth, eye infection, eye pain, constipation, intestinal infection, eye allergy, face acne body pain, abdominal pain, newborn infection, intestinal worm, hair fall, and scabies [47]. Roy and Janbandhu [44] studied *A. vera* in Maharashtra and Saphale village of India and reported traditional uses of *A. vera* juice which was used to cure dental ailments and applied as a wound healing remedy. Oladeji *et al.* [43] in Nigeria documented that the decocted leaves of *A. vera* are used against malaria as it inhibits the growth of *the Plasmodium falciparum* strain.

Asif [48] reported that *A. vera* is a wound-healing remedy which has been utilized against skin ulcers, gastric ulcers, mouth ulcers, psoriasis, skin injury, herpes simplex virus, and age-related problems. Mutie *et al.* [49] reported different uses of *A. vera* plant gel from Kenya, where it was used against cough, swelling of the diaphragm, peptic ulcers, malaria, and wounds. A recent study in Tanzania showed that the *A. vera* leaves were used to treat malaria, general stomach problems, hernia, typhoid, wounds, ringworms in humans, and new castle disease in chickens [45]. *Aloe latcritia, Aloe secvndiflora,* and *Aloe duckcri* were the most frequently used species against diseases without any side effects whereas *A. secundiflora* was reported to cause death if taken in high doses.

As per the collected data of this review, the most commonly used parts of the *A. vera* plant as traditional medicine includes leaves (70 %), followed by the flowers (20 %), and roots (10 %) (Figure 4). It is found that despite of industrial use of *A. vera* it is also being used by people to treat almost more than ~20 different health problems.

3.3. Medicinal Uses of *A. vera*

The ancient *Aloe* plant has been proven to be medicinally significant by various research groups [1, 2, 4, 29- 31]. Manvitha and Bidya [39] reported various phytochemicals from the *Aloe* plant useful in medicine such as curing sunburn, tumor formation, and inflammation, maintaining cell growth and

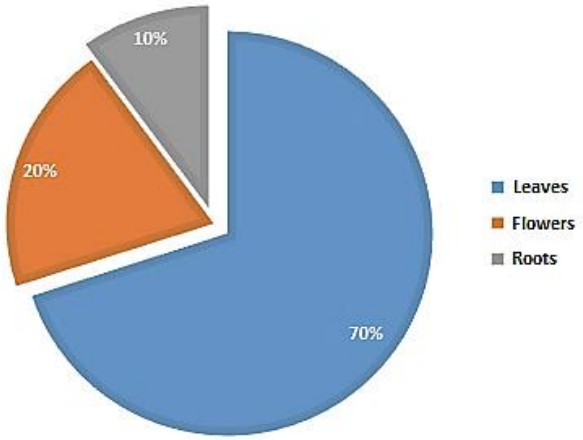


Fig. 4. Percentage of parts used in ethnobotantanical studies of *Aloe* species

development in the body, and maintaining sugar levels in diabetic patients. According to Grace *et al.* [29], natural products extracted from *A. vera* leaves such as carbohydrates, can be applied to treat skin diseases and a liquid matrix, can be used as an effective purgative or in veterinary medicines.

Gupta and Rarawt [30] reported the effectiveness of *A. vera* in reducing joint pain, muscle-related tendonitis, and other injuries. *Aloe* juice reduces stress (oxidation stress) and biological and physical alterations in the body. Tiwari and Upadhayay [31] reported that the latex in the leaves of *A. vera* contains anthraquinones which stimulate bowel contraction. Particularly, emodin found in latex act as an anticancer drug for lung, prostate, and skin cancers. Asif [48] studied the sugar-controlling ability of *A. vera* and showed improvement in carbohydrate metabolism, anti-hyperglycemic and anti-hyperchloremia effects in diabetic patients and hence maintaining the blood sugar, body fat, and body weight.

According to Danish *et al.* [1], *Aloe* contains high water content and can be used as a body moisturizer. Moreover, it cures thermal burn, stomach ailments, sunburn, and wounds caused by radiation and helps in cell growth, and provides relief against constipation. Sayar *et al.* [2] reported that *Aloe* possesses many anti-microbial and inflammatory properties and can cure gingivitis (a mild redness, irritation, and swelling in gums) and reduces bleeding and swelling of the gums.

Farid *et al.* [4] reported medicinal uses of the *A. vera* plant due to its exceptional features. It is considered a revolutionary weapon against various diseases in medical treatments and services. Traditionally *A. vera* has been used to cure dermal disorders but presently, more advanced advantageous therapeutic uses of this plant in bone marrow BM-MSC (mesenchymal stem cells) transplantation has been investigated which will help in curing liver complications.

3.4. Phytochemistry of *A. vera*

A lot of studies have reported diverse classes of phytochemicals (Table 2) in *Aloe* species using different methods [17, 25-27, 51-58]. Sathyaprabha *et al.* [51] found tannins, saponins, terpenoids, flavonoids, steroids, cardiac glycosides, phlobatannins, squalene oleic acid and dodecanoic acid in *A. vera*. In their study, some other phytochemicals including n-hexadecanoic acid, 1,2-benzenedicarboxylic acid, eugenol, phenol, 2,4-bis(1-phenylethyl) diisooctyl ester were also reported in *A. vera* using the GC-MS procedures.

Wintola and Afolayan [59] studied *Aloe ferox* leaf gel and quantified phenols (70.33 mg/g), flavanols (35.2 mg/g), proanthocyanidins (171.06 mg/g), alkaloid 60.9 mg/g) in the acetone extracts. In the ethanol extract, phenols (70.24 mg/g), flavanols (12.53 mg/g), proanthocyanidins (76.7 mg/g), and alkaloids (23.76 mg/g) were also quantified. Patel *et al.* [52] described the complexity of the *Aloe* gel extracted from the whole plant and analyzed using HPLC. The phytochemicals quantified were alkaloids, carbohydrates, tannins, steroids, triterpenoids, glycosides, flavonoids, and phenols.

Raphael *et al.* [33] and Kumar *et al.* [60] performed different tests for the analysis of the phytochemical constituents in the aqueously extracted gel from the leaf of *A. vera*. They reported tannins, phlorotannins, saponins, flavonoids, anthraquinones, terpenoids, steroids, and alkaloids in the extracts of *A. vera.*

Cardralli *et al.* [61] studied the *Aloe marlothii* and *Aloe melanacatha* to analyze the phytochemicals in the methanolic extracts of gel from leaves through Mass spectrophotometry. Aloeresins, *Aloe* resin A (843.4 g/100g), anthraquinones aloin (0.66- 4.96 g/100g), and hydroyaloins were found with a major part of gallic acid and polyphenols, flavonoids and flavonols as a phytochemical constituent in the studied *Aloe* species.

Dharajiya *et al.* [56] reported phytochemicals like saponins, alkaloids, tannins, cardiac glycoside, sterols, flavanoids, and phenol in the four different types of extracts (ethyl acetate, hexane, methanol, aqueous) from the fresh leave of *A. vera* by using thin layer chromatography.

Mahendiran *et al.* [62] showed the presence of active chemical compounds in the aqueous extract of *A. vera* contained flavonoids, phenolic compounds, alkaloids, gums and mucilages, carbohydrates, tannins, saponins, and terpenoids by using FT IR

Table 2. Phytochemistry of *Aloe* plants reported from different regions of the world

***Aloe* spp.**	**Part used**	**Extract used**	**Phytochemicals/ derivatives**	**Detection method**	**Reference**
A. vera	Leaves	Aqueous	Phlobatannins, tannins, saponins, steroids, flavonoids, terpenoids, and cardiac glycosides, dodecanoic acid, squalene oleic acid. 1,2-benzenedicarboxylic acid ester, Eugenol, n-hexadecanoic acid, and phenol, 2,4-bis (1-phenylethyl)	GC-MS	[51]
A. ferox	Leaves	Aqueous and ethanol	Phenols, flavanols, pro-anthocyanidins, and alkaloids	Spectrophotometry	[59]
A. vera	Leaves	Chloroform and aqueous	Alkaloids, tannins, flavonoids, terpenoids, carbohydrates	ND	[33]
	Leaves	Ethanol	Alkaloids, carbohydrates, tannins, steroids, triterpenoids, glycosides, flavonoids, phenols	HPLC	[52]
	Leaves	Ethanol	Chromone, anthraquinone, or anthrone derivatives	HPLC	[69]
	Leaves	Methanol	Glycosides, alkaloids, tannins, reducing sugars, steroids and phenolic compounds, terpenoids, flavonoids, and saponin glycosides	Colorimetric method	[60]
	Dried leaves	Aqueous	Phenolic acids, catechins, flavonoids, proanthocyanidins, quinones, tannins, coumarins, alkaloids, amines, betalains vitamins, nitrogen compounds, terpenoids, and carotenoids	FTIR	[70]
	Leaves	Aqueous, ethanol, and methanol	Alkaloids, glycosides, flavonoids, steroids, reducing sugar, terpenoids, carbohydrates, phenolic compounds, amino acids, tannins, and saponins	Colorimetric method	[22]

***Aloe* spp.**	**Part used**	**Extract used**	**Phytochemicals/ derivatives**	**Detection method**	**Reference**
Aloe marlothii and *Aloe melanacatha*	Leaves	Methanol	Aloeresins, *Aloe* resin A, anthraquinones aloin and hydroyaloins	Mass spectrophotometry	[61]
A. vera	Leaves	Hexane, methanol, ethyl acetate, and aqueous	Saponins, cardiac glycoside, tannins, sterols, flavonoids, alkaloids, and phenols	TLC	[56]
	Leaves	Aqueous	Alkaloids, flavonoids, carbohydrates and saponins, gums, mucilages, phenolic compounds, terpenoids, and tannins	FT IR spectroscopy	[62]
	Leaves	Methanol	Flavonoid compounds such as merictin, quercitrin, apiginin, quercetin, rhamentin, naringin, kampferol, rutin, and phenolic compounds (ferulic, caffic, p-coumaric, vanillic, cinnami, chlorogenic, ellagic acids)	HPLC technique	[63]
	Leaves and flowers	Methanol	Flavonoids, phenols, saponins, terpenoids, carbohydrates, sterols, alkaloids, proteins, tannins, and triterpenes	RP-HPLC	[50]
	Leaves	Aqueous	Chromone, anthraquinone, flavonoids, phenylpropanoids, and coumarins phenylpyrone, phenol derivatives, and phytosterols	ND	[64]
	Leaves	Aqueous and methanol	Alkaloids, carbohydrates, tannin, steroids, tri-terpenoids, glucose, and galactose	HPLC and TLC	[26]
	Leaves	Aqueous	Alkaloids, saponin, tannins, and glycosides	ND	[65]
A. vera	Leaf	Ethanol	*Aloe*-emodindiglucoside, (S-2'-oxo-4'- hydroxypentyl2 (ß-glucopyranosyl-oxymethyl), aloenin 10-hydroxyaloin B, chromone, aloveroside B, aloenin B, aloin B, isoaloerisin D, 10-hydroxyaloin A, aloin A, and aloenin-2`-pcoumaroyl ester	LC-MS	[67]
A. vera	Leaf	Ethanol and aqueous	Alkaloids, saponins, flavonoids, terpenoids, glycosides, and tannins	ND	[71]
Aloe vacillans	Flower	Methanol	Flavonoids, carbohydrates, phenolic compounds, protein, and sterols	TLC	[72]
A. vera			Saponin, carbohydrate, flavonoid, steroids, protein, and phenolic compounds		

**LC-MS= Liquid chromatography-mass spectrophotometry, ND= Not Defined, TLC= Thin layer chromatography, FTIR= Fourier transform infrared spectrometer, HPLC= High-performance liquid chromatography, RP-HPLC= Reverse phase high-performance liquid chromatography*

spectra. Faid *et al.* [63] showed the occurrence of different active complexes including flavonoid compounds such as merictin, quercitrin, apiginin, rutin, quercetin, rhamentin, naringin, kampferol; phenolic compounds (ferulic, caffic, p-coumaric, vanillic, cinnami, chlorogenic, ellagic acids) in the methanol extract of *A. vera* leaves using HPLC technique.

Kahramanoğlu *et al.* [64] reported six different classes of chromone, anthraquinone, flavonoids, phenylpropanoids, and coumarins phenylpyrone and phenol derivatives; and phytosterols in *A. vera.* According to Babu *et al.* [50], flavonoids, phenols, saponins, terpenoids, carbohydrates, sterols, alkaloids, proteins, tannins, and triterpenes were present in the methanolic extracts of *A. vera* leaves and flower evaluated with RP-HPLC.

Usman *et al.* [65] also reported phytochemicals in *A. vera* leaves skin and gel extracted by the aqueous extraction method. They confirmed the presence of alkaloids (31.067 g/100g), saponin (10.67 g/100 g), tannins (25.66 g/100 g), glycosides (0.060 g/100 g), and minerals like phosphate and magnesium in the *Aloe* plant.

In a study, it has been validated that the extracts of *Aloe* species possess complex phytochemical components [66]. Tannins, phenolics, alkaloids, flavonoids, and tri-terpenes were present in the ethanolic extract from the gel made from the leaves of *A. barbadensis*. The n-hexane fraction showed only phenols, alkaloids, and flavonoids. The petroleum ether showed tannins, phenolic, and flavonoids. The chloroform fraction showed tannins, phenolics, saponins, alkaloids, and flavonoids. Tanning agents, saponins, alkaloids, and flavonoids were visible in the dichloromethane fraction. Tannins, flavonoids, phenolics, saponins, alkaloids, and tri-terpenes could be seen in the acetone fraction. Additionally, tannin, phenolics, alkaloids, and flavonoids were present in the methanol fraction [66]. Hamdeni *et al.* [17] evaluated the phytochemistry of *A. vera* and reported the presence of phenols, flavonoids, flavonols, and condensed tannins using various techniques.

Bendjedid *et al.* [67] reported phytochemicals in the leaves of *A. vera* by extracting the gel using ethanol as a solvent using the LC-MS method. They verified the presence of isoaloerisin D, aloenin B, aloenin 10-hydroxyaloin B, aloenin 10-hydroxyaloin A, aloveroside B, aloin B, aloe-emodindiglucoside, (S-2'-oxo-4'-hydroxypentyl2 (ß-glucopyranosyl-oxymethylene) chromone, aloin A, and aloenin-2 in the ethanol extract of *A. vera* leaves gel. Arsene *et al.* [68] reported phytochemicals like aloesin, 8-o-methyl-7-hydroxyaloin B, chlorogenic acid (3-o-caffeoylquinic acid), trans-5-p-coumaroylquinic acid, luteolin 6,8-di-c-glucoside, cis-5-p-coumaroylquinic acid, trans-5-O-caffeoylquinic acid, 8-o-methyl-7-hydroxyaloin A, aloe-emodin-glucoside, aloinoside B, aloinoside A, 2′-O-feruloyaloesin, aloin B, aloe-emodin-glucoside, aloin A, 2′-p-methoxycoumaroylaloeresin B, 6′-malonylnataloin, *aloe*-emodin-glucoside, aloe-emodin" in the methanolic extract of *A. vera* leaves using HPLC–MS/MS technique.

Overall, for the phytochemistry of *A. vera,* more than 40 diverse groups of chemicals have been reported with potential effects against numerous health-related disorders, however, still there is a possibility to quantify more novel groups of compounds in *Aloe* species using advanced analytical techniques to unveil the medicinal significance of individual compound. Some of the essential phytochemical groups reported from *Aloe* species are illustrated in Figure 5.

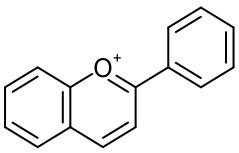
3.5. Biological Activities of A. vera

Some of the essential biological activities of *A. vera* extracts and chemical constituents are illustrated in Figure 6 and the details are discussed below.

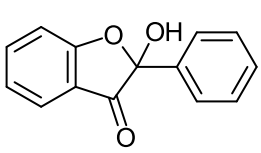
3.5.1. Antioxidant Activity

The activity of preventing oxidation (formation of free radicals), lowers the chances of tumors and various heart diseases. Antioxidants are a group of compounds that inhibits the free radicals and lipid oxidation reactions in the body [36, 77, 78]. A lot of studies have described the antioxidant activity of the *A. vera* plant [27, 57, 58, 70, 79-82] and the details of antioxidant activity conducted globally using various methods are given in Table 3.

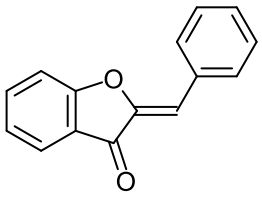
Vega-Gálvezv *et al.* [83] reported the effect of hydrostatic pressure on the antioxidant activity of *A. vera* gel ethanol extracts with DPPH (2,2′-diphenyl-



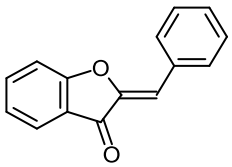
Anthocyanins



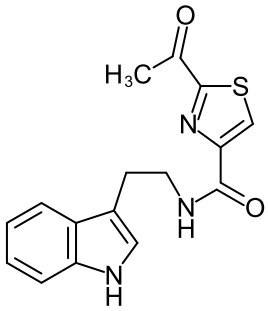
Auronols



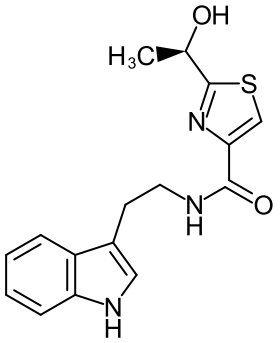
Aurones



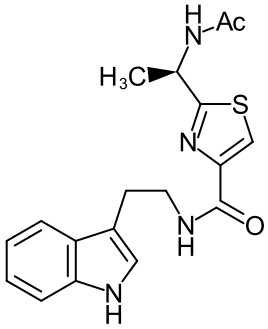
Cetophenones



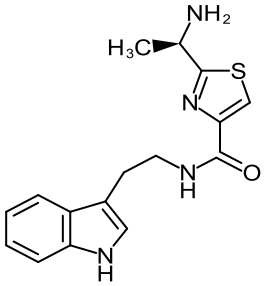
Bacillamide A



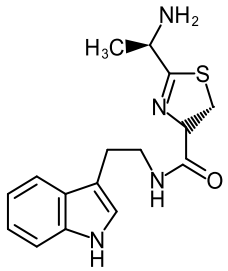
Bacillamide B



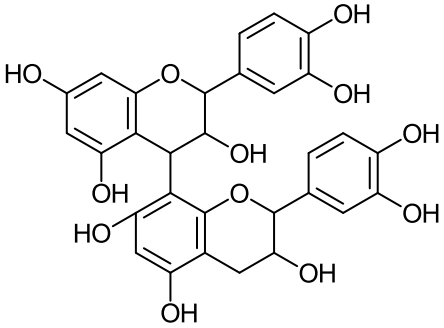
Bacillamide C



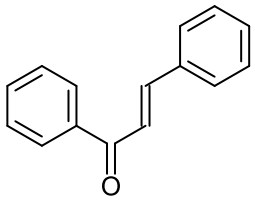
Bacillamide D



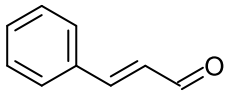
Bacillamide E aldehyde



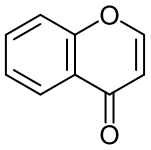
Biflavonoid procyanidin



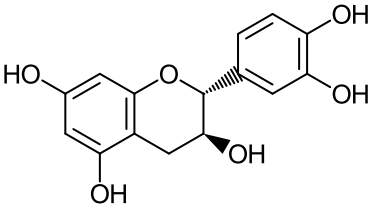
Chalcones



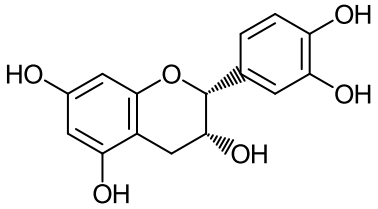
Cinnamic



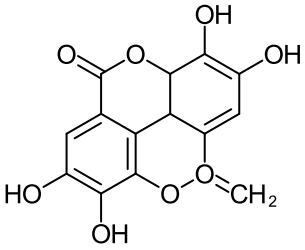
Chromones



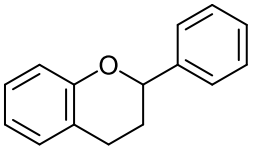
Catechin



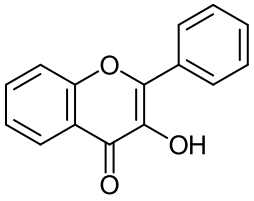
Epi-catechin



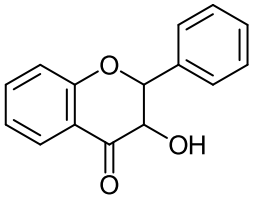
Ellagic acid



Flavonoids



Flavones



Flavon-3-ols

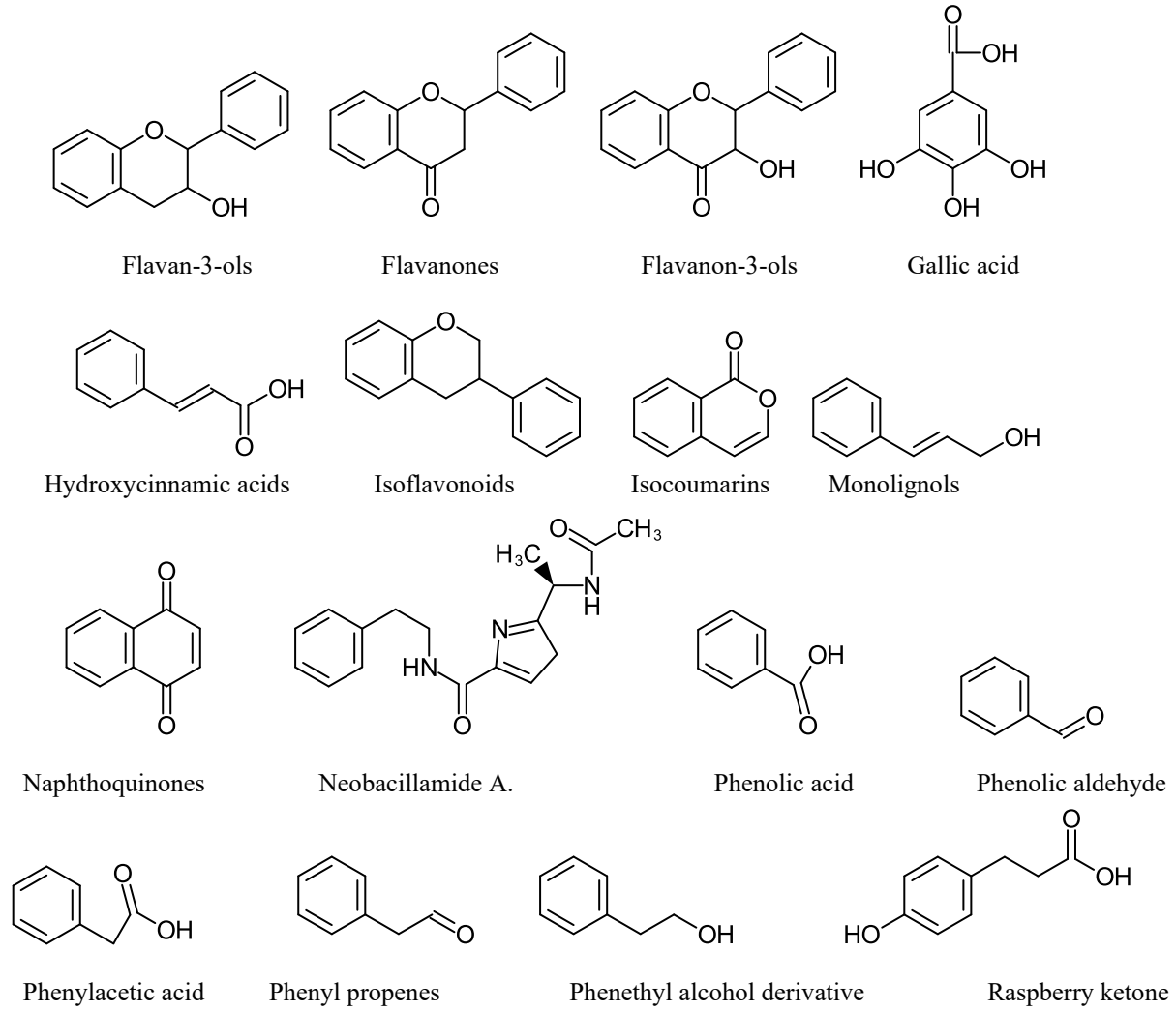


Fig. 5. Phytochemical groups and derivatives of flavonoid, phenols, tannins, and alkaloids from *Aloe* species. Adapted from: Multia *et al.* [73], Uivarosi *et al.* [74], Tsimogiannis *et al.* [75] and Vaca *et al.* [76]

1-picrylhydrazyl) activity and observed the activity range of 13.47 ± 0.72. Another study reported the potential antioxidant activity of methanol extract of *A. vera* gel was reported through hydrogen peroxide, DPPH, metal chelating, reducing power assay, and β carotene-linoleic assay [70]. Mahendiran *et al.* [62] documented the antioxidant activity with DPPH; hydrogen peroxide (H_2O_2), ABTS, superoxide radical, and hydroxyl radical scavenging assays in the aqueous extract of *A. vera* gel with a spectrophotometer. Lee *et al.* [84] verified the antioxidant activity of *A. vera* leaves in the methanol, acetonitrile, and aqueous extracts by ABTS and DPPH assays with positive results.

Benzidia *et al.* [79] found that the tannins in *A. vera* are the major phytochemicals that can inhibit free radicals with DPPH assay using three different fractions (F1, F2, and F3) of the acetone-aqueous extracts of *A. vera* gel. The reported ranges of antioxidant activity were 1.9, 3.74, 5.55, and 2.8 mg/ml correspondingly.

Quispe *et al.* [85] evaluated the ethanolic extract of *A. vera* gel, peels, roots, and flowers for antioxidant activity with DPPH, ABTS, and FRAP methods. The highest antioxidant activities of peel recorded were 2.43 mM ET/g MF in the DPPH assay, 34.32 mM ET/g MF in the ABTS assay, and 3.82 mM ET/g MF in the FRAP assay.

Tariq *et al.* [57] validated that the extracts from *A. vera* gel using methanol, ethanol, n-hexane, and aqueous solvents scavenged the free radicals and

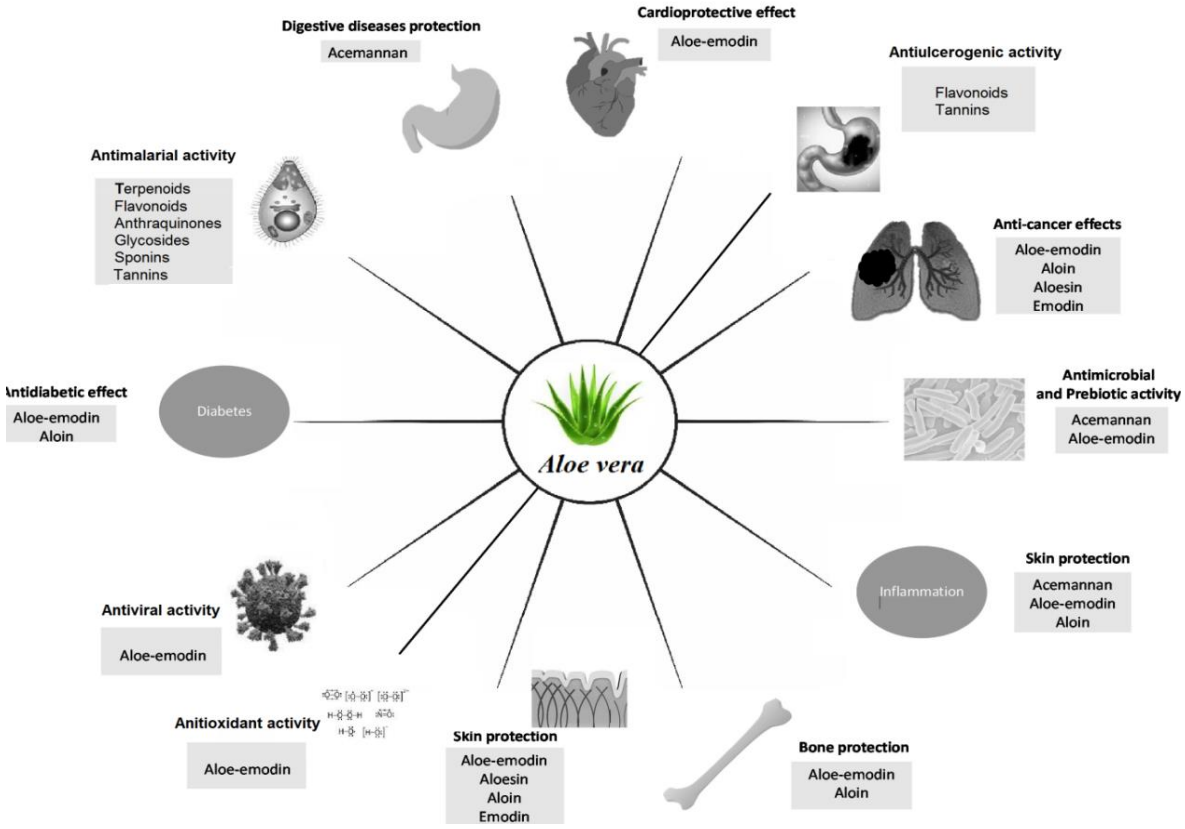


Fig. 6. Biological activities of some major chemical constituents of *A. vera*

Table 3. Anti-oxidant activity of *A. vera* extracts reported from different regions of the world

***Aloe* spp.**	**Part used**	**Extract used**	**Method of antioxidant activity**	**Reference**
	Leave gel	Ethanol	DPPH	[83]
	Leaves	Methanol	DPPH, metal chelating, and hydrogen peroxide	[70]
	Leaves gel	Acetone-aqueous	DPPH	[79]
	Leaves	Acetonitrile, aqueous, and methanol	ABTS and DPPH	[84]
	Peels, flowers, gel, and roots	Ethanol	ABTS, DPPH, and FRAP	[85]
A. vera	Leaves	Aqueous, ethanol, methanol, and n-hexane	DPPH and Ferric ion	[57]
	Leaves	ND	DPPH, FRAP, and TAC	[81]
	Flowers	ND	DPPH, ABTS, and FRAP	[80]
	Leave	Methanol	ABTS and DPPH	[86]
	Fresh latex of leaves	Lyophilized gel	Superoxide ions and hydrogen peroxide	[82]
	Leave gel	Acetone, aqueous, ethanol, and methanol,	DPPH	[58]
	Fresh leaves latex	Aqueous	DPPH	[27]

DPPH= 2,2- diphenyl-1-picrylhydrazyl, FRAP= Ferric ion reducing antioxidant power, ND= Not defined, TAC= Total antioxidant capacity, ABTS= 2,2'-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid

the highest activities of ethanol extract with DPPH (11.82 to 75.54 %) andFRAP (228.9 ± 39.1 µg/mg) were recorded. The overall reported range of total antioxidant activity of different fractions of *A. vera* leaf was 28.77 ± 9.36 to 150.4 ± 25.8 µg EQ/mg. Yadav *et al.* (2020) evaluated *A. vera* leaves with peel (AL-P) and *A. vera* without –peel (AL-WP) where the free radical scavenging activity by FRAP was 156.83 ± 0.659 and 192.66 ± 1.416 mg Fe (II)E/g DWE, DPPH was 48.12 % and 68.88 % at 1mg/mL, NO scavenging potential was 38.43 % and 54.55 % at 0.1 mg/mL, and TAC was 89.66 ± 0.577 and 108.66 ± 1.000 AAE/g DWE.

Martínez-Sánchez *et al.* [80] documented the antioxidant activity of organic extract of *A. vera* flowers at different maturity stages with DPPH, ABTS, and FRAP radicals scavenging assays. The obtained values of antioxidation of free radicals were 179.91 ± 8.16 mg TEA/100g, which were highest in the first immature stage of the flower and decreased as the bud opens. Kaparakou *et al.* [86] reported the antioxidant potency of methanol extract of *A. vera* leaves gel with ABTS and DPPH assays and the range of recorded activities were 1.64 to 9.21 µmol Trolox mL^{-1} and 0.73 to 5.14 µmol Trolox $mL^{-1)}$ respectively. Ojha *et al.* [82] validated that *A. vera* possesses active phytochemicals with a considerable reduction in the oxidation of compounds and a decrease in the concentration of free radical superoxide ions (42.49 + 0.92 %) and hydrogen peroxide (35.95 + 0.97 %) in male albino rate when dosed with 6 mg/kg of extraction of *Aloe* plant.

Aida *et al.* [58] assessed the antioxidant activity of phytochemicals extracted from the *A. vera* gel using various solvents such as methanol (AVGM), ethanol (AVGE), aqueous (AVGW), and acetone (AVGA). Their obtained antioxidant activity results were highest for the *A. vera* gel extracted with methanol (1.015 ± 0.003) followed by AVGE (0.574 ± 0.007), AVGW (0.3525 ± 0.030) and AVGA (0.223 ± 0.008) expressed in Trolox equivalent per gram (mMTE/g). Another inquiry evaluated the *in vitro* antioxidant activity of aqueous extracts of fresh latex of *A. vera* leaves with the DPPH method where the recorded activity was 21.900 ± 0.0594 mg/ml [27].

The data compiled in this review for the antioxidant activity of *A. vera* showed that overall, 9 different organic extracts from various parts of the *A. vera* plant were proven to have potential antioxidant activities and DPPH and FRAP methods were the commonly used methods for the evaluation of antioxidant activity of *A. vera*. Nevertheless, it is proposed that some biological models to evaluate the activities of *A. vera* extracts on lipid peroxidation and activities on different enzymes with oxidizing potentials including adenine dinucleotide phosphate oxidase (NOX), nitric oxide synthase (NOS), monoxides and nicotinic and xanthine oxidoreductase (XO) etc should be explored.

3.5.2. Anti-inflammatory Activity

The activity of reducing redness, swelling, and pain in living organisms is termed anti-inflammatory activity. In many studies, the phytochemicals extracted from *A. vera* were reported (Table 4) to be active in reducing inflammatory effects [87-96]. In a study, Devaraj and Karpagam [88] tested the aqueous extract of *A. vera* leaf for its ability to reduce inflammation. They employed albino Wistar rats as substrates and varied extract concentrations and observed the effects of carrageenan and formaldehyde on rat paw edema to unveil the anti-inflammatory efficacy of *A. vera*. It was confirmed that the leaf extracts at a dosage of 600 mg/kg reduce the development of edoema brought on by carrageenan and formaldehyde with no mortality and gives anti-inflammatory effects.

Vijayalakshmi *et al.* [90] performed MMP inhibition tests on peripheral blood mononuclear cells (PBMC) isolated from heparinized venous blood using the ficoll diatrizoate gradient centrifugation method. *A. vera* aqueous extract was proven to inhibit the MMP-9 in a dose-dependent manner with gelatin zymography and RT-PCR procedures. Williams *et al.* [87] and Huseini *et al.* [89] corroborated that *A. vera* chromones inhibit the cyclooxygenase pathway and reduce the production from arachidonic acid of prostaglandin E2 in rats with carrageenin-induced paw edema. Whereas, mice with Croton oil-induced edema show a significant decrease in inflammation due to

Table 4. Reported anti-inflammatory activity of *A. vera* in different models

Inflammatory condition	Model/cells used	Extract/part used	Result	Reference
Carrageenan and formaldehyde-induced rat paw edema	Albino Wistar rats	Leaves aqueous extract	Decrease in the formation of edema	[88]
MMP inhibition studies on PBMC	Peripheral blood mononuclear cells (PBMC)	Leaves	Inhibition in MMP-9	[90]
HMA ointment on epidermal cell	Albino Wister rats	Leaves	Reduce edema	[91]
Immune-modulation of inflammatory arthritis condition	RBC membrane	Gel homogenate	Prevent the tissue damage & immune-modulation of inflammatory arthritis condition	[93]
Colitis	Albino Wistar rats	Leaves gel	Reduction in inflammatory agents in colonic tissue	[92]
Cytokine during an immune response	Human gingival fibroblasts	Leaves	Inhibition of TNF-α	[96]

TNF-α= Tumor necrosis factor, PBMC= Peripheral blood mononuclear cells, MMP-9= Matrix metallopeptida

β-sitosterol, campesterol, lupeol, and cholesterol in the *A. vera* gel.

Farzadinia *et al.* [91] documented the antiinflammatory effect of *A. vera* using it as honey milk *A. vera* ointment on the burning part of male albino rats. The burn was induced artificially and treated with HMA ointment containing dried *Aloe* gel powder, honey, and dry milk powder displayed observable anti-inflammatory effects, hence reducing edema by drying out, granulation, and closing of the wound edges, and increase in catalase activity. It also lowered the amount of collagen fiber and the hardness of the skin and increased the formation of connective tissue.

Paul *et al.* [93] reported the anti-inflammatory activity of *A. vera* gel homogenate for immune modulation of inflammatory arthritis conditions. It was validated that hypotonicity-induced (74.89 ± 1.26 %) and heat-induced (20.86 ± 0.77 %) RBC membrane lyses can be inhibited by the use of *A. vera* gel homogenate at a concentration of 1000 µg/ml. The same concentration can be used for the *in vitro* inhibition of protein denaturation (39.35 ± 4.25 %). The effect of *A. vera* was also assessed *in vivo*, and the results showed a reduction in tissue damage thus maintaining the normal functioning of TNF-α and Cox-2 gene expressions for the immune-modulation of inflammatory arthritis condition.

Naini *et al.* [92] investigated the anti-inflammatory activity of *A. vera* on experimental colitis (Inflammatory disease) in Wistar rats. Trinitrobenzenesulfonic acid (TNBS) was used for the induction of experimental colitis in rats and the extracts of *A. vera* were administered to the rats orally or rectally. Tumor necrosis factor, interleukin-6, and nitric oxide levels were higher in colonic tissue from rats with experimental colitis, and malondialdehyde and myeloperoxidase concentrations were also higher. *A. vera* treatment had a healing impact with an anti-inflammatory effect in rats.

Villarreal *et al.* [96] confirmed the anti-inflammatory effect of *A. vera* with ELISA assays (enzyme-linked immunosorbent assay) and reported an increase in the expression of cytokine and IL-1β (released during immune-response) levels in human gingival fibroblasts. The *A. vera* gel extract was able to decrease cyclooxygenase-2, 5-lipoxygenase biosynthesis, inducible nitric oxide synthase (iNOS), and TNF-α concentration hence proving a major role in lowering inflammation.

Overall, 9 organic extracts from *A. vera* were used *in vitro* and *in vivo*, showing anti-inflammatory action by inhibiting enzymes and factors such as TNF-α in humans. It is further proposed that extracts from *A. vera* should be evaluated against

more factors other than TNF-α and inflammation-causing pathways at the cellular level should be targeted to better explore the anti-inflammatory potentials of this plant.

3.5.3. Anticancer Activity

The activity of controlling the proliferation of cells or reduction of cancer-developing cells by *A. vera* extracts was reported by various authors [80-100] as shown in Table 5. Jose *et al.* [94] performed an MTT test to assess how well flavonoids derived from *A. vera, Mimosa pudica,* and *Phyllanthus niruri* inhibits the growth of the human breast carcinoma cell line (MCF-7) to evaluate their potent effects. The extracts of *A. vera* (IC_{50} = 54.970.36 g/ml), *P. niruri* (IC_{50} = 35.520.50g/ml), and *M. pudica* (IC_{50}= 35.520.50 g/ml) showed the highest inhibition against the tested cells. It was proposed that the extracts could be utilized effectively to treat cancer. In another study, the *in vitro* anticancer activities of *Calligonum comosum* and *A. vera* extracts against HepG2 cells by MTT test, the cells' viability was evaluated [96]. The cytotoxicity against HepG2 cells was individually boosted by the extracts in a time and dose-dependent manner. It was found that the extracts could, at least in part, via modulating apoptosis have anti-hepatocarcinogeniceffects.

Mahendiran *et al.* [62] reported the anti-cancer activity of aqueous extracts of *A. vera* gel against three cancerous cell lines such as cervical (HeLa), human breast adenocarcinoma (MCF-7) and epithelioma (Hep-2), and one normal human dermal fibroblast (NHDF) cell lines with MTT assay and found positive results.

Karpagam *et al.* [97] investigated human cancer cell lines HeLa, HepG2 (liver cancer cell line), and A549 were used to test the anticancer properties of the ethanolic leaf extract of *A. vera* using the 3-(4, 5-dimethylthiazole-2yl)-2, 5-diphenyl tetrazolium bromide assay (human lung adenocarcinoma epithelial cell line). Their outcomes confirmed the presence of several active compounds with anticancer potential in the ethanolic leaf extract of *A. vera*. In another study, *A. vera* gel was used as a capping and reducing agent for the creation of Ag@TiO2 nanoparticles. It was also found that the nanoparticles containing *A. vera* gel have activity against lung cancer cell lines (A549). After being administered systemically *in vitro*, the Ag@TiO2 NPs produced a significant amount of reactive oxygen species (ROS), which completely suppressed the development of cancer cells [98].

Mohamed and Masry [99] reported that *A. vera* gel extract and sunlight were used to synthesize silver nanoparticles with anti-cancer activity. After 72 hours of incubation with AgNPs-AV *in vitro*, a rapid fall in breast cancer cells was observed. This greenly synthesized nano-formulation offers a lot of promise to be studied from a variety of angles.

Ahmad *et al.* [101] evaluated the methanolic extracts of the healing herbs *Aloe castellorum* and *Aloe pseudorubroviolacea* to reduce the human colon cancer, cell line (HCT-116). The methanolic extract of *A. castellorum* has more cytotoxic activity than *A. pseudorubroviolacea* against HCT-116, which was confirmed with the GC-MS technique. Murugesan *et al.* [100] developed a potent and efficient anti-carcinogenic gel material that represents a cutting-edge medicine delivery technique. The cytotoxic tests revealed that a loaded phospholipid *A. vera* was biocompatible and was

Table 5. Anticancer activity of *A. vera* against various cancer cell lines

Cancer type	Study model/ assay	Cell line	Reference
Breast cancer	MTT assay	MCF-7	[94]
Liver cancer	*In vitro*	HepG2	[95]
Breast adenocarcinoma, cervical and epithelioma cancer	MTT assay	MCF-7, HeLa and Hep-2	[62]
Liver cancer	*In vitro*	HepG2, HeL, and A549	[97]
Blood cancer	Gas chromatography	HCT-116	[101]
Lung cancer	*In vitro*	A549	[98]
		MCF-7	[100]

MTT= 3-(4, 5-dimethyl thiazolyl-2)-2, 5-diphenyltetrazolium bromide

effective against the MCF-7 cancer cell line. The outcomes demonstrated that phytosome carriers have the potential to enhance *A. vera* oral delivery by opening the door for its use in the treatment of cancer. Overall, for the anticancer activity of *A. vera*, the data reviewed here substantiated that *A. vera* possesses a broad-spectrum anticancer activity by preventing the uncontrollable division of specific types of cancer cells like HCT-116, HepG2, HeLa, A549, MCF-7 and hence reduces the chances of blood cancer/leukaemia, liver cancer, breast and lung cancer. However, it is believed that the bioactive compounds present in different extracts of *A. vera* should be isolated and screened extensively against further cancer types, and interpreting the anticancer mechanism of action of *A. vera* extracts at the molecular level could be a possible research line to be explored for *Aloe* based cancer therapies.

3.5.4. Antidiabetic Activity

The high sugar level in the blood of the organisms causes diabetes and different health effects and high cholesterol in the body can be controlled by the extract of the *A. vera* plant. A lot of studies have reported the activity of *A. vera* extracts against diabetes [50-107] and the details are given in Table 6.

Mohamed [102] conducted an experiment on diabetic and control rats to delineate the consequence of *A. vera* gel extract in the control of diabetes. Experimentation was conducted on 4 groups of forty rats, and the result showed that oral administration of *A. vera* gel extract can reduce serum glucose, total cholesterol, and triacylglycerols. The hypoglycemic effect of *A. vera* gel extract may be due to the occurrence of hypoglycemic trace elements such as Cr, Zn, and Mn which potentiate insulin action. *A. vera* gel extract possesses an antidiabetic effect due to an increase in serum cholesterol and tri-acylglycerols.

In another inquiry, Babu *et al.* [50] studied the flower and epidermis extract of *A. vera* that possesses amylase and alpha-glucosidase with anti-diabetic properties. The flower and gel have phytoconstituents like proteins, phytosterols, carbohydrates, and mineral components making it suitable for use as an anti-diabetic drug. Their outcomes also validated that the gel and flower of *A. vera* are more anti-diabetic than the epidermis of leaves. Muñiz-Ramirez *et al.* [103] applied different assays for the evaluation and determination of methanol extract of *A. vera* for the prevention of diabetes caused by AGEs (Advanced glycation end products) formation. AVM was found to be effective

Table 6. Activity of *A. vera* plant extracts on different types of diabetes

Type of diabetes	Part used	Extract used	Concentration of extract	Results	Reference
All types of diabetes	Whole plant	Gel extract	ND	Decreased serum glucose, total cholesterol, and triacylglycerols	[102]
	Flower			Regulated blood glucose levels	[50]
AGEs induced diabetes	Whole plant	Methanol extract (AVM)	5 mg/ml	Inhibition of diabetes caused by AGEs	[103]
Streptozotocin induced diabetes	Leaves	Gel extract	ND	Restoration of the FPG and insulin levels	[104]
Type 2 diabetes				Normalized hyperglycemic conditions	[105]
Streptozotocin-induced diabetes mellitus	Whole plant			Anti-hyperglycemic in STZ-induced diabetic models	[107]
Diabetes	Leaves			Controlled blood glucose homeostasis	[108]
All types of diabetes	Whole plant	*A. vera* juice		Controlled blood sugar level	[106]

FPG= Fasting plasma glucose, ND= Not defined

against the formation of AGEs as well as carbonyl protein, CML, and fructosamine.

The best results were obtained at the concentration of 5 mg/ml of AVM. AVM also worked for the inhibition of enzymes like α-amylase and α-glucosidase. Whereas thiol group content was found to be increased with the period within 4 weeks. It was found that AVM is effective against AGEs and inhibits the formation of postprandial glucose, so reducing the chances of diabetes associated with AGE.

Babu *et al.* [104] observed the mechanisms involved in the mitigation of streptozotocin-induced diabetes in rats by using *A. vera* gel extract through the proteomics approach. They validated that *A. vera* extract (AVE) alleviates diabetes by regulating the pathways involved in the development of diabetes. In AVE, both the components carbohydrate fraction and polypeptide fraction (CF & PPF) synergistically work to regulate the insulin fraction. Hasan and Abdulla [105] studied the gel extracted from *A. vera* with polysaccharide which regulates the blood sugar level. The gel was prepared by dissolving 7.5 g *Aloe* gel in 100 ml distilled water.

Their outcomes confirmed that the hyperglycaemic state could be normalized by the treatment of *A. vera* extracts as they possess phytochemicals, minerals, and many primary metabolites that regulate blood glucose levels.

Haghani *et al.* [107] assessed the chemical properties of *A. vera* and its effects on Streptozotocin-induced diabetes mellitus. *A. vera* can control blood glucose, recover plasma insulin, decrease oxidative stress, and stimulate the production of collagen and elastin fibers and hence leading to the healing effect of STZ-induced diabetic ulcers. *A. vera* inhibits oxidative stress due to its properties of inducing antioxidant enzymes and glutathione levels. *Aloe vera* is also capable of reducing the manufacture of inflammatory mediators, thus causing the suppression of inflammatory responses in STZ-induced diabetic models.

Deora and Venkatraman [108] evaluated the active components of *A. vera,* which possess hypoglycaemic and hypolipidemic activities beneficial to cure diabetes. It not only reduces the hypoglycaemic and hypolipidemic activities beneficial to cure diabetes. It not only reduces the chances of diabetes but also maintains a healthy life by reducing the adverse impact of diabetes on the liver. It was found that the oral administration of *A. vera* gel extracts improves blood glucose homeostasis and imparts variations in glucose-lowering effects.

Ankita *et al.* [106] confirmed that by taking *Aloe* juice, a person can maintain sugar levels in the blood due to the presence of polysaccharides that can control blood sugar and cholesterol levels. The possession of polysaccharides makes it suitable for the treatment of all diabetic situations as it exhibits hyperglycemia properties and increases the glycerin level.

Overall, in this review, the collected data on the antidiabetic activity of *A. vera* validated that the extracts from different parts of the *Aloe* plant help in controlling blood glucose and restoring insulin levels. It is believed that the isolation of novel compounds with the exploration of their antidiabetic mechanisms of action at the cellular level from *A. vera* extracts is a promising research mark for *Aloe-based* diabetes treatment.

3.5.5. Antimicrobial Activity

The inhibition of the growth of microbes like bacteria, fungi, viruses, etc., or prevention of the formation of microbial colonies and their destruction can be controlled by the phytochemicals obtained from the extracts of various plant species [109, 110]. There is a huge literature available with reported antimicrobial activities of *A. vera* on various pathogenic microbial strains [22, 111-118] (Table 7).

Stanley *et al.* [111] reported the antimicrobial activities of ethanolic, methanolic, and aqueous extracts of *A. vera* against *Escherichia coli, Staphylococcus aureus,* and *Candida albican* through the agar diffusion technique. Gentamycin was considered a positive and dimethyl sulfur oxide (DMSO) was considered a negative control. Of all, methanol extract was best with maximum inhibitory effects followed by ethanolic and aqueous extracts of *A. vera*.

Table 7. Antimicrobial activity of *A. vera* against different pathogenic microbial strains

Aloe spp.	Part used	Extraction method	Microorganism tested	Result	Reference
A. vera	Gel from leaves	Ethanolic, methanoic, aqueous extraction	*E. coli, S. Aureus* and *Candida*	+	[111]
		Aqueous, ethanol and methanol	*A. niger* and *Rhizopus*	+	[22]
A. vera, A. volkensi, A. secundriflora		Methanolic extraction	*S. aureus, Bacillus subtilis, E. coli* and *Erwinia carotovora*	+	[112]
A. barbadensis		Methanolic, ethanolc and acetone extraction	*Bacillus* and *Staphylococcus*	+	[113]
		ND	*S. Aureus, E. coli, Pseudomonas* and *Enterobacter*	+	[114]
		Methanol	*E. coli, S. aureus, P. aeruginosa* and *Yeast*	*E. coli* showed positive results while *S. Aureus, P. Aeruginosa, yeast* showed negative results	[116]
		Ethanol with other fractions	*Salmonella tylhi, E. coli* and *Aeromonas*	*Aeromonas* showed positive results while *S. tylhi* and *E. coli* showed negative results	[115]
A. vera		Methanol	*H. pylori*	+	[118]
		Ethanol	*S. aureus, Streptococcus, E. coli* and *Salmonella*	Inhibits only gram-positive bacteria	[117]

+ = Represents positive result, – = Represents negative result, E. coli= Escherichia. coli, S. aureus= Staphylococcus aureus, P. aeruginosa= Pseudomonas aeruginosa, H. pylori= Helicobacter pylori

Dharajiya *et al.* [56] reported the anti-microbial activity of *Aloe* gel against strains of various types of bacteria and fungi such as *Bacillus cereus, Serratia marcescens, Pseudomonas aeruginosa, Aspergillus flavus, E. coli, Aspergillus oryzae, Trichoderma viride, Penicillium chrysogenum* with thin layer biography method. Their outcomes showed that the *A. vera* gel has inhibitory effects on *S. marcescens* and three species of *Aspergillus*.

Malik *et al.* [22] documented methanol and ethanol extracts of *A. vera* with significant inhibitory actions as compared to the aqueous extracts which showed lower inhibition against *A. niger* and *Rhizopus.* Their outcomes recommended *A. vera* leaves extracts using ethanol and methanol solvents as better antifungal agents.

Waithaka *et al.* [112] revealed antimicrobial properties of the methanolic extract of leaves of *A. vera*, *A. volkensii,* and *A. secundriflora*. Mueller Hinton agar and potato dextrose agar for bacteria and fungi was used respectively containing *S. aureus, Erwinia carotovora, Klebsiella pneumonia, E. coli, Bacillus subtilis, Candida albicans, Fusarium oxysporum*.

These extracts showed positive results with various zone of inhibition on the tested strains. Gorsi *et al.* [113] showed that gel from *Aloe barbendis* leaves extracted with ethanol, methanol, and acetone inhibits the growth of bacterial strains

like *Salmonella typhi, E. coli, B. subtilis,* and *S. aureus*. Among the tested extracts, ethanol extract was found to be more effective with 20.33 and 18.63 zone of inhibition for *Bacillus* and *Staphylococcus*, whereas, acetone extraction was found to be better than aqueous extract with 13.60 mm inhibition zone for *S. typhi.*

Anju *et al.* [114] proclaimed that silver nanoparticles made from *A. vera* can be used to inhibit microbial growth. After extraction of *A. vera* gel naturally containing acemannan, the formation of silver nanoparticles (AgNPs) was analyzed through a spectrophotometer with 400 nm absorption. Their results showed that AgNP has anti-microbial properties against both gram-positive (*S. aureus*) and gram-negative (*E. coli, P. aeruginosa,* and *Enterobacter*) bacteria. These activities were analyzed through the disk diffusion technique.

Ahmed *et al.* [116] evaluated the inhibitory effects of *A. vera* gel on different biological agents like bacteria, yeast, and fungi strains. For this purpose, the gel was extracted from leaves of *Aloe barbadensis miller* and after blending it properly powder was formed by the cold maceration method. Using the solid medium method anti-microbial activities were analyzed through nystatin and gentamycin tests for fungi and bacteria respectively. *E. coli* showed inhibition zones whereas *S. aureus* and *P. aeruginosa* showed resistance against *A. vera* gel extract. On the other hand, lower activity was observed against yeast i.e., *Candida albicans.* Bajalanlou and Pakbin [115] obtained the transparent gel from *A. barbadensis* using methanol, aqueous, dichloromethane (DSM), acetone, chloroform, n-hexane, and PET solvents and analyzed the antimicrobial activity through the disk diffusion method. The ethanol extracts displayed the overall highest inhibitory activity against *Aeromonas* with a 9.6 mm inhibition zone. The *n*-hexane extract showed the least anti-bacterial activity with 0.12 mm inhibition zones. Other extracts such as PET, chloroform, DCM, and acetone showed 0.25 to 2 mm inhibition zones whereas methanol and aqueous extracts showed 0.93 to 3.75 mm inhibition zones. *Aeromonas* showed resistance in PET fraction and *Pseudomonas* and *Chryseobacterium meningospticum* were resistant to the aqueous fraction of *A. barbadensis.* Yahya *et al.* [118] studied the methanol extracts of *A. vera* and found effective results against *Helicobacter pylori* which causes gastric infection in humans.

Vadiati Saberi *et al.* [117] evaluated the effect of *A. vera* gel on the growth of gram-positive and gram-negative bacteria. The acetoacetate compound extracted from the ethanol extract of the gel repressed growth of gram-positive bacteria as compared to gram-negative bacteria.

Overall, the collected data here for the antimicrobial activity of *Aloe* species authenticated that the *Aloe* extracts are effective against almost 12 bacterial and 7 fungal species. It is still believed that the elucidation of proper mechanisms of action of *Aloe* extracts against pathogenic microbes is a possible research line for advanced *Aloe-based* natural antimicrobial therapies.

3.6. *Aloe*-Based Food and Cosmeceutical Products

There is extensive data available on the uses of *A. vera* in cosmetic items such as night creams, soaps, cleansers, shampoos, suntan creams, and lotions (Table 8). In a study, Pounikar *et al.* [119] prepared a cosmetic herbal hydrogel using the inner part of *A. vera* leaf by mixing other substances through heating. Mainly, the mixing of acacia, hydroxyl propyl methyl cellulose (HPMC), and carbopol 934 in a ratio of 1:1:2 done to make a product to increase moisture content in the skin, making skin clear by increasing transparency, and smoothness and reducing skin microbial growth. According to Estrada-Caslillon *et al.* [120], *A. vera* leaves are used in cosmetics to make shampoo, hair dye, and hair health products and are reported to be used in rituals. Rajkumar *et al.* [121] proclaimed the use of *A. vera* gel in goat meat nuggets to enhance the quality of food items in India. Different quantities of 0 %, 2.5 %, and 5 % gel were applied to nuggets before storing them in the refrigerator for 9 days. As a result, 2.5 % of *A. vera* gel in nuggets was said to be preferred because it does not affect the yield and does not decrease the protein content like 5 % of *A. vera*. It enhanced the quality of the item by improving its texture and nutritive value.

Mahmoudi *et al.* [122] developed yogurt by adding *A. vera* extract and *Lactobacillus casei* in cow milk and then stored it for different days like

Table 8. Details of *Aloe*-based food and cosmeceutical products reported globally

Region	*Aloe* spp.	Part used	Food/ cosmetic product	Nutritional or cosmaceutical uses	Reference
India	*A. vera*	Leaves gel	Herbal hydrogel	Moisturise skin, make skin smooth and transparent and inhibit microbial growth	[131]
			Applied in meat nuggets	Increase nutritional parameters	[121]
Iran			Yoghurt	Provide better physiochemical and microbiological properties	[122]
India	*A. barbadensis*		Herbal ice cream	Increase storage capacity with improved taste	[125]
Indonesia	*A. chinensis baker*		Lotion	Reduce irritation, prevent dryness and clear skin	[123]
	A. vera		Face mask	Makes the skin clear, soft and clean and increases vitamin C content	[124]
India			Edible coating on fruits	Increase the shelf life of fruit, delay oxidative browning, increase storage capacity with high total soluble solids	[126]
			Khoa burfi	Increase moisture content with desirable taste for eating	[127]
	A. barbadensis		Peel off mask	Clear skin, UV protection and healing potentials	[3]
			Kulfi	A film of *A. vera* increase thickness and density and reduce water vapor transmission and increases taste	[129]
Pakistan	*A. vera*	Juice	Cookies	High quantity of crude fiber, proteins, *β*-carotene, and low fat with healthy food	[128]
		Leaves gel	Coating on strawberry fruit	Strawberry fruit coated with *A. vera* gel prolongs post-harvest life and maintains nutritional quality	[130]
India	*A. barbadensis miller*	Leaves gel	Mouthwash	Antiplaque and antimicrobial activity	[125]
		Leaves extract	Coating on pink guava	Retention of *β*-carotene increases the antioxidant capacity of guava	[132]

1, 3, 5, 7, and 10 at 4 ^{0}C. The results showed that the concentration of lactobacillus was less in yogurt with *A. vera* extract and did not affect the quality of yogurt when added with 2.5 % concentration rather it improved the physiochemical, microbiological, and sensory properties of yogurt.

Hendrawati *et al.* [123] developed an *A. vera* gel extract mask for improving skin quality. The composition of this *A. vera* mask was polyvinyl Pirolidon K30, poly vinyl chloride, methylparaben, propylparaben, BHT, and water in 7.55, 1.51, 0.10, 0.12 and 90.61 % respectively. The preferred amount of *A. vera* gel extract was 0.15 % for better results and was used to make the skin soft, enhance color and increase vitamin C content.

The same authors Hendrawati *et al.* [124] developed *A. vera* gel extract-based lotion by using gel from leaves. The ingredients in the lotion were heated and then melted by adding different concentrations of gel extract like 33.33 % in 50 ml, 50 % in 775 ml 66.67 % in 100 ml and 100 % in 150 ml. The best concentration with positive effects was said to be 66.67 % in 100 ml. It provided a lot of therapeutic effects on the skin like reducing

irritation, retaining moisture, preventing dryness, and making it clear.

Verma *et al.* [125] prepared herbal ice cream using *A. vera* and mint. *A. Barbandis* species was used to extract *A. vera* juice. The best concentration of ingredients was 10 % fat, 15 % sugar, 0.5 % stabilizer, and emulsifier, 20 % *A. vera* juice, and 0.5 % mint extract. After blending all ingredients, pasteurizing, homogenizing, and cooling, 10 % of *A. vera* juice and mint was added and then stored. The product was analyzed and total solids, acidity, protein, carbohydrates, and ash were determined showing the best chemical characteristics. The product showed good storage capacity and taste and due to *A. vera* juice, it increases blood circulation, and detoxification by improving the digestive system.

Another edible coating of *A. vera* gel was done by Kumar and Bhatnagar [126] on oranges, grapes, sweet cherries, and papaya. The gel coating increased antifungal activity and shelf life along and reduced moisture content and the appearance of brown color due to oxidation. It maintained the time of maturation of fruits by increasing storage capacity. In oranges, it caused no weight loss and increased acidity and total soluble solids.

Chaudhary *et al.* [127] developed Khoa Burfi by adding *A. vera* juice at different concentrations (5, 10, 15 and 20 %). The optimum concentration was found to be 15 % after analyzing moisture, pH, color, and texture. The pH and moisture content were increased due to *A. vera* juice and adhesiveness and hardness were decreased. The addition of *A. vera* showed improved shelf life of burfi.

Masood *et al.* [128] developed *A. vera-based* cookies where the ingredients of the cookies were wheat, butter, sugar, salt, baking soda, and water with 10, 20, and 30 % of *A. vera*. They found improved moisture content, crude fiber, protein content, beta carotene, and reduced fat. Other properties of the product like flavor, texture, and color were also analyzed and the product was found to be very effective for health.

Asthana *et al.* [3] developed a peel-off mask for skin care as a product using gel from *A. Barbadensis* leaves. This mask was prepared by mixing the gel with montmorillonite (MMT) clay which is helpful in external detoxification and polyvinyl chloride (PVC) which is environment-friendly and provides homogeneity to the skin. This product was useful in protecting skin from UV radiation with healing properties. Moreover, it has antiseptic properties and an antiaging effect for batter and shiny skin.

In India, Mahajan *et al.* [129] prepared *A. vera*-based edible film for frozen dairy products using kulfi as a modal product. A standardized file was prepared by mixing carrageenan, glycerol, and gel from *A. barbadensis*. The film reduced lipid oxidation, water vapor transmission, microbial growth, and antioxidant capacity with an increase in storage capacity, thickness, and density.

Haider *et al.* [130] studied the effects of *A. vera* gel coating on the shelf life and nutritional quality of strawberry fruit. They claimed that the use of 15 % *A. vera* gel as a coating material in strawberries prolongs post-harvest life and maintains nutritional quality for a considerable duration.

Pattnaik *et al.* [131] developed a mouthwash using *A. vera* gel which showed antiplaque and antibacterial activities. *A. vera* gel can also be used to prevent the deposition on teeth where bacteria can proliferate which shows its antiplaque activity for healthy teeth.

According to Otalora *et al.* [132], a mucilage extract from *A. vera* leaf is used as coating material with small capsules on pink guava using a spray-drying method. The coating with beta carotene in fruit increased the antioxidant activity and total carotenoid contents by 14 % and 36 %. The coating keeps the fruit healthy, acted as a natural colorant, and increased the content of dietary fiber.

Overall, the data reviewed here validated the applications of different *Aloe*-based food and cosmetics products such as cosmetic herbal hydrogel, lotion, *A. vera* gel extract mask, peel-off mask, mouthwash, and food products including meat nuggets, yogurt, herbal ice cream, edible coating of *A. vera* on fruits, khoa burfi, Kulfi, cookies, coating on pink guava.

It is believed that the consumption of *Aloe-based* food products and the utilization of *Aloe-based* cosmetics can be improved and increased by

adopting suitable procedures. Conversely, the study of complications associated with some specific conditions and with some specific compounds in *Aloe* is the possible research line for the valuation of safe utilization of *Aloe-based* food and cosmetic products.

4. CONCLUSION

In the history of plant-based medications, *A. vera* has a major contribution because of its frequent use for the treatment of various diseases. It is one of the most globally utilized plants as its extracts provide therapeutic effects due to the presence of a wide variety of phytoconstituents. The phytochemicals from *A. vera* extracts are employed against reactive oxygen species, diabetes, cancer, inflammation, and bacterial and fungal microbes. The data presented here on *A. vera* could be very vital to advance the research of clinical uses and the development of *Aloe-based* food and medicinal products with improved nutrition and therapeutic effects. It is recommended that more controlled investigations and clinical trials are prerequisites in the future to substantiate the outcomes and efficacies of *A. vera* under different circumstances. Also, some complications associated with specific compounds in *Aloe* should be addressed for the safer consumption of *Aloe-based* food products and the utilization of cosmetic products.

5. CONFLİCT OF INTEREST

The authors declared that there is no competing interests.

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LIST OF ABBREVIATIONS

ABTS=2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid, AOAC= Association of Official Analytical Chemists, *A. vera= Aloe vera*, DPPH= 2,2- diphenyl-1-picrylhydrazyl, DW= Dry weight, FPG= Fasting plasma glucose, FRAP= Ferric ion reducing antioxidant power, FTIR= Fourier transform infrared spectrometer, HPLC= High-performance liquid chromatography, LC-MS= Liquid chromatography-mass spectrophotometry, mg/g= Milli gram per gram, Mg CE/g= Milligram catechin per gram, MgQE/g= Milli gram quercetin/ gram, MMP-9= Matrix metallopeptidase 9, MTT= 3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyltetrazolium bromide, MWCO= membrane molecular weight cut-off, PBMC= Peripheral blood mononuclear cells, TAC= Total alkaloid content, TAC= Total antioxidant capacity, TFC= Total flavonoid content, TLC= Thin layer chromatography, TNF-α= Tumor necrosis factor, TPC= total phenolic content, TTN = Total tannin content, μg CE mg-1= Micro gram catechin per milli gram, μg GAEmg-1= Micro gram gallic acid per gram, μg RE mg-1= Microgram rutin per gram.

Proceedings of the Pakistan Academy of Sciences: B
Life and Environmental Sciences 60(3): 367-376 (2023)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(60-3)905

Pakistan Academy of Sciences

Review Article

Role of *Dof* Transcription Factors under Abiotic Stresses

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Abstract: For the survival of the rapidly growing global population, plant species must exhibit tolerance towards climate change. Plants possess mechanisms to respond to stress by changing their biological processes and stimulating stress-responsive genes. The *Dof* (TFs) family, which binds to DNA with a single finger, reflects a plant-specific group of TFs that play an important part in regulating plants that are facing different types of abiotic stresses which may influence their growth and development. Discovery of this family has made a significant impact on the field of plant sciences. However, the characterization of *Dof* transcription factors in crop plants is currently limited reported. Several *Dof* transcription factors (TFs) of plants have been shown in nature. The transcription factors *TaDofs*, *StDof*, *MnDofs*, *JrDof3TF*, *Va/VvDofs*, *GhDof1*, *OsDof1*, *SmeDof*, *ZmDof*, *CsDof*, *DcDof*, *CaDofs*, *ThDof*, *BraDof*, and *AcDof* are important for abiotic stressors such heat, cold, salt, drought, and heavy metals. In addition, *Dof* transcription factors play a role in the regulation of factors related to yield and quality. Nevertheless, some outstanding issues remain. The review article provides a summary of the role of various stress-responsive *Dof* transcription factors in response to abiotic stresses. Additionally, this study investigates the limitations and possible opportunities associated with *Dof* transcription factors in the development of crops that are capable of withstanding climate change. Therefore, it is recommended to conduct comprehensive research on *Dof* transcription factors (TFs) across many different transcription factors fields to find their potential novel functionality, which will be beneficial to our retention of the delicate biological processes in plants.

Keywords: Abiotic stress, *Dof*, Transcription factor, Salinity stress, Crop improvement

1. INTRODUCTION

Transcription factors (TFs) are essential to plant regulation and signaling networks [1, 2]. TFs control gene expression by binding to promoter DNA sequences [3-5]. In adaptation to abiotic stress, such as osmotic stress, cold, heat, and drought [1, 6-10] plants need more TFs than animals [5]. Drought, salinity, cold, etc. significantly reduce plant productivity. Stresses can reduce major crop yields by 50 %. Genes or other transcription factors (TFs) linked to abiotic stress increase proline content, close stomata to decrease transpiration, enhance stress-protective enzyme synthesis, and enhance abiotic stress tolerance [11-13]. The genetic factors TATA, CAAT Box, ARR1, GATA, AGAAA, CAAT, and DNA-binding are major contributors to genetics. A Zinc Finger (*Dof*) factor that's found in the promoter region of *OsRGLP2* is responsible for regulating several plant processes such as defense, light responses, development, and growth. [14]. Transcription factors (TFs) known as DNA-binding with one finger (*DoF*) are linked to the processes of plant growth and development. The *Dof* transcription factors exhibit a DNA-binding domain (C2/C2) that consists of 52 amino acids and a zinc finger that's capable of binding to the 5-(T/A) AAAG-3 target DNA sequence along with a resilient target DNA sequence [15]. The first Dof gene in maize was successfully identified [16]

Received: May 2023; Revised: July 2023; Accepted: August 2023
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and the interaction of multiple regulatory proteins responsible for the regulation of *Dof* transcription factors via their C-terminal region [17, 18]. Low/high temperatures, high salt treatment, and drought significantly influence plant development and productivity. Abiotic stresses reduce vegetable quality and production [19]. Pepper showed the expression of *CaDofs* as its response to abiotic stress [20], and researchers in the field of tea plant cultivation have carried out investigations into many different transcription factors (TFs). However, the regulatory mechanism of *Dof* transcription factors in tea plants remains difficult to identify [17, 21]. The *Dof* transcription factors (TFs) have significance in various biological processes throughout every stage of the life cycle of plants, and have importance for growth, seed storage protein synthesis, germination of seeds and development, regulatory metabolism, the photosynthesis process, flowering, and responses to stress [22-24]. *Dof* transcription factors regulate secondary metabolic processes like glucosinolate biosynthesis, cell cycle regulation, and flavonoids [25, 26]. They also regulate the cell cycle, phytochrome and cryptochrome signaling, plant hormonal signaling, abaxial-abaxial polarity, nitrogen use, and abiotic and biotic stress tolerance [27, 28]. The *DcDof* transcription factor, as compared to other plant *Dof* factors, has been given little focus in academic research. Furthermore, certain *Dof* genes can regulate both plant growth and stress responses. The enhanced expression of *Arabidopsis CDF3* has been observed to enhance the resistance to drought, cold, and osmotic stress, while also slowing down the start of flowering. This suggests that the gene in concern exerts a regulatory effect over both flowering time and tolerance to abiotic stress [29]. Overexpression of the *Dof* transcription factor *TDDF1* in tomatoes increased flowering-time control gene expression, causing early flowering and increased tolerance to drought, salt, and *Phytophthora infestans* late blight [30]. Under salt stress, the growth of the primary root in rice is decreased. This can be attributed to the suppression of *OsDOF15* expression in the roots, resulting in to decrease in the production of ethylene [31] and studies have shown the importance of *Dof* transcription factors in various biological processes, promoting plant growth and development [32]. Current investigation showed that *Dof* transcription factors play an important role in multiple signaling pathways regulation, in response to different abiotic stresses, plant growth and development, and other biological processes. The results indicate that *Dof* transcription factors have the potential for regulating both lipid metabolism and stress responses. Nevertheless, the members of the *Dof* TF family have currently to be reported. Current research provides an extensive overview of the resistance to abiotic stress, as well as the evolutionary links and the *Dof* transcription factor family.

2. STRUCTURE, FUNCTION, AND MECHANISM OF ACTION OF VARIOUS GROUPS OF *DOF* TRANSCRIPTION FACTORS

Suspendisse Sequence motifs that match DNA binding domains classify TFs. As shown by comparing the principal classes, *Dof* transcription factors have distinct functions in the hierarchy of response, although many interact as part of their response. Plants use several mechanisms to tolerate ecological problems such as drought, salinity, oxidative stress, cold, heat, and other infections that affect plant growth and development. Due to their sessile nature, environmental challenges can cause suboptimal growth conditions, requiring metabolic pathways to be changeable to allow plants to resist, tolerate, or recover from stress. The complex structure of the abiotic stress response in plants results from the polygenic nature and association between events involving signal transduction and stress response factor synthesis during a time of stress. As plants are sessile (immobile), environmental cues including drought, water logging, salt, mineral toxicity, and temperature change (frost, cold, heat) negatively affect their metabolism, growth, and development [33, 34].

In the same way, the upregulation of *ZmDOF36* resulted in a reduction in the levels of reduced sugars and soluble sugars within the endosperm of maize seeds. On the other hand, an increase in soluble sugar levels was found to promote the synthesis of starch. *ZmDOF36* was found to exert a positive regulatory effect on the expression of various genes associated with starch synthesis, including *ZmAGPS1a*, *ZmAGPL1*, *ZmISA1*, *ZmISA3*, *ZmGBSSI*, and *ZmSSIIa*. This regulation was achieved through the binding of *ZmDOF36* to specific motifs located in the promoters downstream

of these genes [35]. The previous study showed that *ThDOF14* enhances the ability of plants to tolerate salt and osmotic stress. This is achieved through an increase in proline levels and an improvement in the plant's ability to scavenge reactive oxygen species (ROS) [36]. Similarly, it has been observed that *ThDOF14* exhibits a specific affinity for the *DOF* motif present in the downstream promoter region of *TheIF1A*. This interaction suggests that *ThDOF14* may play a role in the plant's response to salt stress and osmotic stress by regulating the expression or engaging in molecular interactions with *TheIF1A* [37]. The study conducted by Cai et al. (2016) showed that the tomato *SlDOF22* gene had an impact on the accumulation of ascorbic acid (AsA) and also enhanced salt tolerance in plants [38]. Figure 1 shows how plants respond to various stresses via activating pathways, gene interaction, and molecular "crosstalk". Plants can defend themselves against different types of stress, which makes possible the identification of the most adaptable and resistant varieties for the benefit of producing a plant with desirable characteristics. Abiotic resistance genes usually stimulate a signal transduction pathway to identify pathogens and acquire resistance. The molecular understanding of abiotic stress response primarily depends on genetic engineering techniques, such as over-expression or mutation studies, to provide insight into the sensor, signal transduction factors, and antimicrobial factor genes.

3. ROLE OF *DOF* TRANSCRIPTION FACTORS UNDER ABIOTIC STRESSES

Abiotic stressors like heat, cold, drought, flooding, heavy metals, and salt are affecting crop life due to global warming. Abiotic factors limit maize, cotton, rice, and wheat yields by 50 % [39]. Table 1 shows how *Dof* TFs/genes/proteins affect plant abiotic stress tolerance.

3.1. Drought Stress

Drought stress, which is caused by excessive groundwater, decreased precipitation, and high temperatures, depicts a primary contributor to agricultural problems, which exert an adverse effect on the worldwide economy and the food security of a significant number of people by limiting crop growth and production [39, 58].

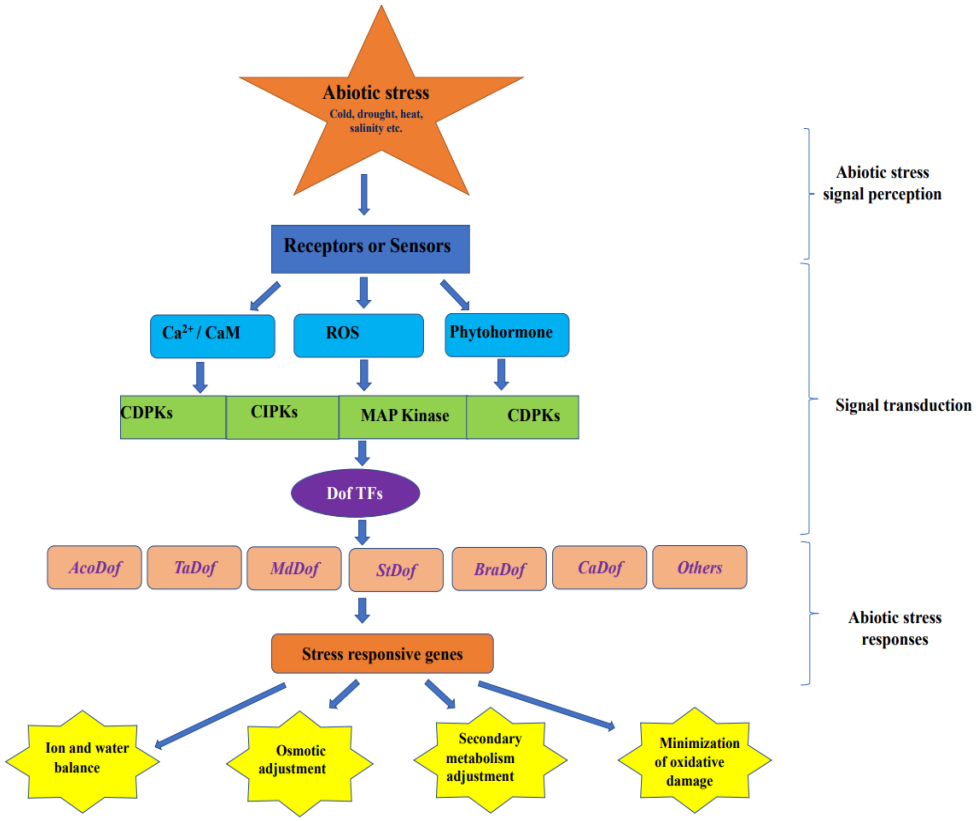

Fig. 1. Model for *Dof* TFs regulation abiotic stress signaling pathways.

Table 1. Role of different *Dof* transcriptional factor gene families in abiotic stress tolerance in plants

Stress	Crop	*Dof* TFs / Genes/ Proteins	References
Salinity, cold, heat, and drought stress	*Ananas comosus*	*AcoDof1, AcoDof12, AcoDof26, AcoDof22, AcoDof9, AcoDof19, AcoDof23, AcoDof11, AcoDof17, AcoDof20, and AcoDof1.*	[40]
Heat, drought, and heavy metals stress	*Triticum aestivum*	*TaDof, TaDof14, TaDof14*	[41, 42]
Salinity, drought, cold stress	*Malus domestica*	*MdDof*	[5]
Salinity, osmotic, heat and low temperature, and drought stresses	*Solanum lycopersicum*	*SlCDFs, SlCDF1-5*	[27]
Drought, salinity, low temperature, and high temperature	*Glycine max*	*GmDof4.2*	
Cold, heat, salinity, and drought stress	*Brassica pekinensis* Rupr	*BraDof*	[18]
Salt and osmotic stress	*Tamarix hispida*	*ThDof, ThelF1A*	[37]
Temperature, salinity, heat, and irritation	*Daucus carota*	*DcDof*	[44]
Heat stress	*Oryza sativa*	*OsDOF27*	[45]
Salinity and drought stress	*Camellia sinensis*	*CsDof*	[17]
Cold stress	*Brassica napus*	*BnCDF1*	[46]
Salt stress	*Nelumbo nucifera*	*NnDofs*	[47]
Heat and salinity stress	*Capsicum annuum*	*CaDofs*	[20]
Salinity and heat stress	*Chrysanthemum morifolium*	*CmDOFs*	[48]
Salinity stress	*Camelina sativa*	*CsDof*	[49]
Drought Salt and stress	*Tamarix hispida*	*WRKY (ThWRKY4)*	[50]
Salinity stress	*Zea mays*	*ZmDof, ZmDof16, ZmDof22, ZmDof36*	[51]
Osmotic, heat, and drought stress	*Populus trichocarpa*	*PtrDofs*	[52]
Cold stress	*Vitis vinifera* grapevine	*VaDof17d*	[8]
Salinity and salt stress	*Gossypium hirsutism*	*GhDof1*	[53]
Heat, cold, heat, and salt stress	*Saccharum spontaneum*	*SsDofs*	[15]
Drought and salinity stress	*Solanum melongena*	*SmeDof*	[54]
Salt and drought stress	*Vaccinium corymbosum*	*VcDof*	[55]
Drought and salinity stress	*Solanum tuberosum*	*StDof*	[24]
Drought and salt stress	*Rosa chinensis*	*RchDofs*	[56]
Salt stress	*Medicago truncatula*	*MtDof32*	[57]

The transcription factor *VyDOF8*, which comes from *Vitis yeshanensis*, a species of Chinese wild grapevine, exhibited a significant rise in expression levels under conditions of drought, cold, and salt-induced stress. *Dof* transcription factors play an important role in improving the responses of plants to drought stress. The overexpression of *VyDOF8* in tobacco plants has the potential to enhance their ability to withstand drought conditions. This has led to the reported increase in abscisic acid (ABA) concentration, root growth, proline, and chlorophyll accumulation, stress response gene expression, and antioxidant activity [59]. *SlCDF1* and *SlCDF3* overexpression in *Arabidopsis* increased

drought tolerance [17, 60]. *TaDofs* are nucleus-based transcription factors that affect growth, development, and abiotic responses. Customized annotation revealed that drought stress produced *TaDofs*, which were associated with defense, phytohormone response, growth, development, and metabolism [61]. The increased expression of *Solanum tuberosum Dof* (*StDof*) genes which can improve growth, development, and abiotic stress tolerance, has been found in response to both drought stress and abscisic acid (ABA) treatments. [24].

3.2. Salinity Stress

The process of global climate change has resulted in several types of environmental stressors, including soil salinity. This abiotic factor is the second largest cause of global productivity in agriculture loss. The adverse impacts of salinity on the growth and productivity of plants are mediated through various physiological, biochemical, and molecular mechanisms. These responses include ion homeostasis regulation, biosynthesis of phytohormones, and antioxidant defense systems activation. Plants respond to salinity by inducing the expression of stress-related genes, proteins, and metabolites that help to alleviate some of the adverse effects of salinity. Increasing salt tolerance becomes essential for maintaining global agronomic productivity. Transcription factors play an important part in the mechanism that causes plants' salt tolerance [62, 63]. *MnDofs* improve lotus salt tolerance [47]. The salinity responses of transgenic tobacco plants have been improved through the application of *BZIP* transcription factors obtained from salt-tolerant lotus root tips [43, 64]. Overexpression of *SICDF1* or *SICDF3* in *Arabidopsis* plants improved salt tolerance, stress-responsive gene expression (*COR15*, *RD29A*, *RD10*), flowering time, and specific target genes and metabolites [65]. *GmWRKY54* overexpression reduced soybean salinity stress [66]. The overexpression of *GhWRKY34* in *Arabidopsis* plants results in a specific absorption of Na^+ or K^+ ions in both roots and leaves, which helps promote the development of salt tolerance [67].

3.3. Heat Stress

Heat stress is a major environmental issue because of its negative impact on plant growth and development. The negative effects of high temperatures on cellular integrity and viability are well-studied. However, it is significant that plants have developed a mechanism of heat shock response that mitigates the negative impacts of abrupt temperature changes. Thermotolerant plants can endure heat stress, which can alter vegetable crop development, yield, and quality [2, 68-70]. Heat stress affects plant growth, health, and physiological, phenotypic, and genetic expressions [71, 72]. The *JrGRAS2* gene was recently identified as a regulator of heat shock protein (HSP) expression, thereby enhancing the capability of plants to tolerate heat stress. Therefore, *JrGRAS2* is considered an important gene for plant genetic engineering that focuses on improving heat response. The *HsfA1* protein is considered to be an important activator of the key response to heat stress [73-75]. Transcription factors have been found to have an adverse effect on gene expression in response to abiotic stress and the quality of grains. Additionally, the number of *Dof* family members has been shown to regulate glycogen and starch production in grains in response to high temperatures (HS) [76]. The findings suggest that *Arabidopsis* plants with an insertional mutant of *Mterf18* exhibit greater resistance to heat stress and elevated levels of HSP transcripts compared to their wild-type plants [77, 78]. Previous study shows that shot1 mutants and ATAD3-disrupted plants exhibit mitochondrial absorption and signaling problems, ultimately resulting in enhanced heat tolerance in plants. The moso bamboo that survived heat stress exhibits a stimulation of LTR retrotransposons, especially *PHRE1* and *PHRE2* [79].

3.4. Heavy Metal Stress

The effect of heavy metal stress (HM) on plants can be seen through different pathways such as growth inhibition, physiological process delays, and decreased crop productivity. These effects occur due to the change in cell membrane integrity, cellular ionic balance, metabolic balance, protein, and enzyme activity [69, 70]. Heavy metals can cause reactive oxygen species (ROS) and affect physiological functions such as photosynthesis, respiration, and vascular and enzymatic activity. Furthermore, a high level of heavy metals can cause difficulties in ion homeostasis [80]. A previous

study showed that *OsHMP09*, *OsHMP018*, and *OsHMP22* exhibited higher expression levels throughout all tissues. Alternatively, *AtHMP20*, *AtHMP23*, *AtHMP25*, *AtHMP31*, *AtHMP35*, and *AtHMP46* showed higher expression levels in the roots and leaves under various heavy metal stresses [81]. The *NtSOD* gene family enhances heavy metal toxicity tolerance in *Nicotiana tabacum* [82]. A previous study showed that *JrDof3TF* improves the heat stress response of *JrGRAS2* and is a heat response candidate gene in plant molecular breeding [75].

3.5. Cold Stress

The distribution, growth, and yield of crops are all significantly affected by cold stress. Sessile plants have many kinds of physiological and biochemical responses to cold stress, resulting in a 40 % decrease in temperate yields for agriculture. Cold stress adaptation is regulated by TFs and proteins [83, 84]. Cold stress causes 51-82 % of global crop output losses [85]. The study found that overexpression of *Va/VvDofs* resulted in improvements in root development, germination rate, and seed development, which led to improved cold resistance. However, the *Dof17d-Ed* mutant exhibited reduced cold tolerance, as evidenced by a decrease in rafnose family oligosaccharides [8] and moreover, in *Gossypium hirsutum GhDof1* overexpression can improved cold tolerance [53]. Previous study showed that *OsDof1* can enhance cold tolerance in rice act as a potential target for rice genetic breeding [86].

4. CONCLUSION AND FUTURE PERSPECTIVE

This review shows how *Dof* TFs can improve plant responses to various abiotic stresses. These studies show how DNA-binding with one finger (*Dof*) transcription factors can combat environmental challenges and increase yield and productivity under stress. Climate change threatens growth, development, and crop yield due to abiotic factors such salt, heat, cold, waterlogging, drought, and heavy metals. By focusing on various genes and or their regulators, it is urgently necessary to create crops that are tolerant to abiotic stress. The considerable number of *Dof* functional studies presented in this review highlights the existence of many unanswered questions regarding *Dof* transcription factors. The functional diversity of *Dof* transcription factors has garnered significant interest in recent years. However, the investigation into the molecular mechanisms through which these transcription factors regulate specific biological processes, such as plant growth, dormancy, and germination, has been restricted to a few model plants. Therefore, additional research must be conducted to further current knowledge in this area. In the future, it is of great interest to understand how these factors contribute to the stimulation of plant defense mechanisms against environmental stresses, ultimately resulting in an increase in crop yield and some members of *Dof* may become potential factors to have quite wide application prospects for the development of the food processing and biofuel industries. Therefore, it is important to conduct comprehensive research on *Dof* transcription factors (TFs) across many different fields in order to find their potential novel functionality, which will be beneficial to our retention of the delicate biological processes in plants.

5. CONFLICT OF INTEREST

The authors declared no contlict of interest.

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Proceedings of the Pakistan Academy of Sciences: B
Life and Environmental Sciences 60(3): 377-382 (2023)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(60-3)749

Pakistan Academy of Sciences

Research Article

An Estimate of Protective Immunity against SARS-CoV2: Comparison of Different Vaccine Types

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Abstract: Several types of vaccines have been approved to prevent SARS-CoV-2 infection. Few studies are conducted on the efficacy of COVID-19 vaccines. Vaccination is important to eliminate and fight SARS-CoV-2 infection and several vaccines have been approved. This study aimed to assess the incidence density of COVID-19 infection among the community, estimate the effectiveness of different types of vaccines (inactivated virus, viral vector or mRNA) and efficiency of incomplete and complete vaccination. In this observational cross-sectional study, a total of 4924 specimens were received from 1st January 2022 to 2nd February 2022 for the detection of SARS-CoV-2. The patient's age, gender, and vaccination data were recorded and S, N, and ORF 1ab genes were amplified after RNA extraction through PCR. out of which 1034 (20.99 %) cases were positive. Among 1034 (20.99 %) positive cases, 418 and 616 patients were vaccinated and non-vaccinated respectively. The cases of SARS-CoV-2 in vaccinated patients were categorized into a sudden infection (≤ 10 days) and late infection (≥10 days) after the incomplete and complete dose of vaccination. Vaccination provides partial protection against SARS-CoV-2 infection. This might be due to the low efficacy and inability to detect recent variations in the protein structure of the virus.

Keywords: SARS-COV-2, Vaccine Effectiveness, COVID-19 Vaccine Types, Ct Value.

1. INTRODUCTION

Coronavirus disease 2019 (COVID-19) is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2); an emerging infectious disease was first reported at the end of 2019 in Wuhan. Globally SARS-CoV-2 infection has occurred in almost every country with millions infected and hundreds of thousands dead [1]. The treatment and prevention of an infection depend on effective drugs and vaccines. In addition to traditional inactivated vaccines, new technologies are being employed to develop COVID-19 vaccines, such as mRNA/ DNA vaccines, genetically engineered vaccines, and vaccines based on adenovirus-based vectors [2]. For COVID-19, mRNA-based vaccines have shown the highest levels of protection, followed by viral vectors, protein subunits, and whole-inactivated viruses [3].

To eliminate and fight SARS CoV-2 infection, vaccination is important and several COVID-19 vaccines have been approved, including BBIBP-CorV (Sinopharm, China) and CoronaVac (Sinovac Biotech, China) an inactivated virus vaccine with aluminum hydroxide adjuvant of 2 doses are given [4]. CanSino Bio Vaccine/ Ad5-nCoV (China), Gam-COVID-Vac/Sputnik V (Gamaleya National Research Center for Epidemiology and Microbiology, Russia) and ChAd0x1 (AZS1222) (AstraZeneca/ Oxford UK) are viral vector vaccines. mRNA-1273 (Moderna US) and BNT162b2 (Pfizer-BioNTech US) are mRNA vaccines [5, 6]. This study was conducted to determine the

Received: September 2022; Revised: Feberary 2023; Accepted: May 2023
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effectiveness of different types of vaccines against COVID-19 and the chances of infection in partially and fully vaccinated individuals.

2. MATERIALS AND METHODS

2.1. Study Design

This observational cross-sectional study was carried out 1st January, 2022 to 2nd February, 2022 at the Department of Pathology, Mardan Medical Complex (MMC). The study population included patients of both genders, age above 20 years, suspected COVID-19 patients (vaccinated and non-vaccinated), Ct values of N, S and ORF1ab genes and those who visited MMC Mardan. Patients having age less than 20 years, asymptomatic, co-infected, or other respiratory disease were excluded from this study.

2.2. Ethical Statement

Ethical approval was obtained from the ethical committee of the hospital, Mardan Medical Complex (MMC) for this study.

2.3. Sample Size

A total of n=1034 COVID-19 positive patients were included in the current study. The patient information such as age, gender, and history of vaccination including vaccine type and vaccination dose (incomplete or complete dose) was recorded through a questionnaire form. COVID-19 positive patients were categorized into 2 groups; sudden infection (≤10 days) and late infection (≥10 days) after the incomplete and complete dose of vaccination.

2.4. Sample Processing

SARS CoV-2 RNA was extracted through an auto extractor (Hangzhou Bigfish Biotech Co Ltd, China) and Real-Time Polymerase Chain Reaction (RT-PCR) was performed for amplification of S, N and ORF 1ab genes (Rotor-Gene, Qiagen, Germany). The cycle threshold (Ct) values of the study patients were categorized as high (Ct 28–34.9) and low (18–27.9). The Ct values were compared between the patients with severe disease and mild disease.

2.5. Statistical Analysis

Quantitative variables such as mean, standard deviation, categorical variables (frequency and percentages) and significance among variables were calculated through SPSS Version 22.0.

3. RESULTS AND DISCUSSION

3.1. Study Population and Characteristics

A total of 4942 specimens of the suspected COVID-19 patients were screened for the detection of SARS-CoV-2. The SARS CoV-2 was detected in 1034 (20.99 %) specimens of the suspected patients through RT-PCR. The demographic data of all the COVID-19 positive patients is tabularized in Table 1. Significant (p value <0.0001) was observed for vaccinated and non-vaccinated patients. The infection rate was significantly high in females than in males, while a non-significant difference was observed between the different age groups (Table 1).vaccinated patients. Incomplete and complete vaccination dose among the vaccinated patients and COVID-19 within ≤ 10 days and after ≥ 10 days of vaccination among incomplete and complete vaccinated patients are shown in Table 2.

3.2. COVID-19 in Vaccinated Patients

Among the Vaccinated patients n=174 (41.62 %) BBIBP-CorV, n=158 (37.79 %) CoronaVac, n=26 (6.2 %) Cansino, n=22 (5.26 %) Sputnik V, n=18 (4.3 %) ChAd0x1 (AZS1222), n=8 (1.91 %) mRNA-1273 and n=12 (2.87 %) BNT162b2.

3.3. N, S and ORF1ab Gene Amplification in COVID-19 Patients

Table 3 shows the ct value (high and low) of different genes; N, S and ORF1ab among the vaccinated and non-vaccinated COVID-19 patients with a p-value of 0.01 (significance). Out of 616 non-vaccinated patients, 504 (81.81 %) had a low Ct value and the rest 112 (18.18 %) had a high Ct value while out of 418 vaccinated patients, 105 (25.11 %) had low Ct value and 313 (74.88 %) had high Ct value (Table 3).

Table 1. Demographic data of COVID -19 vaccinated and non-vaccinated patients

Variables	n (%)	p. value
Total patients	4942	
COVID-19 positive	1034 (20.99)	
COVID-19 negative	3908 (79.366)	<0.0001
Vaccination History		
Vaccinated patients	418 (40.42)	
Non-Vaccinated patients	616 (59.57)	<0.0001
Gender among Positive COVID-19		
Male	667 (64.50)	
Female	367 (35.49)	<0.0001
Vaccination among Positive COVID-19		
Incomplete /Partially vaccinated (1 dose)	198 (47.36)	
Complete /Fully vaccinated (2 doses)	220 (52.63)	0.0152
Gender among Vaccinated patients		
Male	270 (64.59)	
Female	148 (35.40)	<0.0001
Age group of vaccinated Patients		
20-40	126 (30.14)	
41-60	150 (35.88)	0.314
60 & above	142 (33.97)	0.5037
Mean age	**52.58**	

This observational cross-sectional study was carried out from 15 November 2021 to 15 January 2022). We screened a total of 4924 specimens of the suspected COVID-19 patients for detection of SARS-CoV-2, with the incidence of positive samples n= 1034 (21.6 %) patients, out of which n=667 (64.50 %) were males and n=367 (35.49 %) were females. The COVID-19 patients were categorized in three groups according to age: group 1 (21-40 years), group 2 (41-60 years), and group 3 (60 and above). Each group comprised of 126 (30.14 %), 150 (35.88 %) and 142 (33.97 %) patients respectively. The highest number of positive patients were observed in group 2 followed by groups 3 and group 1. The mean age for each group was 30 years, 50.17 years, 75.18 years for group 1, group 2, and group 3 respectively. For all the vaccinated patients the mean age was 52.58 years. A previous study reported that SARS CoV-2 infection was predominant in patients ranging age group 25-44 years followed by the age group higher than 45 years and less than 25 years [7].

Out of 1034 patients, a total of n=418 (40.42 %) were vaccinated and n=616 (59.57 %) were non-vaccinated. Among the vaccinated patients, n=184 (44.01 %) were incomplete vaccinated while n= 234 (55.98 %) were fully/ complete vaccinated. Among the total n=174 (41.62 %) patients vaccinated with BBIBP- CorV vaccine, n= 64 (36.78 %) patients were partially vaccinated (single dose) and n=110 (63.21 %) patients were fully vaccinated (2 doses). A total of n=158 (37.79 %) patients were vaccinated with CoronaVac vaccine (n= 78 (49.36 %) partially vaccinated and n=80 (50.63 %) patients were fully vaccinated). Twenty-six (6.2 %) patients were administered Cansino vaccine. Similarly, n=22 (5.26 %) patients were vaccinated with Sputnik V (n=20 (90.90 %) patients were partially vaccinated and n=2 (9.09 %) patients were fully vaccinated), and n=18 (4.3 %) patients were vaccinated with ChAd0x1 (AZS1222) (n=16 (88.88 %) patients were partially vaccinated and n=2 (11.11 %) patients were fully vaccinated). Eight (1.91 %) patients were vaccinated with mRNA-1273 and none of the patients completed their vaccination dose with mRNA-1273 vaccine, similarly, 12 (2.87 %) patients were vaccinated with BNT162b2 and none of the patients completed their vaccination dose. A similar response was

Table 2. COVID-19 among vaccinated patients (n= 418)

Vaccination Type (n; %)	**COVID-19 infection after Vaccination**	
	Infection within ≤10 days n (%)	**Infection** after ≥ 10 days n (%)
BBIBP- CorV (174; 41.62)		
1 dose (64; 36.78) 2 doses (110; 63.21)	28 (43.75) 2 (1.81)	36 (56.25) 108 (98.18)
CoronaVac (158; 37.79)		
1 dose (78; 49.36) 2 doses (80; 50.63)	70 (89.74) 4 (5)	8 (10.25) 76 (95)
Cansino (26; 6.2)		
1 dose (26; 100)	0	26 (100)
Sputnik V (22; 5.26)		
1 dose (20; 90.90) 2 doses (2; 9.09)	0 0	20 (100) 2 (100)
ChAd0x1 (AZS1222) (18; 4.3)		
1 dose (16; 88.88) 2 doses (2; 11.11)	0 0	16 (100) 2 (100)
mRNA-1273 (8; 1.91)		
1dose (8; 100)	0	8 (100)
BNT162b2 (12; 2.87)		
1 dose (12; 100)	0	12 (100)

observed in another study in patients who were not vaccinated with an mRNA vaccine had 2.34-fold higher odds of reinfection compared with fully vaccinated adults [8].

Among BBIBP-CorV vaccinated patients with incomplete dose, 28 (43.75 %) patients were diagnosed with COVID-19 within ≤ 10 days and n=36 (56.25 %) were diagnosed with COVID-19 after ≥ 10 days of vaccination while BBIBP-CorV vaccinated patients with complete dose were 2 (1.81 %) within ≤ 10 days and 108 (98.18 %) after ≥ 10 days of vaccination.

Out of n=78 CoronaVac partially vaccinated patients, n=70 (89.74 %) patients were diagnosed with COVID-19 within ≤ 10 days and n=8 (10.25 %) were diagnosed with COVID-19 after ≥ 10 days of vaccination whereas CoronaVac fully vaccinated patients, 4 (5 %) patients were diagnosed COVID-19 within ≤ 10 days and 76 (95 %) after ≥ 10 days of vaccination.

None of the patients were infected with SARS CoV-2 after the incomplete and complete dose of Sputnik V vaccination within ≤ 10 days. All patients n=20 (100 %) and n=2 (100 %) were COVID-19 positive after ≥ 10 days of vaccination, similarly, none of the patients was COVID-19 positive after the incomplete and complete dose of Astrazeneca vaccination within ≤ 10 days. n=16 (100 %) and n=2 (100 %) had COVID-19 after the incomplete and complete dose of ChAd0x1 (AZS1222) vaccination after ≥ 10 days. Patients incomplete vaccinated with mRNA-1273 (n=4) and BNT162b2 (n=6) were COVID-19 positive after ≥ 10 days. Another study has reported COVID-19 vaccines to offer protection against SARS CoV-2 variants, including Delta, after completion of the vaccination series, and the effect of partial uptake of vaccines is found to be suboptimal [9,10].

Ct values of gene N, S and ORF1ab were calculated among all the positive COVID-19 patients (vaccinated and non-vaccinated). The Ct values of patients with high viral load were significantly lower than patients with low viral load. Out of 616 non vaccinated patients with high viral

Table 3. Comparison of mean Ct values between vaccinated and non-vaccinated patients

COVID-19 Patients (n; %)	Genes	High Ct value Mean	Low Ct value mean	Significance testing
Non-vaccinated (n=616; 59.57)	N	31.647	25.291	p=0.01
	S	32.011	26.132	
	ORF1ab	32.821	26.781	
Vaccinated (n= 418; 40.42)	N	32.196	26.543	p=0.01
	S	32.733	26.962	
	ORF1ab	33.228	27.341	

load, n=504 (81.81 %) had Ct value <27 (n= 113 (22.42 %) Ct value 18-22.9 and n=391 (77.57 %) Ct value 23-27.9) and n=112 (18.18 %) with low viral load had Ct value > 27 (n=50 (44.64 %) Ct value 28-30.9) and n=62 (55.35 %) Ct value 31-34.9).

In total of 418 vaccinated patients, n=105 (25.11 %) had Ct value <29 (n=40 (38.09 %) Ct value 24.1-27.9 and n=65 (61.90 %) had Ct value 28-29.9), while 313 (74.88 %) low viral load patients had Ct value > 29 (n=63 (20.12 %) with Ct 28-30.9 and n=250 (79.87 %) with Ct 31-34.9). A significant difference was found in viral load and Ct values of the vaccinated and non-vaccinated patients (p=0.01). In the current study we categorized Ct values of as high (>29) and low (<29). Another study has grouped patients on the basis of Ct values as high (Ct 31–40), moderate (21–30) and low (11–20) [11].

4. CONCLUSION

Vaccination provides partial protection against SARS COV-2. This might be due to the low efficiency and potential to detect recent variations in the protein structure of the virus. Furthermore, incomplete vaccination in the community also increases the risk of COVID-19. The study also concluded that chances of SARS-CoV-2 infection (sudden and late) are high in patients vaccinated with inactivated vaccines (Sinopharm and Sinovac). To our knowledge, this is the first study to describe the effectiveness of different types of vaccines against COVID-19 and reported COVID-19 within ≥ 10 days or more ≤ 10 days among incomplete and complete vaccination doses.

5. LIMITATIONS

The limitations of this study were the lack of follow-up of all the vaccinated patients, study duration was short, lack of clinical correlation among the vaccinated patients and COVID-19 variants were not detected and Ct values among incomplete and complete vaccination in vaccinated patients were not calculated.

6. RECOMMENDATIONS

Vaccines needs to be updated periodically to increase clinical efficacy against SARS CoV-2 variants and detection of SARS CoV-2 infection and other complications at the time of vaccination is needed to overcome the efficiency and effectiveness of vaccine among COVID-19 patients.

7. CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Proceedings of the Pakistan Academy of Sciences: B
Life and Environmental Sciences 60(3): 383-392 (2023)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(60-3)765

Pakistan Academy of Sciences

Research Article

Durum-21: A New High-Yielding and Good Quality Durum Wheat Variety Suitable for Pasta Production

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Abstract: Durum-21 (D-21) is a high-yielding, disease resistant with better-quality traits variety, developed by Wheat Research Institute Faisalabad. This variety is mainly developed for industrial purposes for pasta production. Worldwide, durum wheat is utilized for pasta production; but in Pakistan, due to a lack of research work and non-availability of quality seeds of durum wheat, bread wheat is being utilized for pasta production. D-21 is developed with the breeding code of D-21 having parentage of FKN/3/2*FR//KAD/GB/4/BB/CHA/5/AS-2002 with pedigree as PB20733-1a-2a-2a-0a-0a-19a-0a. The candidate line D-21 was developed by crossing a germplasm accession with approved bread wheat variety AS-02. The genotype was further evaluated over multiple locations in Punjab Pakistan for yield and yield-related attributes in the station, provincial, and national uniform durum yield trials executed by Wheat Research Institute (WRI), Faisalabad during 2015-20. The promising line out yielded the two commercial check varieties D-97 and Fsd-2008 by 1.42 % in the provincial yield trial and 4.2 % in the national yield trial. D-21 had desirably medium to tall plant height (96-100 cm) without anthocyanin pigment. It has erected to semi-erect growth habit at the seedling stage. Its color is green with medium waxiness on stem and yellowish-white at maturity. Its 1000-grain weight ranged from 38.9 to 39 g while the test weight remained from 69 to 78.5 g. The protein contents were 13.1 to 14.95 %; which is higher than the two checks (D-97 and Fsd-2008). Due to its better grain yield and promising nutritional and quality parameters, it was approved in the year 2021 for cultivation all over the country.

Keywords: Durum, Adaptability, Pasta, Pigmentation

1. INTRODUCTION

Durum wheat (*Triticum turgidum* L.) is a tetraploid species of wheat that is primarily grown for industrial purposes. It is mainly grown in Mediterranean countries, including the Middle East, North America, and North Africa [1] Durum wheat offers a great advantage over bread wheat in a number of aspects. Durum wheat has A and B genomes with 28 chromosomes while bread wheat contains A, B, and D genomes with 46 chromosomes [2] Durum wheat is much harder than bread wheat which requires more thorough grinding to produce semolina and flour. Durum wheat dough contains a strong visco-elastic protein complex known as gluten which makes it more suitable for pasta production, on the other hand, bread wheat is mainly utilized for domestic purposes to make bread [3]. A typical durum wheat grain is vitreous, very hard, amber in colour, and contains 2-3 % more protein and gluten content as compared to bread wheat [4]. Besides high protein and gluten content it is also rich in carotenoid, folate, iron, calcium, and dietary fibre [5]. Durum wheat was grown on an area of over 16 million hectares with an annual global production of over 38 million tonnes [6]. The largest producing countries of durum wheat are including Turkey and Canada with an estimated area of more than 2 million ha of each followed by Italy, Algeria, and India [7]. Due to its economic importance and unique properties, it is used to make a wide range of food products including semolina, pasta, noodles, burghul wheat, Couscous, and desserts. Durum wheat is milled into a small granular form that is

Received: October 2022; Revised: April 2023; Accepted: June 2023
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known as semolina and that is mainly utilized for pasta production [8].

Different value-addition industries of wheat require specific quality wheat grains. The most widely used wheat in pasta products is durum wheat [9]. The international food industry, involved in pasta products uses durum wheat flour for making quality products. Pakistan has a transforming economy hosting many western food chains across the country. Changing lifestyles and tastes of the population suggest increasing demand for pasta products. Currently, durum wheat is being imported by all pasta industry stakeholders on account of the non-availability of high-yielding durum wheat variety and milling units. Due to increasing demand for pasta products and their premium price, milling units are being established across the country and research work has been initiated for the development of good quality durum variety. The provision of high-quality durum variety will help to reduce the import bill through local production of durum wheat flour. The current research work was conducted to develop high-yielding, better quality, and disease-resistant durum wheat variety that can be utilized in industry for pasta production.

2. MATERIALS AND METHODS

The present research work was conducted at Wheat Research Institute, Faisalabad Pakistan along with various locations in the province of Punjab during 2015-2020. The D-21 has parentage of FKN/3/2*FR//KAD/GB/4/BB/CHA/5/AS-2002 with pedigree as PB20733-1a-2a-2a-0a-0a-19a-0a. Durum wheat line D-21 is developed by crossing a germplasm accession with approved bread wheat variety AS-02. The proposed variety has an edge in yield over Durum-97 with genetic resistance to diseases. This variety is suitable for planting from 1st to 20th November in Punjab province. The D-21 was evaluated for higher grain yield, disease resistance, and other attributes at station yield trials. Station yields trials further consist of preliminary and regular yield trials. Both these trials were conducted at WRI, Faisalabad. Based on higher yield performance in generation trials D-21 was tested in a preliminary yield trial conducted during 2016-17. The trial was consisting of 10 entries along with one check variety D-97 following RCBD design. The experimental plot size was maintained at 8.1 m^2 with 6 rows and three replications. The R×R distance was kept at 27 cm and the length of each row was maintained by 5m. This line is then promoted into a regular yield trial that was comprised of 16 entries along with 2 check varieties Fsd -2008 and D-97. The trial was laid out under RCBD, by maintaining the net plot size of 8.1 m^2 during 2017-18. The promising line was then promoted into Provincial Uniform Durum Yield Trial (PUDYT) to check the adaptability and stability over multiple locations throughout the province of Punjab. The D-21 was compared with two local checks Fsd-08 and D-97 in Provincial Uniform Durum Yield Trial. This genotype was again sent in National Uniform Durum Yield Trial (NUDYT) in the next two consecutive years (2018-19, 2019-20) to test the adaptability and yield stability of a mega environment throughout Pakistan. The grains of D-21 were sent to Crop Disease Research Institute (CDRI), NARC, Islamabad to screen out against the indices of leaf and yellow rust during the NUDYT testing years. All other management practices were accomplished as per crop recommendation. Seed samples of the candidate line were taken from (PUDYT 2017-18) for the assessment of the quality analysis from Cereal Technology Laboratory WRI, Faisalabad according to the standard protocol of American chemists [10] & [11]. The crop development stages were observed continuously until crop maturity and grain retentions in mature spike during every crop cycle. The brief development history of D-21 is mentioned in Table 1.

3. RESULTS AND DISCUSSION

3.1. Preliminary Yield Trial (2015-16)

The preliminary yield trial was conducted at Wheat Research Institute Faisalabad during 2015-16. Ten (10) entries along with three replications were tested and compared with one local check variety durum-97 as shown in Table 2. The advanced line D-21 yielded 3931 kg/ha^{-1}. It performed better than the check variety in the preliminary yield trial and gave 8.90 % higher yield than D-97. Therefore, it was re-evaluated for yield and stability in regular yield trials (B- trial).

Table 1. Development history of "D-21"

S. No.	Year	Generation
1	2007-08	Hybridization
2	2008-15	F_1 to F_7
3	2015-16	Preliminary Durum Wheat Yield Trial under the code D-21
4	2016-17	Regular Wheat Yield Trial at Wheat Research Institute, Faisalabad, under code D-21
5	2017-18	Punjab Uniform Durum Wheat Yield Trial
6	2018-19	National Uniform Durum Yield Trial
7	2019-20	National Uniform Durum Yield Trial

3.2. Regular Yield Trial (2016-17)

The regular yield trial was conducted during 2016-17 at WRI, Faisalabad to check the yield performance of candidate line D-21. The results of this trial indicated that the advanced line D-21 produced 3856 kg ha^{-1} with an 8.90 % higher yield than D 97. Moreover, the disease data also indicated that the advanced line was ultimately resistant to leaf and yellow rusts as mentioned in Table 2. Therefore, it was promoted to Punjab Uniform Durum Yield Trial during 2017-18 for yield stability and adaptability evaluation.

3.3. Punjab Uniform Wheat Yield Trial (2017-18)

Based on the better performance of two consecutive years in station yield trial, the advanced line D-21 was tested for wider adaptability across a mega environment in a provincial uniform durum yield trial (PUDYT) over 20 locations in irrigated areas of Punjab during 2017-18. The overall yield performance of different advanced lines at various locations in Punjab is given in Table 3. The proposed line D-21 out yielded the check variety D-97 by 1.45 % at different locations in Punjab. Moreover, the candidate line also showed resistance against leaf and yellow rust. On the basis of tabulated and diseased screening data, this line was further tested in National Uniform Durum Yield Trial (NUDYT) during 2018-19.

3.4. National Uniform Durum Yield Trial

A good variety is that which perform better with respect to yield and yield-related attributes under a diverse environment. Development of higher

Table 2. Grain yield of "D-21" in station yield trials

Sr. No.	Year	Trial name	Grain Yield (kg ha^{-1})	
			D-21	**Durum 97**
1	2015-16	PYT (Preliminary yield trial)	3937	3531
2	2016-17	RYT (Regular yield trial)	3856	3625
		Average	3896	3578
		% Increase Over Durum-97 (Check)		**8.90**

Table 3. Grain yield of "D-21" in PUDYT during 2017-18

Sr. No.	Location	Grain yield (kg ha^{-1})	
		D-21	**D-97**
1	Govt Seed Farm, Dhakkar Pakpattan	1831	1396
4	MMRI, Sahiwal	2978	2729
5	PSC Khanewal	1474	1289
6	ARF Sargodha	1779	2399
7	RRI Kala Shah Kaku	2020	2123
	Average	3361	3312
	% Increase over check		**1.45**

grain yield across mega environments is very critical. Development of characters in a genotype require favorable genetic recombination along with multilocation testing at the national level against a wide range of variation in biotic and abiotic stress factors. The promising line D-21 gave a higher yield in two consecutive years as compared to local checks Fsd-2008 and D-97 during (2018-19 and 2019-20) by 3.5 and 4.8 % respectively (Tables 4 and 5).

3.5. Agronomic Trials

To optimize sowing time, seed rate and fertilizer levels of advanced line D-21, the agronomic trials were conducted at WRI, Faisalabad during 2018-20. The promising line D-21 performed better with respect to planting time with a notable margin of 9.7 % in 2018-19 and 12.8 % again in 2019-20 over the check varieties as mentioned in Table 6. To assess the best seed rate for D-21 another agronomic trial was performed at WRI, Faisalabad. The advanced line D-21 performed better at the seed rate of 150 Kg ha^{-1} in both the years. It out yielded the local checks by a margin of 2.8 and 4.7 % respectively as indicated in Table 7. The third agronomic trial was conducted related to fertilizer application. To optimize the level of fertilizers, different doses of fertilizers were maintained. Line D-21 produced better at the rate of N-P-K 120-114-60 NPK (Kg ha^{-1}). It showed 13.9 & 9.3 % more yield in 2018-19 and 2019-20 as compared to local checks (Table 8).

Table 4. Grain yield of "D-21" in NUDYT during 2018-19

S. No.	Location	Grain Yield (kg ha^{-1})	
		D-21	**FSD-08**
1	CCRI, Nowshera	2473	2658
2	WRI, Faisalabad	3948	4124
3	ARI, Quetta	3273	3262
	Average	3231	3348
	% Increase over check		**3.5**

Table 5. Grain yield of "D-21" in NUDYT during 2019-20

S. No.	Location	Grain Yield (kg ha^{-1})	
		D-21	**Durum-97**
1	Arid Zone Researc Institute, Bhakkar	1764	1715
2	Wheat Research Institute, Faisalabad	4059	4194
3	Nuclear Institue Agricultur, Tandojam	3911	4827
4	Wheat Program, NARC, Islamabad	4031	3263
5	Barani Agricultural Research Institute, Chakwal	3170	4507
	Average	3996	3812
	% Increase over check		**4.8**

Table 6. Grain yield of "D-21" in sowing date trials

S. No.	Grain Yield (kg ha^{-1})				
	2018-19			**2019-20**	
	Date	**D-21**	**Local Check**	**D-21**	**Local Check**
1	Nov 01	4109	3919	3454	3280
2	Nov 20	4020	3512	3976	3312
3	Dec 10	3360	3035	3291	2908
	Average	3829	3488	3573	3166
	% Increase over checks		**9.7**		**12.8**

Table 7. Grain yield of "D-21" in seed rate trials

S. No.		Grain Yield (kg ha^{-1})			
		2018-19		2019-20	
	Seed rate (kg ha^{-1})	D-21	Local Check	D-21	Local Check
1	100	3795	3587	3675	3496
2	125	4080	3989	3967	3800
3	150	4100	4069	4080	3899
	Average	3991	3881	3907	3731
	% Increase over checks		**2.8**		**4.7**

Table 8. Yield response of D-21 to different fertilizer doses

S. No.		Grain Yield (kg ha^{-1})			
		2018-19		2019-20	
	NPK (kg ha^{-1})	D-21	Local Check	D-21	Local Check
1	85-85-0	2955	2400	3210	2980
2	120-114-0	3502	3060	2978	2567
3	120-114-60	4200	3895	3998	3767
	Average	3552	3118	3395	3104
	% Increase over checks		**13.9**		**9.3**

3.6. Disease Screening Studies

The advanced line D-21 was screened against leaf and yellow rust by Cereal Disease Research Institute Islamabad (CDRI) by the inclusion in National Wheat Disease Screening Nursery (NWDSN) at various locations for two consecutive years 2018-19 and 2019-20. The advanced line D-21 showed high resistance against yellow and leaf rust for two consecutive years. Moreover, it also showed zero % terminal reaction as compared to commercial check variety which showed high susceptibility with terminal rust reaction of more than 50 S in 2018-19 and more than 20 S in 2019-20 as illustrated in tables 9 and 10. The obtained disease screening results of line D-21 revealed that this genotype was resistant to yellow and leaf rust and could be used to develop leaf and yellow rust resistance sources in future breeding programs.

3.7. Quality Related Characteristics

Different quality-related parameters of D-21 were evaluated. Durum wheat is mainly utilized in the industry for pasta production as it contains

Table 9. Yellow rust data of LDSN at different locations

Variety	Year	Faisalabad	Islamabad	Bahawalpur	Kala Shah Kaku
D-21	18-19	0	0	0	0
Morocco		90S	100S	80S	40S
D-21	19-20	0	0	0	0
Morocco		80S	100S	70S	50S

Table 10. Leaf rust data of LDSN at different locations

Variety	Year	Faisalabad	Khanewal	Bahawalpur	Kala Shah Kaku
D-21	18-19	0	0	0	0
Morocco		60S	30S	70S	50S
D-21	19-20	0	0	0	0
Morocco		70S	20S	50S	40S

more amount of gluten and protein content. High percentage of gluten and protein in durum grain makes it better for pasta production. Its 1000-grain weight was 38.9-39 g and the test weight remained 69-78.5 g. The protein contents were 13.1 to 14.95 % which was higher than the two checks D-97 and Fsd-2008. Moreover, its pasta and noodles making quality parameters were also promising as compared to local checks variety Fsd-2008 and D-97 (table 11).

3.8. Botanical Attributes of D-21

The new variety D-21 had desirably medium to tall (96-100 cm). Anthocyanin pigment was absent at seedling as well as at maturity. It has an erect to semi-erect growth habit. Its color is green with medium waxiness on the stem and yellowish-white at maturity. Its stem is stiff enough that provide resistance against lodging. A number of tillers vary from 350 to 450 m^2. It contains a waxy flag leaf with an erect orientation of 27 to 30 cm in length and 1.9 to 2.1 cm in width. Hairiness auricle was present with weak anthocyanin pigmentation. It has awns of medium length, shattering resistant, dense ear with small to medium in size having 55-65 seeds per ear. Rachis is 10-12 cm in length with 16-18 segments. It takes 110-120 days for heading and it matures in 150-60 days. Its glume length is about 13.5-14.5 and its width is 3.8-4.0 cm with strong attachment. Shoulder of the glume was narrow to medium and glume beak was straight and its pubescence was absent. The glume surface was smooth and without internal hairs and imprints. It has a medium sized seed with an ovate shape and amber in color with a medium brush and opaque surface with an intermediate groove. The germ size of the seed was medium. Its bread and chapatti-making quality were good to very good as mentioned in Table 12. Moreover, it is highly resistant against the leaf and yellow rust races and gave good yield under different locations in Punjab and Pakistan. It also showed a good response against fertilizer applications having better adjustment to different ecological zones. Similar observations were reported by Nilusha *et al.* [9]. Botanical description of Durum-21 is given in Table 12 and a pictorial view of the plant, grain, and spike is presented in Figure 1.

3.9. DNA Finger Printing of Durum-21

The Cluster analysis was carried out among check variety Durum-21 along with advanced lines viz. D-18801, D18802, D-18810, D-18812, D18815, D-18830, D18840, D-18847, D-18708, D18721, D-17728 and D-97 against the 50 SSRs markers to draw a dendrogram based on similarity/dissimilarity coefficient using UPGMA algorithm which showed variable genetic similarity 0.76 to 0.90 among different durum genotypes (Figure 2). The Cluster analysis differentiated all the genotypes into two major clusters, cluster one having genotypes viz. D-18801, D-18812, D-18815, D-18840, D-17728, D-97, D-18847 and D-18708. However, genotypes D-18802, Durum-21, D-18721, D-18810, and D-18830 were placed in the second major cluster. Durum-21 was found significantly different from many of the other durum genotypes i.e., 29 % dissimilar from D-18801, 10 % from D-18802, 16 % from D-18810, 25 % from D-18812, 31 % from D-18815, 18 % from D-18830, 30 % from D-18840, 25 % from D-18847, 24 % from D-18708, 12 % from D-18721, 23 % from D-18778 and 20 % from Durum-97. These dissimilarity values confirmed that Durum-21 is a distinct variety and possesses a diverse genetic background [12, 13].

4. CONCLUSION

Wheat Research Institute Faisalabad is one of the renowned research institutes of the world as it is

Table 11. Quality characteristics of D-21 in NUDYT 2018-19

Quality characters	NUDYT 2018-19			Standards
	D-21	Fsd-08	D-97	
1000-grain weight (g)	38.9-39.0	35.1-37.6	37.25-38.10	≥30
Test weight kg/hl	69.0-78.5	66.6-73.6	63.89-64.25	≥68
Protein (%)	13.1-14.95	14.05-14.45	11.9-12.3	≥12
Pasta quality	Good	Poor	Fair	
Noodles quality	Good	Poor	Fair	

Table 12. Botanical description of Durum-21

Botanical description of Durum-21					
1.	***Variety***	**D-21**	10.2	Length	Medium
1.1	Parentage	FKN/3/2*FR//KAD/GB/4/BB/CHA/5/AS-2002	10.3	Color	Yellowish whit
1.2	Pedigree	PB20733-1a-2a-2a-0a-0a-19a-0a	10.4	Habit	Erect
1.3	Species	*Triticum durum* L.	**11**	***Anther***	
1.4	Breeder	Hybridization	11.1	Anther color at flowering	Yellow
1.5	Maintainer	WRI, Faisalabad	**12.**	***Rachis***	
1.6	Comparable variety	Durum-97	12.1	Hairiness of convex surface of the apical segment	Medium
1.7	Area of adaptation	All Punjab	12.2	Hairiness of margin	Medium
1.8	Approval status	Approved	12.3	Length	5.2-6 cm
2.	***Maturity duration***	150-160 Days	12.4	Width	3.8-4.0 mm
3.	***Sowing time***	November 01 to December 10	12.5	No. of segments	16-18
4.	***Seedling anthocyanin***	Absent	**13**	***Glume***	
4.1	Coleoptile color	Colorless	13.1	Length	13.5-14.5 mm
5.	***Plant***		13.2	Width	3.8-4.0 mm
5.1	Growth at seedling	Semi erect	13.3	Attachment	Medium
5.2	Growth at booting	Semi erect	13.4	Shoulder width	Narrow
5.3	Color at booting	Green	13.5	Shoulder shape	Elevated
5.4	Tillers per m^2	350-450	13.6	Beak length	2.5-3.0 mm
5.5	Height	96-100 cm	13.7	Beak shape	Straight
6.	***Stem***		13.9	Pubescence	Absent
6.1	Waxy bloom	Medium	13.10	Surface	Smooth
6.2	Anthocyanin	Absent	13.11	Internal hair	Absent
6.3	Wall thickness	Intermediate	13.12	Internal imprint	Absent
6.4	Stiffness	Intermediate	13.13	Keel spicules	Present
6.5	Color	Green	**14.**	***Seed***	
6.6	Diameter	3-4 mm	14.1	Color	Amber
6.7	Peduncle length	42-45 cm	14.2	Shape	Ovate
6.8	Nodes/stem	4-5	14.3	Length	6.6-7.5 mm
7.	***Flag leaf***		14.4	Width	3.5-3.9 mm
7.1	Attitude	Erect	14.5	Thickness	2.7-2.8 mm
7.2	Twist	Medium	14.6	Size	Medium
7.3	Length	27-30 cm	14.7	Germ size	Medium
7.4	Width	1.9-2.1 cm	14.8	Brush	Medium
7.5	Sheath waxy bloom	Medium	14.9	Groove	Intermediate
8.	***Auricle***		14.10	Hardiness	Hard
8.1	Hairiness	Absent	14.11	Surface	Opaque
8.2	Anthocyanin	Weak	14.12	1000 kernel weight	37.13-39.24 g
9.	***Ear***		14.13	Seeds/ear	55-65
9.1	Emergence	110-120 Days	14.15	Protein content	12-13%
9.2	Waxy bloom at anthesis	Medium	**15.**	***Baking***	
9.3	Color at maturity	Yellowish white	15.1	Chapati	Fair
9.4	Size	Medium	15.2	Bread	Fair
9.5	Shape	Parallel	**16.**	***Resistance to***	
9.6	Density	Dense	16.1	Lodging	Resistant
9.7	Awnedness	Awned	16.2	Shattering	Resistant
9.8	Supernumerary spikelet's	Absent	16.3	Diseases	
9.9	Speltoides	Absent	16.3.1	Stem rust	Resistant
9.10	Shattering	Resistant	16.3.2	Leaf rust	Resistant
9.11	Kink/twist	Absent	16.3.3	Stripe rust	Resistant
10.	***Awns at maturity***		16.3.4	Stem rust	Resistant
10.1	Distribution	Whole			

considered the founder of the green revolution. Up till now, it has released more than 60 high-yielding varieties of wheat for general cultivation in the province of Punjab as well as in Pakistan. But D-21 is the first variety of durum which is particularly developed for pasta production in Pakistan as it contains better quality traits from the industrial point of view. It was concluded from the current research that the newly developed durum variety D-21 has superior quality traits for pasta production, high yielding, resistance to leaf and yellow rust, and gave a good performance in multi-location of Punjab as well as Pakistan.

5. ACKNOWLEDGMENTS

Wheat Research Institute Faisalabad is highly thankful to "CIMMYT" for the provision of basic breeding material to strengthen the durum wheat breeding program at WRI, Faisalabad. Moreover, Wheat Research Institute

Durum-21: Field view at heading stage

Durum-21

Durum-21: Spikes with grains

Durum-21: Spike

Fig. 1. Pictorial view of Durum-21 (Plant, grains, and spike)

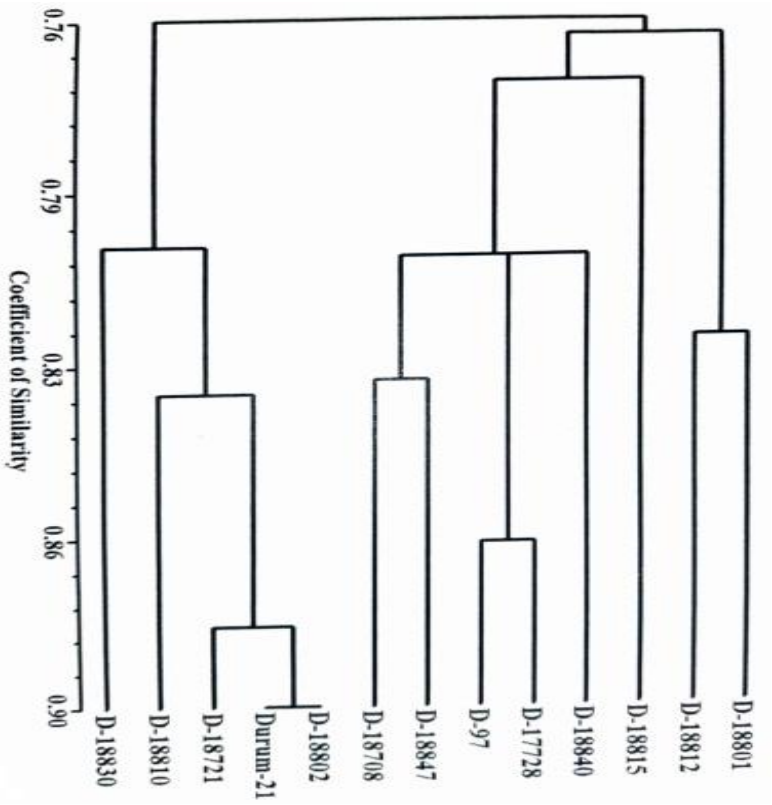

Fig. 2. Dendrogram of different Durum on the basis of similarity

Faisalabad acknowledges the "PARB" for the provision of funds for the development of the newly released variety "D-21".

6. CONFLICT OF INTEREST

All the authors declared no conflict of interest.

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Proceedings of the Pakistan Academy of Sciences: B
Life and Environmental Sciences 60(3): 393-402 (2023)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(60-3)788

Pakistan Academy of Sciences

Research Article

Efficiency Evaluation of Silver Nanoparticles in the Controlling of the Fungi Associated with the Date Palm Offshoots

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Abstract: Recently, Iraq has imported large numbers of tissue culture date palm offshoots from different countries. It is to build new orchards of date palm trees or plant them with the old orchards and some of them in the home's gardens. As a result of the widespread of many symptoms associated with these offshoots, this study was conducted in Basra Governorate, Iraq. To examine the capability of silver nanoparticles in controlling pathogens. The 36 fungi species were isolated from the shoot system of tissue culture date palm offshoots. *Alternaria* sp. was recorded at a high frequency compared to the *Cladosporium* spp. and *Ulocladium* spp. *Neodieghtonia phoenicum*, *Scytalidium lignicola*, and *Neoscytalidium dimidiatum* caused black scorch. Moreover, *Phoma costarricensis* has been recorded as causing the leaf spot disease. The roots infected by wilt disease have shown three various fungi, *Fusarium solani*, *Fusarium proliferatum*, and *Fusarium fujikuroi*. The study also illustrated that silver nanoparticles possessed a high ability to inhibit fungi growth in the laboratory

Keywords: Silver nanoparticle, leaf spot, black scorch, wilt disease.

1. INTRODUCTION

The Date palm (*Phoenix dactylifera* L.) is mostly found in the Middle East countries such as Iraq; dates are of great nutritional importance because they contain energy sources and vitamins as well as antimicrobial, antioxidant, anti-inflammatory, mutagenic, anticancer, and stomach and liver protection activities [1]. It can be infected by many diseases, whether the vegetative or root systems. Most of the reported date palms diseases are attributed to fungal pathogens such as Bayoudh disease wilt disease, black scorch, and leaf spot disease [2]. Nanotechnology can potentially decrease many challenges in disease control by reducing chemical inputs and enhancing the fast detection of pathogens [3]. Silver nanoparticles are the first to be used in plant disease control because of their antimicrobial activity [4].

This is because Iraq imported large numbers of tissue culture offshoots from different countries, complete orchards were formed from it, and others were planted overlapping with the old orchards and some home gardens. Because of the spread of many disease symptoms associated with these offshoots such as death and wilt of tissue culture offshoots, leaf spots, black scorch, and due to the lack of adequate studies on the fungi associated with the date palm offshoots produced by using the tissue culture technique [5, 6]. One of the modern strategies for controlling pathogens is the use of silver nanoparticles. This study aimed to detect the pathogenic fungi on date palm offshoots and secondly to evaluate the role of silver nanoparticles in its control.

2. MATERIALS AND METHODS

2.1. Isolation of Tissue Culture Offshoots Leaves and Roots

Samples were collected from the date palm offshoots produced from the tissue culture grown in the different areas of Basrah, Iraq showing symptoms of leaf spots and black scorch. The plant parts were taken from the leaves. These leaves were cut into smaller pieces and sterilized with 10 % sodium hypochlorite for two minutes. After that, these parts were washed with sterile distilled water

Received: November 2022; Revised: March 2023; Accepted: May 2023
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to get rid of the chloride effect. Then, the samples were dried with sterilized filter paper. Every four pieces were transferred by sterile forceps to Petri dishes, containing Potato Dextrose Agar (PDA) which was sterilized with an autoclave instrument. The antibiotic Chloramphenicol 250 mg/L was added to the dishes and incubated at 25 ±1 °C for 5-7 days. The fungal growths were examined using a microscope. The fungi were purified on a PDA media for morphological diagnosis (genus and species level). The isolates were kept in test tubes containing a PDA culture medium at sloping. Then the samples were saved in the refrigerator until use. As for isolating from the roots, samples were taken from the roots of date palm tissue culture offshoots less than three years old showing symptoms of yellowing and wilting, and the same steps were taken for isolation from the leaves. Fungi were morphologically identified based on the following taxonomic keys [7-11].

2.2. Molecular Identification of Isolated Fungi

DNA of isolated fungi was extracted by using (gSYNC TM DNA Extraction Kit). Primers F: TCCGTAGGTGAACCTGCGG: and R: TCCTCCGCTTATTGATATGC were used to amplify the region of the ITS1-ITS4 gene. The amplification process, electrophoresis technique was to isolate the fungal DNA. The gel was checked with a gel documentation instrument to examine the quality of the bands in the gel and determine the success of the DNA amplification process [12]. A 20 µl of the amplification product for each isolate was sent to the Korean company Macrogen for the nitrogenous base sequences and investigated by the National Center for Biotechnology Information.

2.3. Pathogenicity Experiment of Fungi Isolated from the Shoots

The separated leaves method was used with some modifications [13]. This method involves a 20 cm long piece taken from the fronds of the third basement of the date palm Al-Sayer cultivar. At first, the leaves were washed with tap water after removing the leaflets, then sterilized with 70 % ethanol, washed with sterile distilled water, and dried with filter paper then three holes were punched in each piece using a sterile 0.5 cm cork borer to put in each hole. A 0.5 cm disc taken from the edge of a seven-day-old colony of fungi isolated and grown on PDA. Each hole was wrapped with cling film, for two days after inoculation with the pathogenic fungi. The pieces of leaves were placed in 1-Liter flasks containing 30 mL of sterile distilled water. The nozzles of the flask were blocked with sterile aluminum foil. The flasks were incubated in the incubator at a temperature of 25 ± 1 °C for 21 days. The development of the spot was observed on the rachis pieces every 7, 10, 15, and 21 days. The radius average of the damaged tissue around the site of injury was measured by the ruler, the experiment was carried out using three replications. The control treatment included a placing of 0.5 mm disc PDA in the holes. The development of symptoms was monitored and the diameters of resulting spots were measured.

2.4. Pathogenicity Experiment of Fungi Isolated from Roots

This experiment was carried out using date palm seedlings resulting from the cultivation of the seeds of the Hillawi cultivar. The soil and peat moss mixture (2:1) was sterilized by the autoclave at 121 °C for 15 minutes. After one day of sterilization, it was placed in plastic pots of 1 kg in equal quantities, the soil was contaminated with isolates of *Fusarium* spp., which grown on Millet with ratio of 1 % w/w [14]. The control treatment was sterilized. The millet seeds were added to the same ratio, and then the soil in the plastic pots was moistened for planting using the seeds of the cultivar Hillawi. The pots were planted at a rate of 10 seeds per pot. The Control treatment included the cultivation of seedlings of the Hillawi cultivar in the sterile soil that does not contain the previous fungi. The experiment lasted for two months, during which the percentage of germination and death of seedlings for all pots was recorded according to the following equations:

$$\text{Germination \%} = \frac{\text{number of germinated seeds}}{\text{Total number of seeds}} \times 100$$

$$\text{Seedling's death \%} = \frac{\text{Number of dead seedlings}}{\text{Total number of seedlings}} \text{ x } 100$$

The experiment was performed based on the complete randomized design (CRD) with three replications for each treatment.

2.5. Evaluation of the Efficiency of Silver Nanoparticles in Inhibiting of the Growth of Pathogenic Fungi

The silver nanoparticles AgNPs with a size of 20 nm were obtained from the Chinese company Hongwu International Group Ltd. A 1000 parts per million standard solution was prepared in 1-liter flasks containing 500 ml of potato dextrose Agar (PDA) to obtain the various concentrations (0, 25, 50, 75, and 100 ppm) of silver nanoparticles. The flasks were shaken well with the amount of Agar to PDA, and then the amount of the standard solution of silver nanoparticles was added. PDA was poured into petri dishes with a size of 9 cm. After solidification media, the center of each plate was inoculated with a 0.5 cm disc from the culture of each pathogenic fungus taken from the edge of a 7-day-old newly grown colony. The dishes were incubated at a temperature of 25±1 °C until the growth of the pathogenic fungi in the control treatment was completed. The experiment was carried out in three replications for each treatment, thereafter the radial growth of the fungi was measured according to the equation:

$$\text{Inhibition\%} = \frac{\text{the colony diameter in treatment} - \text{the colony diameter in cont}}{\text{the colony diameter in control}} \times 100$$

All laboratory experiments were carried out with a complete random design (CRD) procedure, and the averages were compared with the LSD. Test. Below the 0.01 probability level [15]. The data were analyzed statistically using the SPSS statistical program.

3. RESULTS AND DISCUSSION

3.1. Isolation of Fungi from Date Palm Shoots

The results of fungal isolation from plant parts infected with leaves spot and black Scorch diseases on the shoots (Table 1) showed the isolation of 36

Table 1. Fungi Isolated from Leaf Spot, Black Scorch and Wilt Disease of Date Palm Tissue Culture Offshoots. Where: A=Leaf Spot Disease, B=Black Scorch Disease, C=Wilt Disease

Isolate name	A	B	C	*Isolate name*	A	B	C
Alternaria alternata	+*	+	**-	*Rhizopus sp.*	-	-	+
Alternaria aspera	+	+	-	*Thielaviopsis paradoxa*	-	+	-
Alternaria botrytis	+	-	-	*Aspergillus restructus*	-	+	-
Alternaria chartarum	+	+	-	*Aspergillus peniciliodes*	-	+	-
Alternaria chlamydospora	+	+	-	*Penicillium spp.*	-	+	+
Alternaria concatenata	+	+	-	*Chaetomium sp.*	-	+	-
Alternaria dianthicola	+	+	-	*Rhizoctonia solani*	-	+	-
Alternaria longipes	+	+	-	*Fusarium solani*	-	-	+
Alternaria penicillata	+	-	-	*Fusarium proliferatum*	-	-	+
Alternaria Petroselini	+	-	-	*Fusarium fujikuroi*	-	-	+
Alternaria radicina	+	+	-	*Nigrospora sphaerica*	+	-	-
Alternaria tenuissima	+	+	-	*Neoscytalidium dimidiatum*	+	+	-
Bipolaris australiensis	+	+	-	*Aspergillus spp.*	-	-	+
Cladosporium cladosporoides	+	+	-	*Ulocladium sp.*	-	+	-
Cladosporium elatum	+	+	-	*Ulocladium alternariae*	+	+	-
Cladosporium herbarum	+	+	-	*Ulocladium atrum*	+	+	-
Cladosporium oxysporum	+	-	-	*Stemphylium sp.*	+	+	-
Drechslera biseptata	+	+	-	*Scytalidium lignicola*	-	+	-
Fusarium chlamydospora	+	+	-	*phoma exigua*	-	+	-
Fusarium oxysporum	+	+	-	*Phoma costarricensis*	+	-	-
Neodeightonia phoenicum	-	+	-	*Trichoderma sp.*	-	-	+

*Isolated ** non-isolated

species of fungi, 12 of them belong to the genus *Alternaria*, four belong to the genus *Cladosporium*, and three species belong to the genus *Ulocladium*. Thirty-one (31) species were isolated from palms affected by leaf spot disease, and 32 species were associated with black Scorch symptoms [16]. Mentioned the above fungi as major genera associated with the symptoms of date palm leaf spot disease in Basrah. The results of the study also showed the isolation of new species of fungi associated with date palm leaf spots disease, such as *A. petroselini*, *A. penicillata*, *A. botrytis*, *Cladosporium oxysporum*, and *P. costarricensis*. Most of these fungi were registered as fungi accompanying disease states for many plants [17]. isolated *C. oxysporum* from tomato plants infected with leaf spots in greenhouses. Misawa and Kurose [18] showed that *A. petroselini* was isolated from parsley which showed symptoms of leaf blight and stem rot.

No studies refer to the isolation of the fungus *P. costarricensis* as a cause of date palm leaf spot disease, as it is one of the common fungi on the Arabica coffee plant (coffee) in South American countries such as Costa Rica and Brazil, and it causes spot and blight diseases [19]. As for Black Scorch Disease, *T. paradoxa* was isolated, and this is consistent with previous studies of this disease, as it was recorded by Klotz [20] as a cause of black scorch disease on date palms for the first time. After that, many researchers in Iraq mentioned this fungus as a cause of black Scorch, terminal bud rot, and deterioration of date palms [21]. In addition to the fungi above, many fungi were isolated, the most pathogenic of which was the fungus *N. phoenicum*, which was isolated and recorded for the first time in Iraq as a cause of black Scorch This is consistent with many studies that indicated the pathogenicity of this fungus on palms, as the genus *Neodeightonia* is an important fungus belonging to the family Botryosphaeriaceae, which is characterized by causing important and common diseases on plants. Abbas *et al* [22] recorded the fungus *N. Phoenicum* on date palms in Greece, *N. phoenicum* in Qatar was also found by Ligoxigakis *et al.* [23] on date palm trees. The results of fungi isolation from the roots of plants infected with wilt disease showed the isolation of several types of fungi, most of which belong to the genus *Fusarium*, namely *F. solani*, *F. proliferatum*, and *F. fujikuroi*. This result is consistent with previous studies in which it referred to the isolation of one or several species of the fungus *Fusarium* with disease states similar or close to the wilt cases studied such as *F. oxysporum*, *F. monliforme*, *F. proliferatum*, and *F. solani* [24-26]. These results are in agree with many previous studies in which it was mentioned the relationship between the *Fusarium* species with date palm wilt diseases such as *F. solani* [6]. Khazaal and Ameen [27] Isolated *F. proliferatum* and *F. fujikuroi*, in addition to other species of the same genus, from the roots of date palms affected by sudden Decline syndrome in Basrah Governorate. Alananbeh *et al*. [28] Indicated that *F. proliferatum* is associated with yellowing and death of date palms in Jordan. In the United Arab Emirates, it was reported that the fungus *F. solani* was isolated from shoots infected with wilt, death, and drying of palm fronds [29].

3.2. Pathogenicity of Fungi isolated from Date Palm Shoots

The results of the pathogenicity test (Figures 1 and 2) showed the ability of the tested fungi to cause infection and the appearance of disease spots after 21 days of inoculation. The fungus *N. phoenicum* recorded the highest infection rate of 2.8 cm, an increased rate of 0.2 cm/day, with a statistically significant difference for all tested fungi except for *P. costarricensis*, which achieved an infection rate of 2.65 cm, with an increase of 0.189 cm/day, and *A. concatenata* and *S. lignicola* each achieved an infection rate of 2.55 cm and 2.48 cm and an increased rate of 0.182 cm/day and 0.177 cm/day for both fungi, respectively. As for the rest of the tested fungi, the rates of infection spread on the leaves (spots size) ranged between 1.5 to 1.1 cm. *A. restructus*, *A. peniciliodes*, *Penicillium spp*., *Chaetomium spp.*, and *R. solani* have recorded no infection on date palm leaves. Many fungi that have proven their ability to cause infection and the emergence of disease spots were recorded in previous studies as pathogens on date palms [16]. Recorded the fungi *A. alternata*, *B. australiensis*, *C. herbarum*, *F. oxysporum*, and *T. paradoxa* as the causative fungi for date palm leaf spot disease. Alasadi and Alnajim [30] Recorded the fungus *A. dianthicola* as the cause of leaf spot disease on date palm and canary palm. The fungus *A. radicina* was recorded as a cause of black spot disease on date palm leaves in Basrah [31].

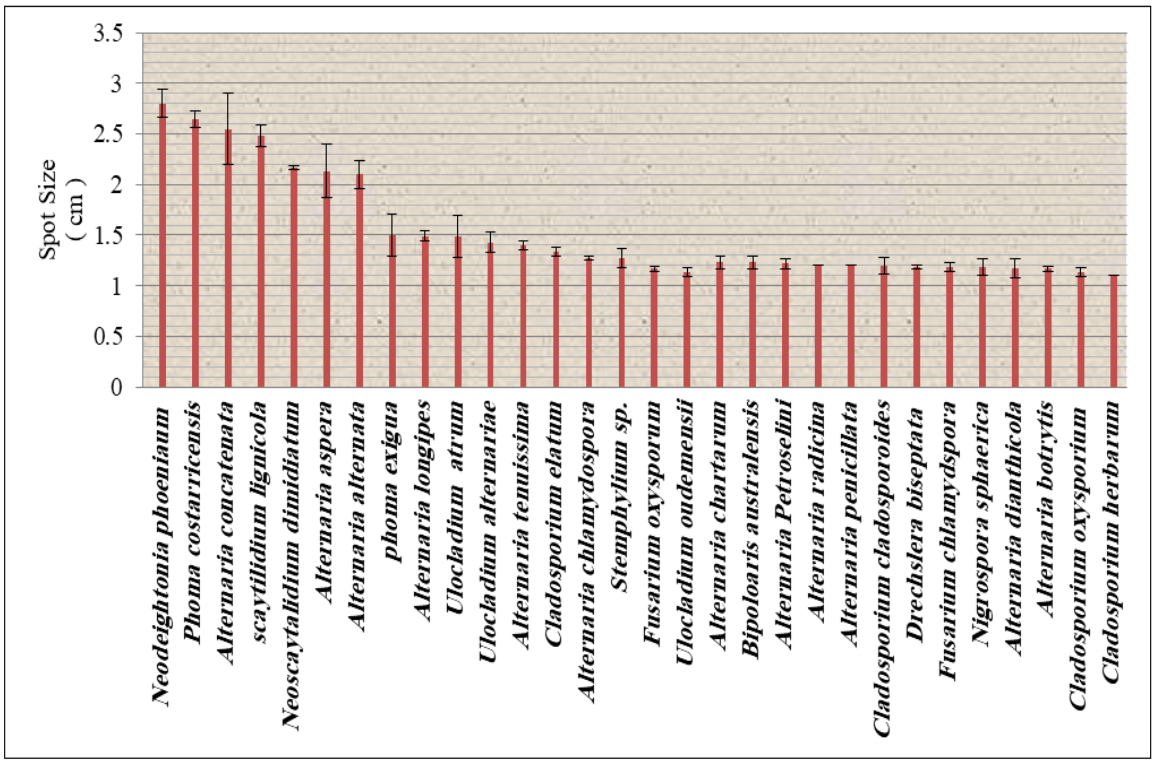

Fig. 1. The size of the diseased spot in centimeters after 21 days of inoculation with fungi isolated from the shoot system.

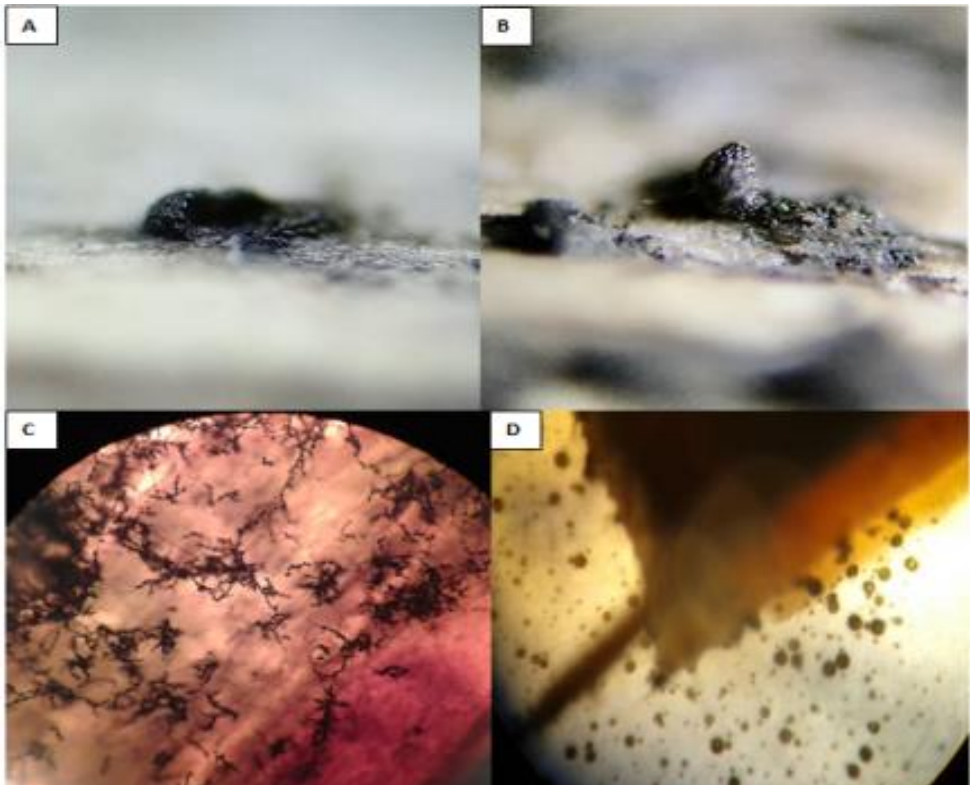

Fig. 2. The structures formed by some fungi on the surface of the plant tissue (leaf) after conducting a pathogenicity experiment. A. The pycnidium of *N. phoenicum*, *B. Aggregation* of conidia after releasing from pycnidium of *N. phoenicum*, *C. Conidia* of *A. alternata*. D. Pycnidia of *P. costarricensis*.

3.3. Pathogenicity Test for the Tungi Root System

The results of this study in Figures (3, 4, and 5) indicated that *Fusarium spp*. Have a negative effect on the germination of date palm seeds. The percentage of germination was 53.33 % for isolate *F. fujikuroi* F3 and *F. solani*, and it reached 56.67 % for isolate *F. proliferatum* F1, *F. fujikuroi* F4, and *F. proliferatum* F7, while it reached 93.33 % in the control. The results revealed significant differences in the percentage of seedling death of date palms, which amounted to 76.67, 76.67, 80.00, 76.67 and 83.33 % for the fungus *F. proliferatum* F1, *F. fujikuroi* F3, *F. fujikuroi* F4, *F. solani* and *F. proliferatum* F7. respectively, while the control treatment (free from pathogens) amounted to 16.67 %. These results were consistent with previous studies that confirmed the role of the fungus *Fusarium* spp, in reducing the germination of seeds of many different plants, including date palm seeds [32-34]. The variation in the percentage of seed infection with *Fusarium* species may be due to the genetic variance among the species of the genus *Fusarium* spp. or to the ability of fungal isolates to produce various enzymes or to secrete many different toxic substances. The ability of the fungus *Fusarium* spp. to cause the death and rotting of seedlings in many different plants may be due to its ability to the production of cell wall-degrading enzymes such as Chitinase, Polygalacturonas, and

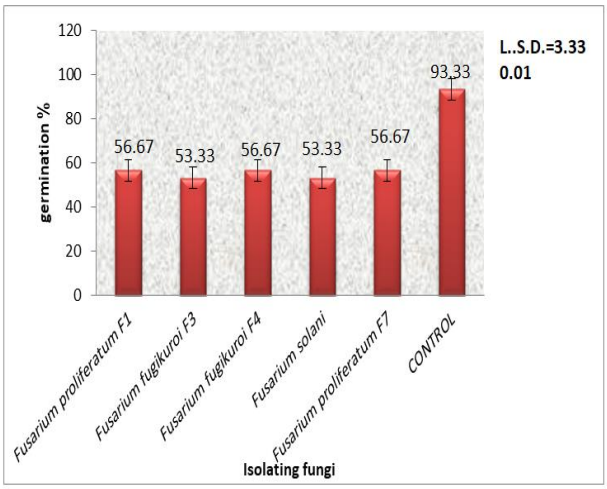

Fig. 3. Effect of fungi isolated from the roots on the percentage of germination of date palm seeds.

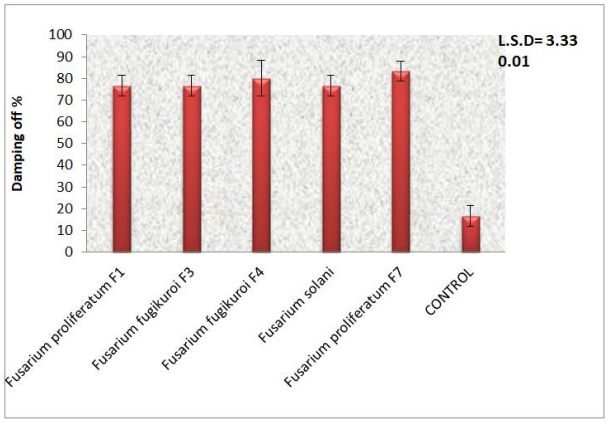

Fig. 4. Effect of fungi isolated from roots on Damping off percentages of date palm seedlings.

Fig. 5. Symptoms of infection with *Fusarium* species on palm seedlings. 1=Control, 2=*F. proliferatum* F1, 3=*F. fujikuroi* F3, 4=*F. fujikuroi* F4, 5=*F. solani*, 6= *F. proliferatum* F7.

Cellulase [35] in addition to its production of many toxins such as Fusaric acid, Dehydro Fusaric acid and Lycomarsmin, which are among the main pathogenicity factors of this fungus [36].

3.4. Molecular Identification of Pathogenic Fungi of the Vegetative and Root System

The results of molecular identification were in agreement with the phenotypic identification Figure (6). Molecular identification is based on the amplification of the gene region ITS1-ITS4 showed that the fungal isolates F1 and F7 belong to the *F. proliferatum*, the isolate F1 was recorded in the NCBI under accession number OM535259.1, and the isolate of this fungus matched with the isolate registered under accession number MN871570.1, and the similarity percentage was 100 % between the two isolates. The second isolate F7 of the fungus *F. proliferatum* was recorded with the accession number OM535261.1 and it was identical to isolate MT509801.1 with a similarity percentage of 98.41 %. The results also showed that isolate F3 and F4 belong to *F. fujikuroi* and were recorded in the gene bank with accession number OM535264.1 and OM535265.1 respectively. The isolate of the *F. solani* was recorded with accession number OM535266.1, Molecular diagnosis of other fungi and their registration numbers in the gene bank are shown in (Table 2) Several previous studies indicated the pathogenicity of this fungus to the date palm [6, 22, 37, 38].

3.5. Effect of Different Concentrations of Silver Nanoparticles on the Radial Growth of Fungi Causing Date Palm Wilt Disease

Figure 7 showed that the percentage of growth inhibition of the tested fungi increased with increasing concentration of silver nanoparticles. The percentage of growth inhibition of the fungus *F. proliferatum* F1 at the concentration (25, 50, and 100 ppm) was 62.20, 65.86, 67.73, and 72.56 % respectively. While the percentage of growth inhibition of *F. fujikuroi* F3 was 48.50, 50.33, 64.40, and 66.60 %, respectively. It was 53.67, 54.40, 57.70 and 65.50 %, respectively, for *F. fujikuroi* F4, while the percentage of growth inhibition of *F. solani* was 53.30, 52.90, 70.57 and 77.70 %, respectively. While the percentage of growth inhibition of *F. proliferatum* F7 was 48.13, 63.26, 66.23, and 69.23, respectively. The results of this test were similar to the results of previous studies that indicated the ability of silver nanoparticles to

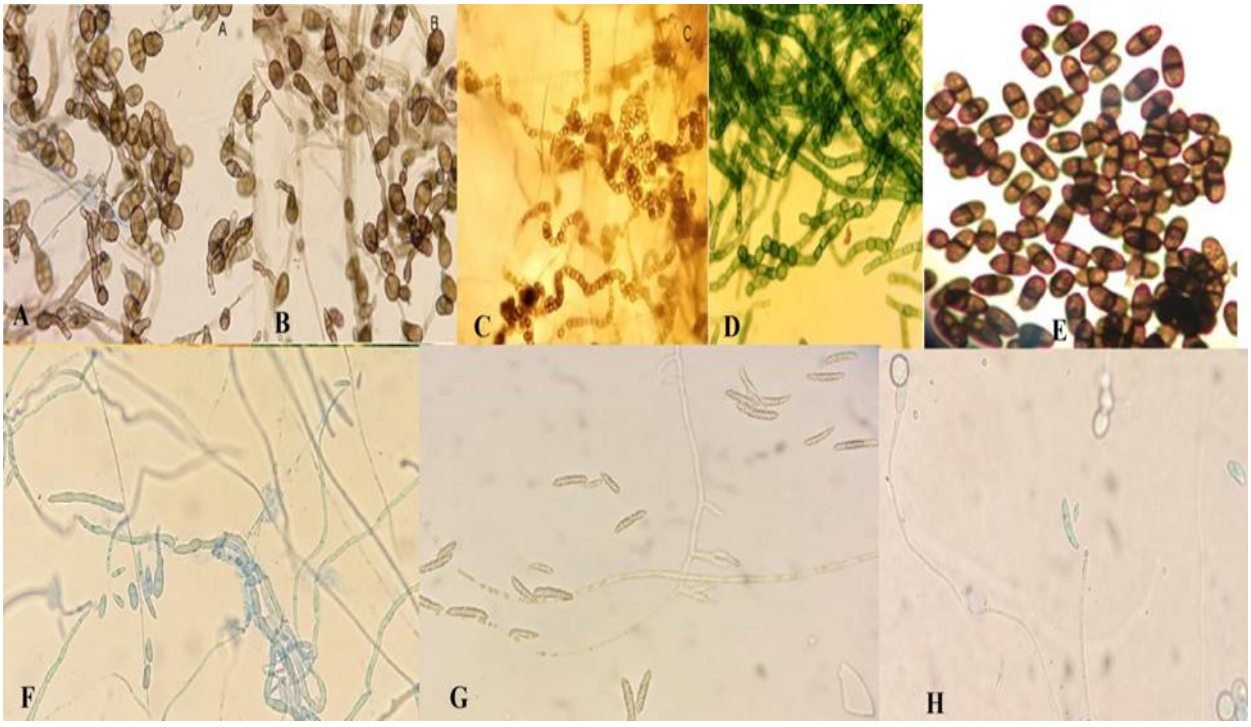

Fig. 6. Morphology of some fungi isolated from leaves and roots of date palm offshoots. *A: Alternaria tenuissima, B: Alternaria alternata, C: Neoscaytalidium dimidiatum, D: Scytalidium lignicola, E: Neodeightonia phoenicum, F: F. proliferatum G: F. fujikuroi H: F. solani*

Table 2. Molecularly identification of fungi that are registered in the gene bank (NCBI).

Scientific Name	Accession number	Accession Number of Matching Isolates	Percent Identity %	Query cover %
F. proliferatum F1	OM535259.1	MN871570.1	100	100
F. fujikuroi F3	OM535264.1	MG543727.1	99.80	100
F. fujikuroi F4	OM535265.1	MT603294.1	100	100
F. solani F6	OM535266.1	MG932644.1	94.85	100
F. proliferatum F7	OM535261.1	MT509801.1	98.41	99
A. alternata	OK235483.1	MW008974.1	100	100
A. tenuissima	OM562280.1	MK534954.1	100	100
N. dimidiatum	OM562604.1	MK480470.1	100	93
N. phoenicum	MZ675601.1	NR_111325.1	99.62	100
S. lignicola	OM585626.1	MG672013.1	97.99	87
P. costarricensis	OK255499.1	KT881552.1	95.34	99

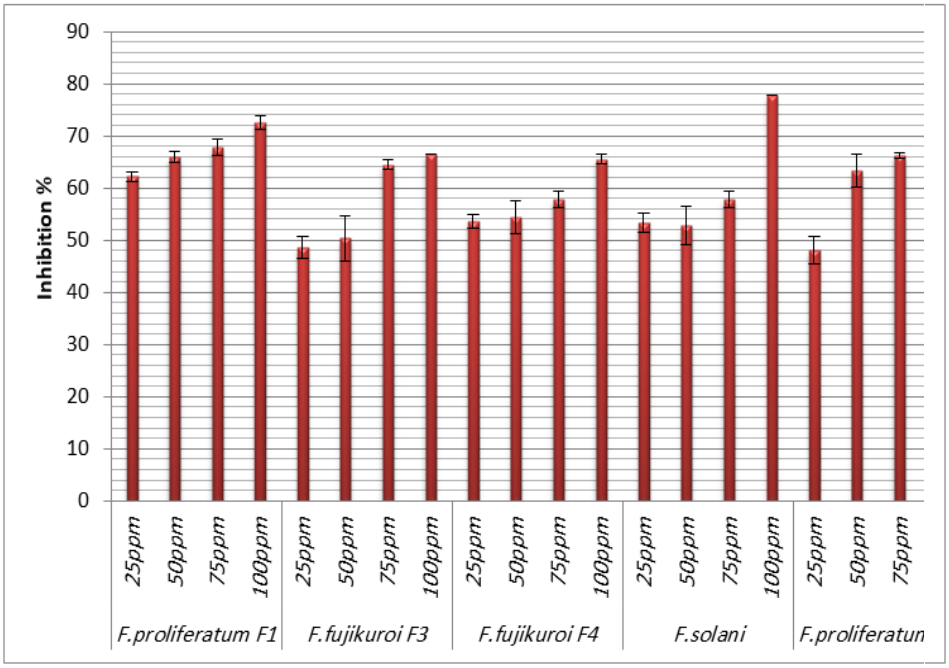

Fig. 7. Effect of different concentrations of silver nanoparticles on the radial growth of fungi causing date palm wilt disease.

inhibit the growth of different pathogenic fungi, such as *F. oxysporum* f.sp. radicis-lycopersici, *A. alternata*, *Botrytis cinerea*, and *Macrophomina phaseolina* [39, 40]. The results also showed that the effect of silver nanoparticles in inhibiting the growth of fungi increases with the increase in the concentration of Silver Nanoparticles used.

The effectiveness of silver nanoparticles is due to their ability to penetrate cells of microorganisms and disrupt the transport systems in cells, including ion exchange, which in turn affect important vital processes such as respiration and metabolism [41]. Silver ions may interact with oxygen, damaging cells and causing damage to proteins and fats, and nucleic acids [42]. Silver nanoparticles also cause an increase in the permeability of cell membranes by inhibiting the action of enzymes associated with cell membranes and affecting the process of gene expression [43-45] It is also found that Silver Nanoparticles disrupt the nucleic acid replication, which leads to disruption of the gene expression [46].

4. CONCLUSION

Most of the fungi recorded on the shoot and root systems of date palm trees resulting from tissue culture are similar to those isolated from date palm trees resulting from other methods of reproduction, such as propagation by offshoots. *P. costarricensis*, *N. phoenicum*, *S. lignicola*, and *N. dimidiatum* as new pathogens for the first time on date palm in Basra-Iraq. The silver nanoparticles in the laboratory played a major role in inhibiting the growth of pathogens.

5. CONFLICT OF INTEREST

There is no conflict of interest among the authors for publishing this manuscript.

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Proceedings of the Pakistan Academy of Sciences: B
Life and Environmental Sciences 60(3): 403-412 (2023)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(60-3)795

Pakistan Academy of Sciences

Research Article

Filamentous Fungi for Bioremediation of Oily Effluents of a Local Ghee Industry in Pakistan: An Environmental Perception

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Abstract: Mycoremediation is emerging as a potential approach for eco-friendly, cost-effective, and the most natural attenuation due to the biodegradation of polluted effluents from oil effluents which affect human health and the ecosystem. This work dealt with the analyses of the biodegradation capability of some potential indigenous fungal isolates viz., *Aspergillus flavus*, *Aspergillus niger,* and *Rhizopus stolonifer,* against oil effluents collected from a local ghee industry in Pakistan. Percentage reduction potential in different parameters i.e., pH, Electrical Conductivity (EC), Total Suspended Solids (TSS), Total Dissolved Solids (TDS), Biological Oxygen Demand (BOD), and Chemical Oxygen Demand (COD), confirmed that these fungi had the potential to degrade oily effluents. *Aspergillus niger* showed the highest reduction potential, while *A. flavus* and *R. stolonifer* had the least reduction potential to treat oil pollution. This indicates the potential of these identified fungi as biosorbents for removing high oil contents from industrial and wastewater discharge.

Keywords: Micromycetes, Mycoremediation, Eco-friendly, Waste management, Biodegradation

1. INTRODUCTION

Our ecosystem is under constant threat from continuing anthropogenic activities on the water by domestic, industrial, agricultural, shipping, radioactive, and aquaculture wastes. Industrial effluents are the major cause of pollution of water and soil in which these are discharged. This discharge belongs to various classes such as pesticides, fertilizers, hydrocarbons, phenols, plasticizers, biphenyls, detergents, oils, greases, pharmaceuticals, etc. These industrial effluents can have negative environmental impacts, causing climate change, loss of natural resources, air and water pollution, and extinction of species. These threaten the global environment as well as economic and social welfare. Thus, disturbing the ecological balance of the environment [1].

Oil industries are not only a significant source of energy but also a major supplier of raw materials for numerous products made from petroleum. On the other hand, the most hazardous and pervasive result of oil industry activities is pollution from oil and grease as toxic organic wastes, which not only destroy ecosystems but also cause biodiversity loss [2]. The layer of these discharged effluents on the water and soil surface reduces the amount of dissolved oxygen [3]. Maximum pollution levels in water bodies increase total suspended solids, total dissolved solids, chemical oxygen demand, and biological oxygen demand. After entering the ecosystem, they interact intricately with a variety of organic and inorganic pollutants, including waste from paper mills, sewage from the food and pharmaceutical industries, leaks from septic tanks, pesticides from agricultural runoff, and heavy metals from the mining and metal industries [4] This

Received: January 2023; Revised: May 2023; Accepted: June 2023
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increase makes water inappropriate for irrigation, drinking or any use for any other purpose [5].

Compared to traditional remediation methods, bioremediation (i.e., Mycoremediation) is more environmentally friendly. Fungal enzymes, in particular laccase and oxidoreductases, are widely used to remove contaminants from freshwater, marine, and terrestrial environments [6-9]. For instance, the ability of fungi to break down petroleum hydrocarbons, including alkanes, aromatic, and nitrogen-sulfur-oxygen-containing compounds, highlights the potential of fungi in bioremediation processes [10-13]. Fungi isolated from the environment of an oil spill can mitigate oil pollution. *Aspergillus* is naturally occurring in contaminated sites; its habitat's hazardous material levels are automatically reduced. As a result, *Aspergillus* strains might be thought of as organic cleansers that the environment uses to do bioremediation [14].

Due to their huge biomass and diversity, microbial communities play a vital role in the formation of activated sludge and serve as the main decomposers in wastewater treatment systems. They are particularly important in the processes of organic matter biodegradation and nutrient cycling [15]. The scope of the current study was to propose a suitable, cost-effective, and environment-friendly bio-treatment process for the small-scale biodegradation of ghee industry effluents, keeping in mind the significance of bioremediation. The objectives of the present investigation were the isolation and identification of some indigenous micromycetes of oily effluents and the evaluation of their potential efficacy in the remediation of pollutants.

2. MATERIALS AND METHODS

2.1. Sample Collection

The samples for this investigation were taken at regular intervals of every two weeks from various distances of the direct discharge point of the selected factory in Lahore, Punjab, Pakistan (i.e., from the main point, at a distance of 2 and 4 meters). Three samples were collected at each sampling point as well and their physicochemical parameters were analyzed. Electrical conductivity is a physical parameter while pH, Total Suspended Solids, Total Dissolved Solids, Chemical Oxygen Demand, and Biological Oxygen Demand are chemical parameters. The samples were further divided into two groups for pre and post-treatment analysis and were stored in sterile glass bottles [16]. All of the samples were transferred to the lab for additional examination.

2.2. Fungal Isolation and Identification

The fungal strains were isolated from the samples that were collected by using the pour plate method. Each effluent was poured into a separate, empty Petri dish in an amount of 100 microliters. The samples in the Petri dishes were then covered with the autoclaved Malt extract agar, which had been cooled to 50 °C. To ensure proper mixing, the Petri dishes were immediately swirled. Each sample was divided into three samples, which were then incubated at 27 °C for a few days to allow the colonies to reach maturity after which fungal development could be seen. Each pure fungal isolate was then sub-cultured onto a new medium containing ampicillin to prevent bacterial contamination. Taxonomic identification was performed based on the standard morphological characteristics of the isolated fungi at the species level by Molla *et al.* [17].

2.3. Treatment of Samples with Fungal Isolates

Most dominant taxa isolated were then cultured on collected samples from the Shan Ghee Industry. Fifty milligrams per milliliter of sample was aliquot in a beaker followed by aseptic inoculation of each of the fungal cultures separately. To prevent any contamination, aluminum foil was used to cover the beakers [18].

2.4. Parameters Analyzed for Mycoremediation

The parameters i.e., pH, Electrical Conductivity (EC), Total Suspended Solids (TSS), Total Dissolved Solids (TDS), Chemical Oxygen Demand (COD), Biological Oxygen Demand (BOD), and temperature were assessed in the laboratory. These parameters were selected for the analysis of effluents because the permissible limits of NEQS are available for them. Standard procedures were used to determine all the parameters [19]. All the

indicated parameters were analyzed both at pre and post-fungal treatment.

2.5. Determination of Biodegradation Potential of Selected Fungi

The reduction potential (Percent) of *Aspergillus flavus, Aspergillus niger,* and *Rhizopus stolonifer* were noted from post-treatment reading differences. In this way, the most efficient fungal strain was selected for further analysis. All obtained data was analyzed statistically to determine the percent reduction.

3. RESULTS

3.1. Micromycetes Isolated from Effluents

Nineteen fungal species (Fig. 1) were recorded during this study of which only three taxa were selected based on their occurrence frequency. *Aspergillus niger* was observed as the most abundant species, followed by *Aspergillus flavus* and *Rhizopus stolonifer* (Fig. 2).

3.2. Efficiency of Fungal Species in Controlling pH

The pH measures the amount of free hydrogen and hydroxyl ions present in water, which serves as an indicator of acids and bases. The pretreated sample collected from a direct source point showed a pH of 8.84 (Alkaline). Notably, the pH remains alkaline even after treatment with fungi while post-treatment of the direct source sample with *A. niger* has exhibited maximum capability to reduce pH to 8.64 (Table 1, 2, and 3), for a period of 6 days, as per the permissible limits of the National Environmental Quality Standards (N.E.Q.S) standard (6-10). Similarly, the sample of 2 m and 4 m away showed a pH reduction to 8.76 (Table 1, 2, and 3) and 8.78 (Table 1, 2, and 3) after being treated with *A. flavus* and *R. stolonifer,* respectively as per limits indicated above. *A. niger* exhibited maximum pH reduction (2.26 %) followed by *A. flavus* (0.90 %) and *R. stolonifer* (0.67 %) (Table 4).

3.3. Efficiency of Fungal Species in Controlling Electrical Conductivity (EC)

EC measures how well water can carry an electric current, which in turn is influenced by the ion concentrations in the solution. The EC value shouldn't have exceeded 400 S/cm, as per WHO guidelines. The EC value of the pretreated sample collected from a direct source was observed to be 766 while the post-treatment with *A. niger* revealed a gradual decrease of EC values from day one to day six, respectively soon by average (Table 1, 2,

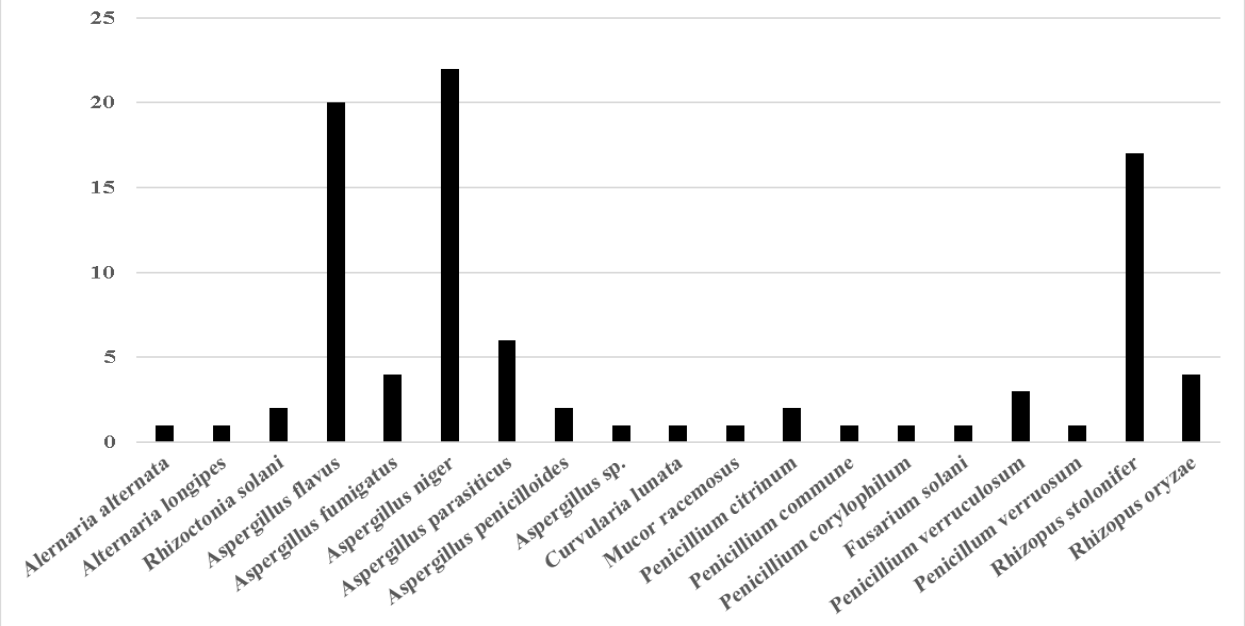

Fig. 1. Frequency of occurrence of fungal taxa isolated during this study from effluents.

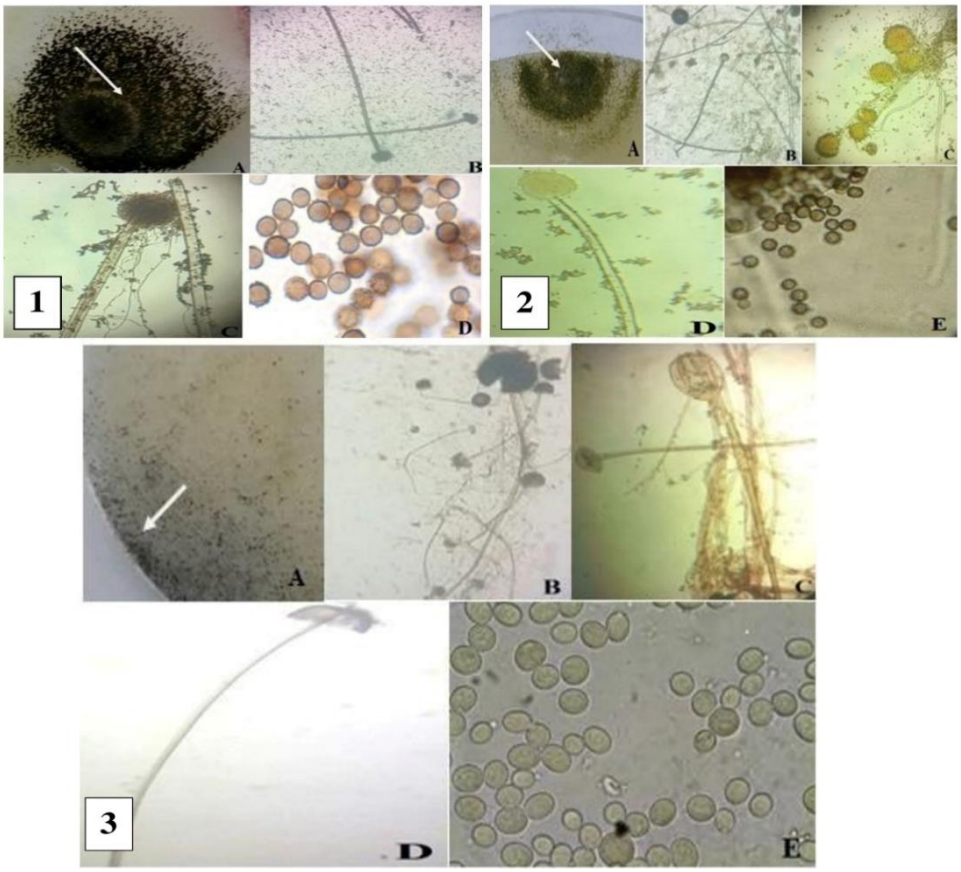

Fig. 2. Morphological characters of (1) *Aspergillus niger Colony,* (2) *Aspergillus flavus,* and (3) *Rhizopus stolonifer*

Table 1. Variation in all parameters in the direct source, 2 and 4 meters away from effluents treated with *Aspergillus niger* (Average of 6 days)

Aspergillus niger												
Parameters	**pH**		**EC**		**TDS**		**TSS**		**BOD**		**COD**	
N.E.Q.S	6-10		680 µS/m		3500 mg/L		150 mEq/L		80 mg/L		150 mg/L	
Effluents Collection	Bf. Trt.	Af. Trt.	Bf. Trt.	Af. Trt.	Bf. Trt.	Af. Trt.	Bf. Trt.	Af. Trt.	Bf. Trt.	Af. Trt.	Bf. Trt.	Af. Trt.
Direct Source	8.84	8.46	766	435	633	150	505	281	485	426	471	421
2 Meter	8.89	8.56	810	508	686	293	523	349	480	233	485	237
4 Meter	8.80	8.52	857	491	706	366	546	409	496	325.	490	330

Table 2. Variation in all parameters in the direct source, 2 and 4 meters away from effluents treated with *Aspergillus flavus (Average of 6 days)*

Aspergillus flavus												
Parameters	**pH**		**EC**		**TDS**		**TSS**		**BOD**		**COD**	
N.E.Q.S	6-10		680 µS/m		3500 mg/L		150 mEq/L		80 mg/L		150 mg/L	
Effluents Collection	Bf. Trt.	Af. Trt.	Bf. Trt.	Af. Trt.	Bf. Trt.	Af. Trt.	Bf. Trt.	Af. Trt.	Bf. Trt.	Af. Trt.	Bf. Trt.	Af. Trt.
Direct Source	8.84	8.76	766	410.17	633	216.7	505	357.17	485	433.67	471	448.33
2 Meter	8.89	8.66	810	635.67	686	275.2	523	380.33	480	313.83	485	313.83
4 Meter	8.80	8.62	857	491.33	706	366.5	546	409.83	496	325.67	490	330.33

Table 3. Variation in all parameters in the direct source, 2 and 4 meters away from effluents treated with *Rhizopus stolonifer (Average of 6 days)*

						Rhizopus stolonifer						
Parameter	**pH**		**EC**		**TDS**		**TSS**		**BOD**		**COD**	
N.E.Q.S	6-10		680µS/m		3500 mg/L		150mEq/L		80mg/L		150 mg/L	
Effluents Collection	Bf. Trt.	Af. Trt.	Bf. Trt.	Af. Trt.	Bf. Trt.	Af. Trt.	Bf. Trt.	Af. Trt.	Bf. Trt.	Af. Trt.	Bf. Trt.	Af. Trt.
Direct Source	8.84	8.78	766	532.67	633	273.17	505	370.00	485	450.83	471	445.5
2 Meter	8.89	8.797	810	618.83	686	332.17	523	381.17	480	469.83	485	448.7
4 Meter	8.80	8.688	857	671.83	706	419.67	546	387.17	496	333.83	490	349.5

and 3). Similarly, the samples treated with *A. flavus* and *R. stolonifer* showed a constant decrease of EC values on a regular basis and were noted within the acceptable limit of NEQS (680 micro-Siemens/cm). *Aspergillus niger* exhibited maximum EC reduction (27.8 %) followed by *A. flavus* (19.8 %) and *R. stolonifer* (12 %) (Table 4).

3.4. Efficiency of Fungal Species in Controlling Total Dissolved Solid (TDS)

Total dissolved solids (TDS) is a measurement of the total amount of inorganic and organic compounds that have been dissolved and are suspended as molecules, ions, or microscopic granules (colloidal sol) in a liquid. *A. niger* has the maximum capability to reduce TDS followed by *A. flavus*, while *R. stolonifer* showed the minimum ability to reduce TDS.

TDS levels within the 50–150 range are often thought to be the most suitable and acceptable range. TDS levels of approx.1000 PPM indicates that the water is hazardous and unfit for human consumption. The standard TDS (Table 1, 2, and 3) value according to N.E.Q.S is 3500 mg/L. The decrease in TDS value that was seen after being treated with *A. niger, A. flavus*, and *R. stolonifer* was within allowable ranges of standard. Similarly, the sample that was 2 meters away from direct discharge showed the reduction (Table 1, 2 and 3); 4 meters away samples reduction values were also within the permissible limits of standard (Table 1, 2 and 3). *A. niger* exhibited maximum TDS reduction

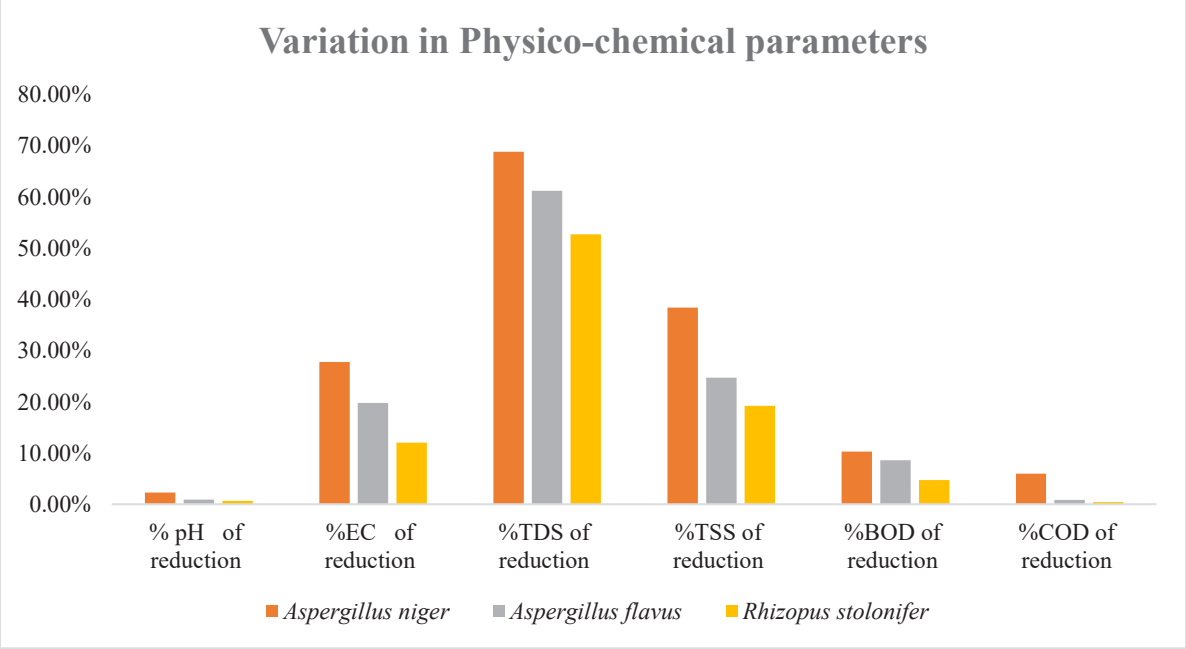

Figure 3. Variation in Physio-chemical parameters of *Aspergillus flavus, A. niger,* and *R. stolonifer*

(68.8 %) followed by *A. flavus* (61.2 %) and *R. stolonifer* (52.7 %) (Table 4).

3.5. Efficiency of Fungal Species in Controlling Total Suspended Solid (TSS)

Total suspended solids (TSS) is the dry weight of undissolved suspended particles that can be measured using a filtering system and captured by a filter. Higher concentrations of bacteria, minerals, pesticides, and metals in the water are frequently indicative of high TSS in a water body. The reduction in TSS values for the direct source sample was observed for a period of 6 days with *A. niger* exhibiting maximum capability to reduce TSS followed by *A. flavus* while *R. stolonifer* showed the minimum ability to reduce TSS. The reduction was noted within the permissible limits and according to the N.E.Q.S standard TSS value (150 mEq/L) (Table 1, 2, and 3). Similar observations in TSS reduction were noted with the sample 2 meters (Table 1, 2, and 3) and 4 meters (Table 1, 2, and 3) away as per the permissible limits of the standard indicated above. *Aspergillus niger* exhibited maximum TSS reduction (38.4 %) followed by *A. flavus* (24.7 %) and *R. stolonifer* (19.2 %) (Table 4).

3.6. Efficiency of Fungal Species in Controlling Biological Oxygen Demand (BOD)

When organic matter is decomposed aerobically (in the presence of oxygen) at a specific temperature, the term "Biochemical Oxygen Demand" (BOD) is used. So, BOD refers to how much oxygen is consumed by bacteria and other microorganisms. Relative reduction of BOD values was observed with a direct source sample for 6 days post-treatment with *A. niger* accounting for the maximum capability to reduce BOD followed by *A. flavus* >*R. stolonifer*. The BOD reduction was noted within the permissible limits and in accordance with the N.E.Q.S standard BOD value (80 mg/L), value means that effluents are severely polluted. Similar observations in BOD reduction were noted with samples at 2 meters (Table 1, 2, and 3) and 4 meters (Table 1, 2, and 3) away following the permissible limits of standards. *A. niger* exhibited maximum BOD reduction (10.3 %) followed by *A. flavus* (8.6 %) and *R. stolonifer* (4.7 %) (Table 4).

3.7. Efficiency of Fungal Species in Controlling Chemical Oxygen Demand (COD)

The Chemical Oxygen Demand (COD) determines the amount of oxygen required to chemically oxidize the organic and inorganic nutrients found in water, such as ammonia and nitrate. Relative reduction of COD values of direct source samples was observed for a period of 6 days with *A. niger* exhibiting maximum COD reduction (5.94 %) followed by *A. flavus* (0.84 %) and *R. stolonifer* (0.42 %) (Table 4) which was within the permissible limits and in accordance to the N.E.Q.S standard COD value (150 mg/L). Similar observations in COD reduction were noted with sample 2 meters (Table 1, 2, and 3) and 4 meters (Table 1, 2, and 3) away in accordance with the permissible limits of standards as indicated above. *A. niger* exhibited maximum COD reduction (5.94 %) followed by *A. flavus* (0.84 %) and *R. stolonifer* (0.42 %) (Table 4).

3.8. Variation in Physico-Chemical Parameters

There is a relative reduction in all the observed parameter values following the treatment with fungal species. The pH reduction was exhibited highest for *A. niger* (2.26 %) followed by *A. flavus* (0.90 %) > *R. stolonifer* (0.67 %). Similar reduction profile of EC was observed with *A. niger* (27.8 %) > *A. flavus* (19.8 %) > *R. stolonifer* (12 %). *A. niger* also exhibited the highest reducing ability of TDS, TSS, and BOD of 68.8 %, 38.4 %, and 10.3 %, respectively in comparison to the other two fungal isolates, where *A. flavus* accounting for a reduction of 61.0 % (TDS), 24.7 % (TSS), and 8.6 % (BOD) followed by the least reducing values observed of 52.7 % (TDS), 19.2 % (TSS), and 4.7 % (BOD) for *R. stolonifer.* (Fig. 3)

4. DISCUSSION

Micromycetes are involved in bioremediation, which is useful for the removal of wastes and harmful materials because these fungi naturally have the potential to decompose many materials [20]. The identification, individual characterization, and use of this fungus in bioremediation were the main reasons for the isolation of these organisms from samples. Fungi are more effective natural degraders

than conventional bioremediation methods, such as bacteria, as demonstrated by Batelle [21].

Aspergillus species were the most prevalent fungus that were successfully isolated from all the samples of fungal isolates. The filamentous fungi are the most frequently reported that can thrive on hydrocarbons. [22-24]. Oil effluents from the ghee industry in Pakistan showed higher BOD, COD, TSS, and TDS levels (above the acceptable limit of the National Environment Quality Standard) [25]. Notably, high BOD and COD values in the effluents cause a huge depletion of DO (Dissolved oxygen) in water which in turn, affects the aquatic life [26-28]. The current study demonstrated that treatment of the oil effluents with fungal isolates lowers the above-indicated parameters within the safety limits of NEQS and WHO [29].

All of the fungal species (tested) were able to degrade the effluents to varying degrees. Notably, *A. niger* was observed to be most abundant and efficient in the degradation of the oil effluents among the three tested fungi. The higher biodegradation efficiency of *A. niger* in comparison to the other two species, is consistent with a previous study [30-32] where the isolated fungal species from the tainted soil were used to bio-remediate crude oil from contaminated environments. Furthermore, the biodegradation capability of *A. niger* was also tested in another study [33] where it has been observed that it can degrade effluents up to 38.0 % in comparison to *A. flavus* and *A. foetidus* that degrades up to 31.20 and 26.1 %, respectively following 60 days' treatment of the effluents. Similar results revealed from another study by Buvansewari *et al*. [34] showed the degradation of sugar mill effluents by *Aspergillus* was the highest in comparison to *Penicillium* and *Rhizopus*.

The outcomes of this are coherent with those of Keren et al. [35], who found that the presence of oil in the soil increased the fungal population. Similar findings were reported at the same time by Jawhari [36], which showed that more frequency of *A. niger* with 100 % in all samples, but *A. fumigatus* and *Penicillium funiculosum* were 83 %. Similar research reports have shown an increase in microbial variety and population.

One of the key factors controlling the composition and activity of fungi is the hydrogen ion concentration [37]. Organic acids and other metabolic products are frequently produced as a result of the microbial degradation of hydrocarbons [38]. The chemistry of the pollutants was directly impacted by temperature, which also had an impact on the diversity of the microbial flora and the biodegradation of hydrocarbons [39]. Oil-contaminated soil and wastewater can be recovered quickly by the culture of fungus (*A. flavus, A. fumigatus*, *A. niger,* and *Penicillium* sp.) which reduces oil pollution to levels that permit the reuse of land and water [36].

The processes used by different refineries result in effluents with various chemical compositions, which depend on the type of treatment they have undergone. [40-43]. In the aquatic ecosystem, fungi and other microorganisms can degrade various pollutants, including crude oil, and use them as a source of nutrients. [41]. *A. flavus, A. fumigatus*, *A. niger,* and *Penicillium* sp. were among the oil-degrading fungi that were isolated from the soil and wastewater, and their density was greater than that of other fungi. These fungi were ideally adapted to break down and make use of both raw and refined oil [36].

The current findings demonstrated that pH remains alkaline both at the pre- and post-fungal treatment with a regular decrease of pH from 8.84 (pre-treatment) to 8.76 and these results are similar to findings [44] where refinery effluents were analyzed. Furthermore, this study indicates a decrease in both COD and BOD values after treatment which corresponds to the literature [45]. Moreover, degradation of TSS and TDS occurs as demonstrated by earlier studies [46-47]. Similarly, the degradation of TDS in crude oil was tested by three fungi individually where *A. niger* has the highest degradation of 53.7 % followed by *Candida* sp. (45 %) and *R. stolonifer* (35 %) [46-47]. To sum up, the current study exhibited that filamentous fungal species, viz., *A. flavus, A. niger, R. stolonifer* contributed efficiently to the reduction of pH, EC, TDS, TSS, BOD, and COD in the oil effluent samples, confirming remediation of effluents.

5. CONCLUSION

It is concluded from our research that *Aspergillus* is the main dominant genus in oily effluent samples which indicates its resistance and highest tolerance index towards oily effluents. The tolerance and the resistance of the isolates depended much more on the fungus tested than on the sites of its collection. This study recommends that the species of *Aspergillus* and *Rhizopus* isolated from effluents should be utilized for the bioremediation process. Fungi have been widely used in bioremediation of industrially polluted soils and waters. The results obtained confirmed the response of isolates towards biodegradation potential, its concentration in the medium, and the isolate under consideration. The results encourage future studies to optimize the tolerance and degradation assay using the isolates that showed the best results, as well as studies on the treatment of environments contaminated with different types of pollutants, including oily effluents.

6. CONFLICT OF INTEREST

All the authors have no conflict of interest.

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Proceedings of the Pakistan Academy of Sciences: B
Life and Environmental Sciences 60(3): 413-430 (2023)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(60-3)833

Pakistan Academy of Sciences

Research Article

Economics of Inter-Cropping: A Case Study of Onion and Tomato at District Muzaffargarh, Punjab-Pakistan

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Abstract: The current research is being conducted in Muzaffargarh district of Punjab, Pakistan to investigate the profitability and land equivalent ratio of intercropping onion and tomato. An economic analysis of intercropping in the Muzaffargarh district can inform farmers about the profitability and sustainability of this practice, aiding their decision-making between intercropping and monocropping. The study utilized a simple random sampling technique to select 45 vegetable growers out of 60, from two major vegetable-growing villages; Hajiwah and Beli Janubi. Descriptive analysis, including frequency distribution, mean, and percentages, was used to analyze the data. The results of the study showed that intercropping had a significantly higher yield (17897 kg/acre) than sole cropping of onions (6075 kg/acre) and tomatoes (16050 kg/acre). Intercropping also had a higher benefit-cost ratio of 1.59, compared to onion sole cropping (1.37) and tomato sole cropping (1.48). The land equivalent ratio was 1.31, which indicated that intercropping was more efficient in terms of land use than sole cropping. The study also revealed that intercropping onions and tomatoes provided additional income to farmers and helped maximize land use. However, farmers encountered challenges such as high seed costs, diseases, low output prices, and high transportation costs. In conclusion, the study suggested that intercropping onion and tomato is a viable agronomic strategy in the Muzaffargarh district, as it improves land-use efficiency and maximizes returns. The study showed that intercropping complemented each other and contributed to increasing yield per unit area and improving nutritional properties.

Keywords: Intercropping, Profitability, Land Equivalent Ratio, Onions and Tomatoes, Muzaffargarh

1. INTRODUCTION

Intercropping is a method of growing two or more crop species in the same field at the same time during the growing season [1] and it is the more efficient use of resources such as soil, water, nutrients, and solar radiation to grow two or more cultivars at the same time on the same land [2]. Intercropping is a traditional but important cropping system approach for increasing total productivity and farmer income, particularly in densely populated countries with limited per capita cropland [3]. Intercropping is effective in generating a variety of crops and is comparable in yield to sole cropping, while also increasing crop resilience, ecosystem services, and nutrient efficiency [4] and climate-resilient intercropping systems have great potential to reduce fossil fuel intensive inputs [5]. Farmers need local expertise and technical assistance based on locally-derived data to achieve optimal intercrop production [6]. Industrial agriculture can be easily diversified through intercropping, which involves the integration of alternative crops or non-crop plants alongside cash crops. This method is relatively simple and effective in promoting diversity within agricultural systems [7] and by incorporating intercropping methods, crop productivity can be significantly enhanced compared to conventional monoculture methods [8].

Received: January 2023; Revised: April 2023; Accepted: June 2023
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A wide range of intercropping has been developed around the world, in places like Indonesia, India, Niger, Mali, Central America, and Western Europe, because it significantly increases land productivity compared to monocultures [9, 10]. Intercropping can improve soil fertility as different crops have different root depths and can therefore access different soil layers, helping to improve soil structure and nutrient availability [11]. To achieve spatial complementarity in intercropping, different plants with varied root patterns should be grown together. For instance, combining deep-rooted and shallow-rooted crops can enable access to distinct soil volumes, enhancing resource utilization and reducing competition for resources [12]. Intercropping increases P availability in the rhizosphere of intercropped plant species [13], as well as improving soil resource utilization [14]. Furthermore, the system increases the land equivalent ratio [15, 16], lowering crop failure risk and increasing food security [17]. The cultivation of particular varieties in intercropping systems has a number of favorable effects [18] and alters the dominant microbial species and soil microbial communities [19, 20].

Higher-income and improved socioeconomic status were the primary drivers of intercropping adoption, and intercropping can be effectively adopted through field training and demonstrations [21]. Intercropping improves farm resource management by increasing total productivity per unit of land and per unit of time and can significantly reduce pest issues [22]. Transitioning to more bio diverse agricultural systems, such as intercropping and agroforestry, can serve as an adaptive measure against climate change. These systems offer a range of benefits at both the farm and ecosystem levels, including biotic, abiotic, economic, and social advantages [23] and intercropping can decrease surface soil evaporation and secondary salinization by increasing the surface coverage of the soil [24]. The practice of intercropping is most prevalent in developing nations [25] and because of its significant yield advantage over sole cropping, it has been recognized as a potentially useful technology to increase crop production [26]. Flexibility, profit maximization, risk reduction, soil conservation, soil fertility improvement, and lower production costs, as well as higher profitability, are some of the main motivations for smallholder farmers to intercrop [27]. Intercrops have the potential to provide a higher yield than sole crops, greater yield stability, efficient use of nutrients [28], reduced disease infestations, and a decrease in the number of pests and weeds [29]. A lot of scientists in the fields of agriculture and ecology are becoming increasingly interested in the intercropping approach to vegetable production as a result of the aforementioned qualities [30]. When lettuce is intercropped with onions, it helps to control Agrotis ipsilon, a significant insect pest that affects lettuce [31] and can enhance the natural suppression of pests [32].

Garlic and strawberry intercropping increase both the gross income and the land equivalent ratio, and intercropping systems had no impact on the production of strawberry pseudo fruits or garlic bulbs [33]. For the effective production of vegetables enriched in selenium, pakchoi and radish can be intercropped to increase selenium accumulation in the edible parts of the crops [34]. The nitrate content of the soil profile decreased because intercropping use soil nutrients more efficiently than sole cropping [35] and intercropping significantly decreased the frequency of forked carrots and increased cauliflower yield [36].

Intercropping maize with legumes has the potential to minimize crop failure risk, increase productivity and income, and increase food security in vulnerable agricultural systems [37] and intercropping also has some issues such as intercropping can lead to competition for resources such as water, light, and nutrients, which can reduce crop yield and quality [38]. Intercropping can be more complex to manage than monoculture systems as it requires careful selection of crop combinations, planting densities, and management practices [39] and some crop species may be incompatible with each other, leading to reduced growth and yield [40]. Both intercropping and rotation are effective methods for enhancing crop productivity and providing ecological benefits [41]. Intercropping can create microclimates that favor the development of certain pests or diseases like increased the incidence of maize stem borers and reduced maize yields compared to monoculture [42] and reduction in the yield of both total and marketable bulbs when onions are intercropped with coriander [43] and a reduction in yields

when leeks are intercropped with carrots [44]. The intercropping system had a significant impact on the yield attributes of different component crops, such as radish, small onion, and vegetable cowpea, but a single stand of component crops produced higher yield attributes than the intercropping system [45]. Muzaffargarh is known for its fertile land and the cultivation of various vegetables, particularly tomatoes. However, the majority of farmers in the region are small landholders with limited resources. Analyzing the cost and returns of intercropping can provide valuable insights into the economic feasibility of this farming practice in the area. The land equivalent ratio (LER) is a useful method for assessing the efficiency of intercropping and determining its effectiveness in optimizing land use and increasing yields. The research conducted in the Muzaffargarh district is a significant effort to understand the farming practices and challenges faced by small-scale farmers in the region, as well as to identify strategies for improving their productivity and economic viability. Overall, this research holds great promise for enhancing the livelihoods of farmers and promoting sustainable agricultural practices in the area. The research was carried out with the following objectives: (i) to examine the socio-economic characteristics and existing agronomic practices of sampled farmers; (ii) to analyze the cost and returns of intercropping for sampled farmers; (iii) to evaluate the land equivalent ratio (LER) to determine the efficiency of intercropping; and (iv) to identify constraints in the production and marketing of sampled farmers in the study area.

2. METHODS AND MATERIAL

2.1. Description of Study Area

Muzaffargarh is a district in the Punjab province of Pakistan that spans over an area of 8,249 km^2. It shares borders with the district Layyah to the north and Bahawalpur and Rahimyar Khan districts to the south, across the Chenab River. As per the 2017 census, the district had a population of 4.32 million people, and the literacy rate was 47 percent. The region is known for its agriculture, with numerous citrus and mango farms in the surrounding areas. The climate in Muzaffargarh is arid, with extremely hot summers and mild winters. The annual rainfall in the district is 127 millimeters, as illustrated in Figure 1.

Fig. 1. Map of the study area (District Muzaffargarh, Punjab-Pakistan)

2.2. Data Collection

The study was conducted as part of the "Strengthening Vegetable Value Chains in Pakistan" (SVVCP) project, which was funded by the Australian Centre for International Agricultural Research (ACIAR). The primary objective of this project was to enhance the value chains of three target vegetable crops in Pakistan (onions, potatoes, and tomatoes) using a community-based approach. The initiative aimed to improve the livelihoods and household incomes of resource-poor communities sustainably, by enhancing the capabilities of value chain actors such as farming families, traders, and intermediaries. The project focused on two villages, Baily Janobi and Hajiwah, in Muzaffargarh district, known for vegetable cultivation. To gather primary data, the Social Sciences Research Institute (SSRI) at NARC conducted a baseline survey, interviewing 45 vegetable growers who were randomly selected from 60 farmers involved in intercropping (onions and tomatoes) and sole cultivation of tomatoes. Of the 45 farmers surveyed, 24 were engaged in sole onion cropping as well. The selected farmers were interviewed using a structured questionnaire that covered topics such as nursery management, intercropping, farm management practices, and production and marketing constraints.

2.3. Data Analysis

To achieve the objectives of the study, a descriptive statistic was used to analyze the percentages, frequency, and mean. The profitability of intercropping was examined on the basis of gross margin, the net return, benefit-cost analysis, and land equivalent ratio.

- To estimate the cost of onion production, the following equations were used:

$$\mathbf{VC = \sum (X_i P_i)}$$

$$\mathbf{TC = TVC + TFC}$$

Where,
TC = Total cost of production (Rs. /acre)
TVC = Total Variable costs (Rs. /acre)
TFC = Total Fixed costs (Rs. /acre)
Xi = Quantity/Number of inputs per acre
Pi = Price of inputs (Rs. /acre)

- To estimate the profitability of onion production, the following equations were used:
Where,

$$\mathbf{GR = \sum (Y_i P_i)}$$

$$\mathbf{NR = GR\text{-}TC}$$

$$\mathbf{GM = GR\text{-}VC}$$

$$\mathbf{BCR = GR/TC}$$

GR = Gross return (Rs. /acre)
NR = Net return (Rs. /acre)
GM = Gross margin (Rs. /acre)
Y_i = Quantity of output (Kg/acre)
P_i = Price of onion (Rs. /kg)

- To find out the economics of the individual intercropping system, the following equation was used:

Land Equivalent Ratio (LER)

LER indicates the proportion or amount of land area that is needed for sole cropping to produce the same yield as intercropping [Mead and Willey, 1980]. The LER was calculated using the following formula to determine the economics of the individual intercropping system.

$$\mathbf{LER = L_1 + L_2 = YI_1/YS_1 + YI_2/YS_2}$$

Where,
L_1 and L_2 = LERs for the individual crops (tomato and onion)
YI_1 and YI_2 = Individual crop yield in intercropping
YS_1 and YS_2 = Yields as sole crops

If LER is > 1, intercropping is considered advantageous; if LER is < 1, intercropping is considered disadvantageous; and if LER = 1, then there is no profit or loss from intercropping.

3. RESULTS AND DISCUSSION

The study consisted of three sections. The first section examined the socioeconomic characteristics of the sampled farmers. The second section focused on farm characteristics, while the third section discussed the farming practices of the farmers.

3.1. Age Group of Sampled Farmers

The respondents' agricultural experience increased

with age, implying that older farmers had more risk-related interactions than younger farmers. According to the data, farmers between the ages of 41 and 50 (33 %) scored the highest, while those beyond 50 (16 %) scored the lowest (Figure 2). The sampled farmers' average age was 40.3 years, indicating that the majority of them were of working age and could increase agricultural production in the field with support and a supportive environment. Overall, the data implies that there is a correlation between age and agricultural experience, with older farmers having encountered and navigated through more risk-related situations. This suggests that elder farmers possess valuable insights and skills from their extensive exposure to diverse agricultural challenges.

3.2. Educational Level of Sampled Farmers

Based on the study findings, 38 percent of the sampled farmers were illiterate, 22 percent had only primary education, 27 percent had high school education, and 13 percent had graduate degrees (Figure 3). The average formal education of the sampled farmers was 5.1 years, which suggests that the majority of the sampled farmers in the research area had little formal education. The results illustrate that most farmers have limited formal education, impacting their ability to embrace new agricultural practices and adapt to changes. This underscores the need for focused educational efforts to narrow the knowledge gap and enhance their engagement with innovations.

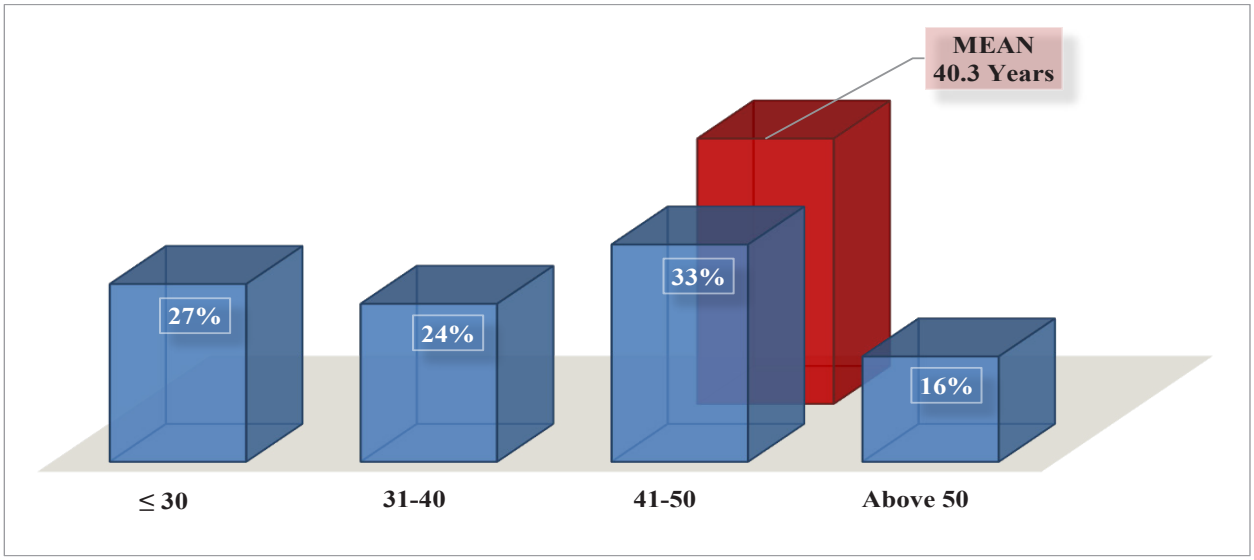

Fig. 2. Age group of sampled farmers

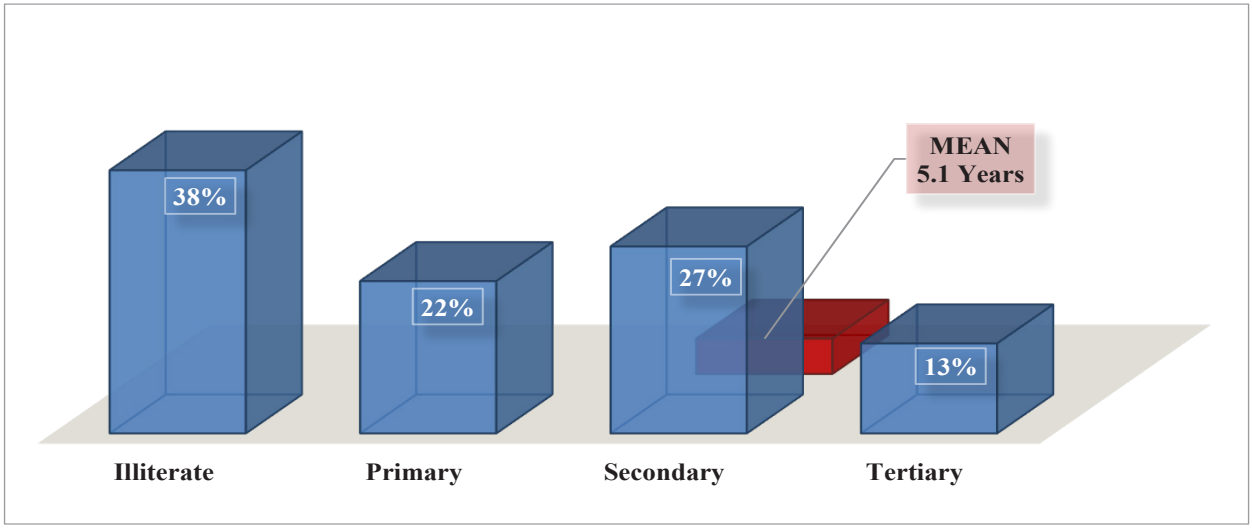

Fig. 3. Educational level of sampled farmers

3.3. Farming Experience of Sampled Farmers

The amount of risk exposure and the implementation of a risk management approach are both influenced by a farmer's level of farming experience. A farmer with numerous years of farming experience has greater knowledge than a farmer with little farming experience. According to Figure 4, the majority (44 %) of the farmers in the research region had a respectable amount of agricultural experience, which varied from 11 to 20 years, whereas (38 %) had less than 10 years of experience. Only 9 % of the farmers chosen had been farming for more than 30 years, while 9 % had been farming for between 21 and 30 years. The sampled farmers had an average of 15.9 years of farming experience. The diversity of farming expertise within the research area influences how individuals are exposed to risks and how they navigate uncertainties. Farmers with greater experience tend to be more skilled at managing agricultural uncertainties by leveraging their accumulated knowledge and making well-informed decisions.

3.4. Household Size of Sampled Farmers

Figure 5 findings regarding household size reveal that more than half (55 %) of the sampled farmers had 6–10 family members, followed by 27 percent who had less than 5 family members. The sampled

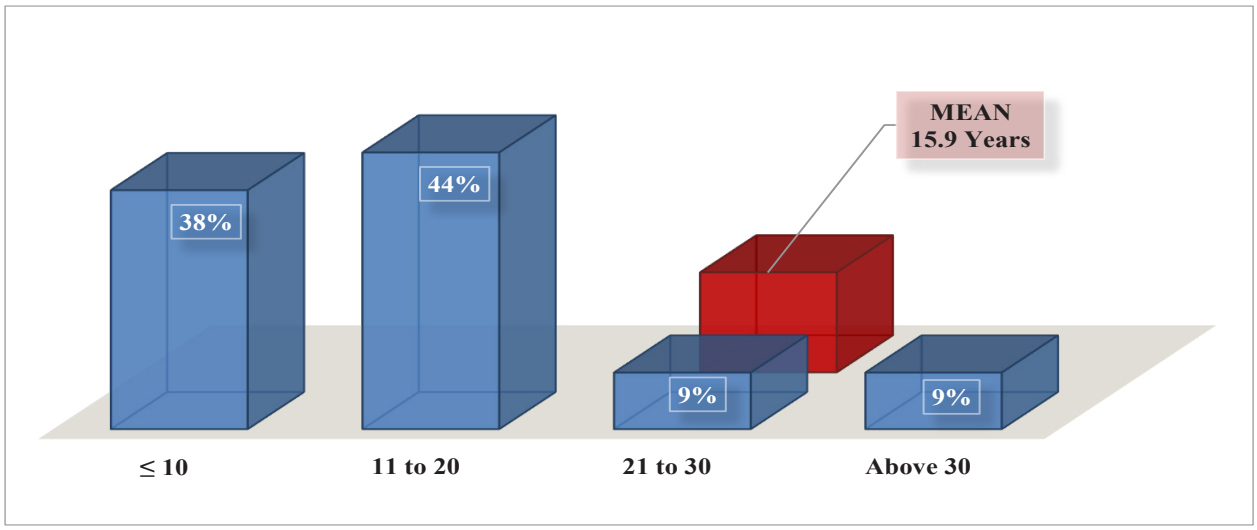

Fig. 4. Farming experience of sampled farmers

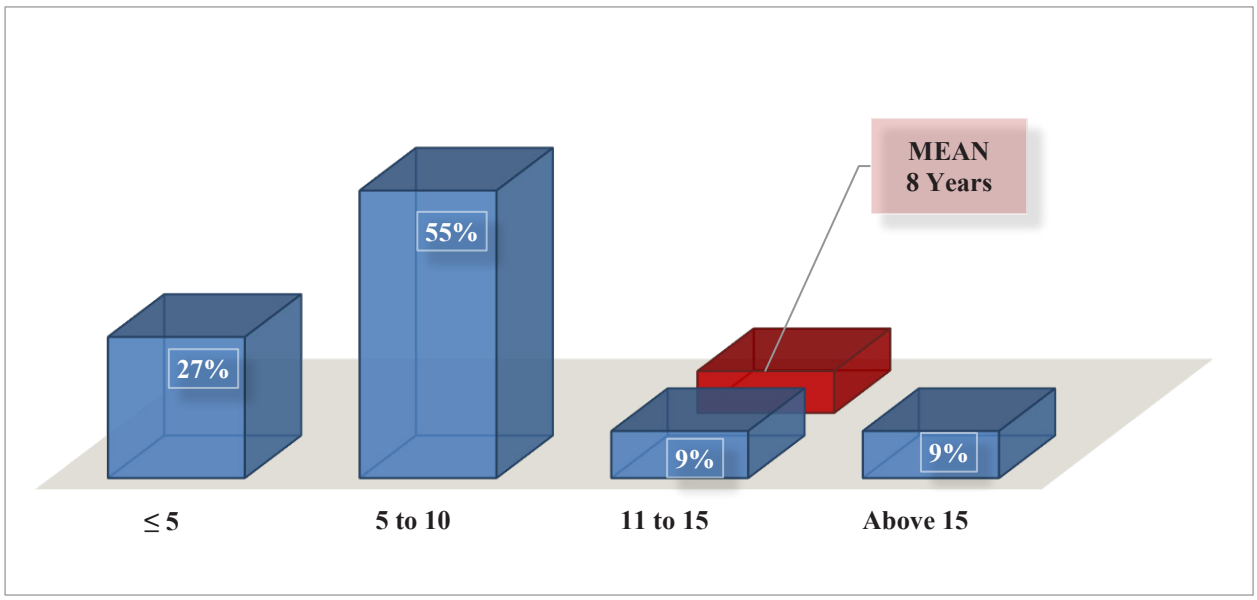

Fig. 5. Household size of sampled farmers

farmers had an average family size of 8 people. Family members of farmers make significant labor contributions to the family labor pool, but this also increases the farmers' reliance ratio. The results show that a substantial number of sampled farmers have larger households, and family members play a vital role in farm work. This boosts productivity but also raises the reliance ratio, making the household more sensitive to economic changes. To address this, it's crucial to manage labor, diversify income, and enhance efficiency to safeguard the welfare of these farming families.

3.5. Family Type of Sampled Farmers

Families are progressively dissolving, and more people are choosing to live separately in order to enhance their standard of living. The majority of the sampled farmers (62 %) were part of nuclear families, whereas (38 %) were part of joint families. Figure 6 demonstrates a rising trend towards nuclear families (62 %), reflecting a preference for independent living, likely motivated by a desire to improve living standards. Concurrently, joint families (38 %) continue to emphasize the lasting importance of strong family bonds and communal living practices in the region.

3.6. Sampled Farmers' Involvement in Farming

Figure 7 depicts the sampled farmers' part-time and full-time participation in farming activities. More than half (51 %) were actively involved in farming, while the remaining 49 percent were only partially involved. The results show a significant number of farmers fully committed to farming, while others engage only part-time. This highlights varying reliance on agriculture for livelihoods, offering insights into rural economies and livelihood approaches in the study area.

3.7. Occupational Distribution of Sampled Farmers

Farming is the primary occupation in rural areas because it provides the majority of the income for those who live there. Figure 8 shows the job descriptions of the sampled farmers. The sampled farmers who rely solely on farming represented 76 percent, while 24 percent also held other employment in addition to farming. The findings indicate that farming is really important in rural areas because it helps people earn a lot of money. The information in Figure 8 also shows that most people mainly depend on farming for their income, while some others have different ways of making money.

3.8. Farm Area Owned by Sampled Farmers

Figure 9 reveals that 27 % of the sampled farmers were tenants and did not have their own land, while the majority (47 %) had less than one acre, followed by 20 % had 1-3 acres, and 6 % had more than 3 acres, while an average farm size was 0.98 acre. The findings illustrate that the sampled farmers were primarily smallholders, often tenants cultivating small plots of land. This inference is strengthened by the average farm size, which was less than one acre, indicating a scarcity of large-scale farming within the sample.

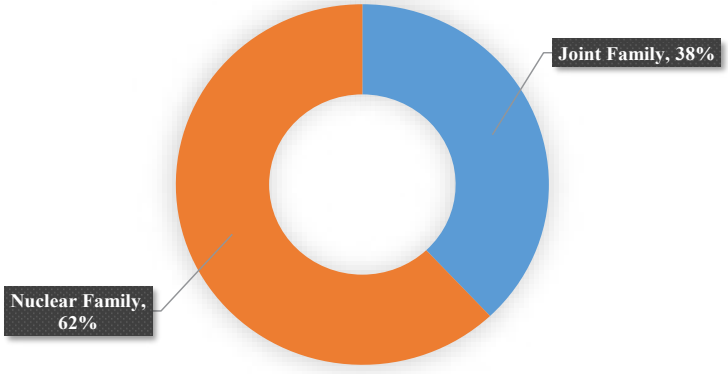

Fig. 6. Family type of sampled farmers

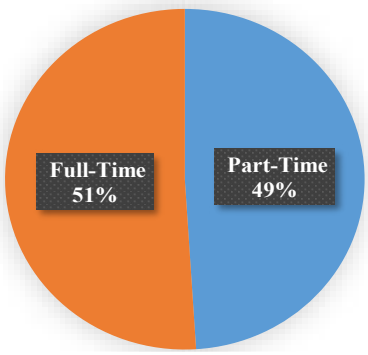

Fig. 7. Sampled farmers' involvement in farming

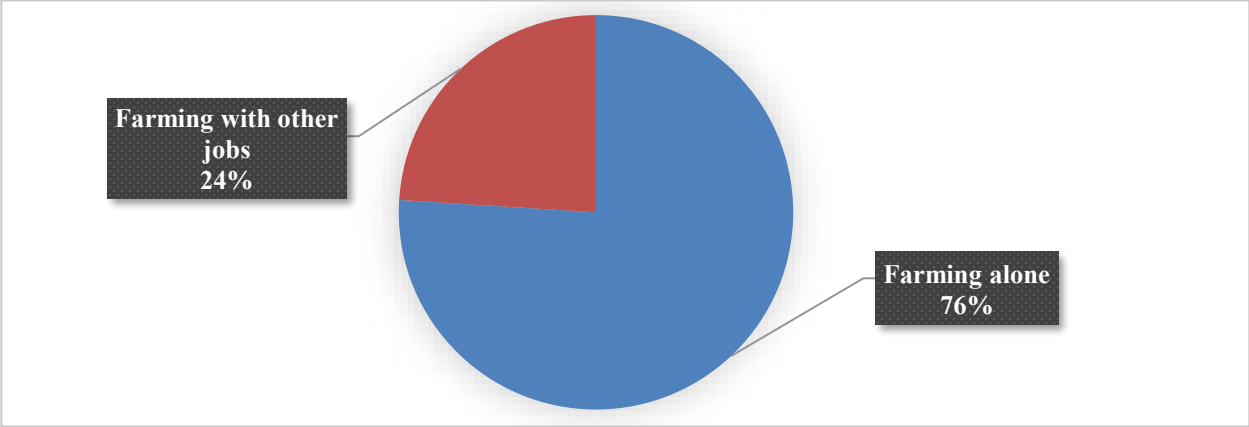

Fig. 8. Occupation of sampled farmers

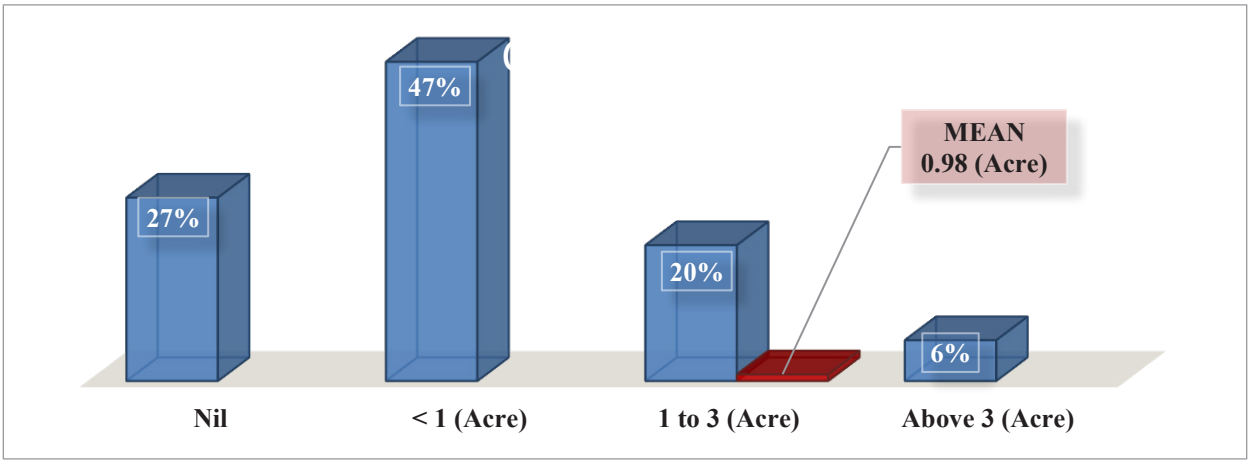

Fig. 9. Farm area owned by sampled farmers

3.9. Tenancy Status of Sampled Farmers

The tenancy status of the sampled farmers is shown in Figure 10. Only 15 % of the sampled farmers were owners; more than half (58 %) were owner-cum-tenants, while 27 % were tenants. Hence, the majority of the sampled farmers had a mixed tenancy status as owner-cum-tenants. This underlines a close interconnection between land ownership and tenancy in this farming community. This diversity implies a variety of economic and property dynamics in action. Further investigation is warranted to comprehend the drivers behind these trends and their potential implications for the agricultural sector.

3.10. Farm Equipment Owned by Sampled Farmers

Table 1 provides information about the farm machinery that the sampled farmers possessed. The study's findings showed that 20 percent of sampled farmers did not possess any agricultural equipment. The majority of sampled farmers (38 %) had their spray pumps; 20 percent had diesel engines; and 16 percent had tube wells. The study revealed a prominent possession of spray pumps and diesel engines among sampled farmers, while essential equipment like tractors, cultivators, and rotavators were less common. Despite limited ownership, the availability of farm equipment for rent in the study area was reported to be sufficient according to farmers.

3.11. Sources of Information

Table 2 shows the major sources of information obtained by sampled farmers in the study area. The majority (76 %) of the sampled farmers indicated fellow farmers as their source of information for agricultural and marketing purposes. Similarly, the other important source were seed dealers,

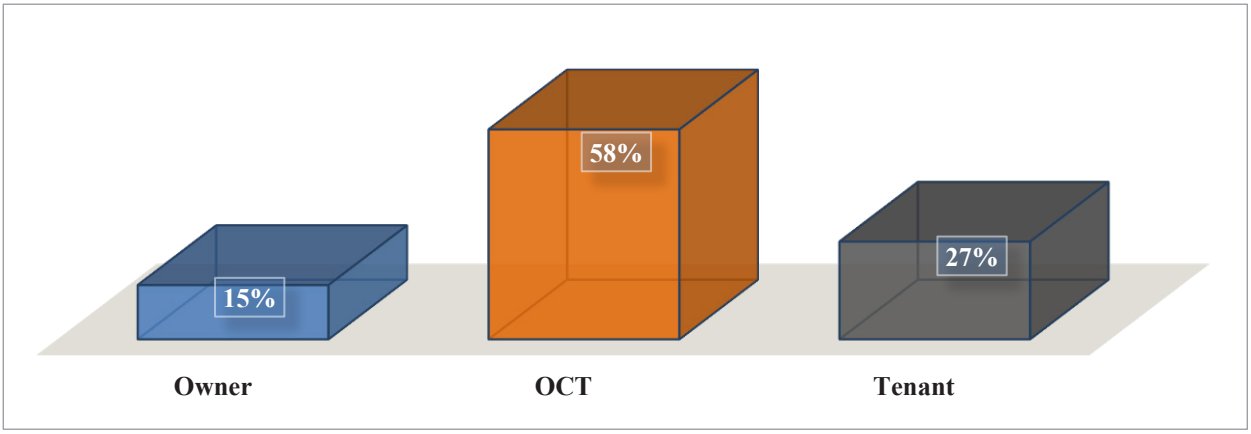

Fig. 10. Tenancy status of sampled farmers

Table 1. Percentage distribution of farm equipment owned by sampled farmers

Farm Equipment (owned)	Frequency	Percentage
Nil	09	20
Tractor	01	02
Cultivator	01	02
Rotavator	01	02
Sprayer	17	38
Tube well	07	16
Diesel Engine	09	20
Total	**45**	**100**

Source: Field survey data, 2019-20

middlemen, and agricultural extension workers, representing (13 %), (7 %), and (4 %), respectively. These findings emphasize the strong reliance on informal networks within the farming community for obtaining relevant information.

3.12. Sources of Irrigation

Table 3 showed the major irrigation sources of the sampled farmers in the study area. The majority (75.6 %) of the sampled farmers were using tube wells, while 24.4 percent were using both canal and tube wells for irrigation purposes. The study underscores the dominance of tube wells as the primary irrigation source underscores the importance of groundwater for sustaining agricultural productivity. The fact that many farmers are using both canals and tube wells for irrigation shows that they are smart and flexible in dealing with water challenges.

Table 2. Percentage distribution of sampled farmers by sources of information

Sources of Information	Frequency	Percentage
Fellow farmers	34	76
Agricultural extension	02	04
Arhti/Middle-men	03	07
Seed dealers	06	13
Total	**45**	**100**

Source: Field survey data, 2019-20

Table 3. Irrigation sources of sampled farmers for selected vegetables

Irrigation Sources	Frequency	Percentage
Tube well	34	75.6
Canal + Tube well	11	24.4
Total	**45**	**100**

Source: Field survey data, 2019-20

3.13. Acquisition of Loan

Farmers used a variety of sources to get agricultural credit to meet their financial needs. The data pertaining to loan acquisition is shown in Table 4. Only 31 % of sampled farmers obtained credit for their farm operations. The low percentage of farmers accessing formal financial institutions, coupled with significant reliance on self-financing and informal sources, emphasizes the need for targeted policies and interventions to improve farmers' access to affordable and timely credit. Addressing these challenges is crucial for promoting sustainable agricultural growth and rural development.

3.14. Sources of Loan

The different loan programs available to farm businesses in the study area are listed in Table 5. More than half (57 %) of the sampled farmers borrowed money from commission agents, who are the most frequent source of debt among them. This is followed by NGOs (22 %), commercial banks (14 %), and relatives (7 %). The distribution of loan programs and sources of debt among farmers in the study area reflects a complex financial landscape. While commission agents dominate as a source of credit, the presence of NGOs and commercial banks, along with borrowing from relatives, underscores the diversity of options available to farmers. Efforts to enhance financial literacy, improve access to formal credit, and regulate informal sources can contribute to a more sustainable and equitable credit ecosystem for agricultural communities.

Table 4. Loan obtained by sampled farmers

Loan Obtained	Frequency	Percentage
Yes	14	31
No	31	69
Total	**45**	**100**

Source: Field survey data, 2019-20

Table 5. Percentage distribution of sampled farmers by sources of loan obtained

Sources of Loan	Frequency	Percentage
Bank	02	14
Commission Agents	08	57
NGOs	03	22
Any others	01	07
Total	**14**	**100**

Source: Field survey data, 2019-20

3.15. Purpose of Loan

Table 6 lists the purpose of borrowed money received by the sampled farmers in the study area. The majority of the sample's farmers (79 %) had taken out loans for crops, followed by loans for livestock (7 %), and then loans for businesses (14 %). Crop-related loans dominate, reflecting the fundamental role of agriculture in these communities. Livestock and business loans showed farmers' efforts to diversify income sources and improve their overall economic well-being. Access to credit for these purposes can play a significant role in promoting sustainable agricultural practices, livestock management, and rural development.

3.16. Loan Size Obtained

Table 7 displays the loan amounts obtained by the sampled farmers in the study area. About half (50 %) of the sampled farmers received loans up to $50,000, 22 % received loans between $50,000 and $1,000,000, and 28 % received loans exceeding $1,000,000 in total. The availability of loans across different amounts states the importance of offering a diverse range of financial products to cater to the unique requirements of farmers at different stages of development.

Table 6. Percentage distribution of farmers according to the purpose of the loan

Purpose of Loan	Frequency	Percentage
Crop	11	79
Livestock	01	07
Business	02	14
Total	**14**	**100**

Source: Field survey data, 2019-20

Table 7. Size of loan obtained by sampled farmers

Loan Size (Rs)	Frequency	Percentage
≤ 50000	07	50
50001-100,000	03	22
Above 100,000	04	28
Total	**14**	**100**

Source: Field survey data, 2019-20

3.17. Profitability of Intercropping

The economics of intercropping presented in Table 8 revealed that the total gross revenue was Rs. 289668/acre, whereas the total cost amounted to Rs. 182756/acre. Similarly, the gross margin was found to be Rs. 124912 per acre, while the net return over the total cost was found to be Rs. 106912 per acre. Hence, the benefit-cost ratio comes to around 1.59. It is evident that the percentage share of the total variable costs is 90.2 percent, and the fixed cost was 9.8 percent of the total cost of production. The variable costs include land preparation (6.8 %), seeds (11 %), nursery raising and transplanting costs (3 %), manures (2.6 %), fertilizers (5.7 %), weeding and hoeing (5.5 %), insecticides and pesticides (6.3 %), irrigation (5.8 %), harvesting and curing (14 %), and transportation and marketing costs (27.2 %) of total production costs. Among the different items of cost, the transportation and marketing cost, harvesting and curing cost, the rental value of land cost, and seed cost were the major items of cost of cultivation in intercropping. The benefit-cost ratio of intercropping was also higher, with a value of 1.59 than sole crops. So intercropping onions with tomatoes was more profitable as compared to sole crops. The findings revealed that the practice of intercropping onions

Table 8. Profitability of intercropping (Rs. /acre)

S. No.	Operating Costs	Cost /Acre	Percent
A	**Variable Costs**	**164756**	**90.2**
a)	Land preparation	12487	6.8
b)	Seed cost (Tomato)	17279	9.5
c)	Seed cost (Onion)	2811	1.5
d)	Nursery raising & transplanting cost	5500	3.0
e)	Farmyard manure	4762	2.6
f)	Fertilizers	14261	5.7
g)	Hoeing and weeding	10137	5.5
h)	Plant protection	11547	6.3
i)	Irrigation	10623	5.8
j)	Harvesting and curing (Tomato)	23500	12.9
k)	Harvesting and curing (Onion)	2040	1.1
l)	Transportation & marketing cost (Tomato)	48095	26.3
m)	Transportation & marketing cost (Onion)	1714	0.9
B	**Fixed Costs**	**18000**	**9.8**
a)	Rental value of land (for 6 months)	18000	9.8
C	**Total Costs (C= A+B)**	**182756**	**100**
D	Yield (kgs/acre) (tomato)	16032	-
E	Sale price (Rs./kg)	15.8	-
F	Gross revenue (Rs./acre) **(D*E)**	253300	-
G	Yield (kgs/acre) (onion)	1865	-
H	Sale price (Rs./kg)	19.5	-
I	Gross revenue (Rs./acre) **(G*H)**	36368	-
J	Total gross revenue **(F+I)**	289668	-
K	**Gross Margin** (Rs./acre) **(J-A)**	**124912**	-
L	**Net Return** (Rs./acre) **(J-C)**	**106912**	-
M	**Benefit-Cost Ratio** (Rs./acre) **(J/C)**	**1.59**	-

Source: Field survey data, 2019-20

and tomatoes was found to be economically favorable. The benefit-cost ratio, which measures the profitability of the venture, indicated that intercropping yielded positive financial returns. Additionally, the comparison of intercropping's benefit-cost ratio with that of sole crops further supports the conclusion that intercropping was more profitable.

3.18. Profitability of Onions (Sole)

The economics of onion (sole) cultivation presented in Table 9 revealed that the gross revenue was Rs. 118463/acre, whereas the total cost amounted to Rs. 86379/acre. Similarly, the gross margin was found to be Rs. 50083 per acre, while the net return over the total cost was found to be Rs. 32083 per acre. Hence, the benefit-cost ratio comes in around 1.37. The percentage shares of variable costs and fixed costs of production were 79.2 percent and 20.8 percent of the total cost of production, respectively. The variable costs include land preparation (13.3 %), seeds (5.9 %), nursery raising and transplanting costs (5.2 %), manures (6.4 %), fertilizers (12.5 %), weeding and hoeing (6.7 %), insecticides and pesticides (1.9 %), irrigation (12.2 %), harvesting and curing (7.8 %), and transportation and marketing costs (6.6 %) of total production costs. Among the different items of cost, the rental value of land, land preparation cost, fertilizer cost, and irrigation cost were the major items of cost of cultivation in onion (sole) production. The data suggests positive economic outcomes for sole onion cultivation, individual farmers should conduct a comprehensive analysis to determine the suitability of this crop within their overall farming strategy.

3.19. Profitability of Tomatoes (Sole)

The economics of tomato (sole) cultivation presented in Table 10 revealed that the gross revenue was Rs. 253590 per acre, whereas the total cost amounted to Rs. 170921 per acre. The gross margin was determined to be Rs. 100669 per acre, with a net return over the total cost of Rs. 82669 per acre. The benefit-cost ratio comes to around 1.48, which shows that the tomato crop is a remunerative

Table 9. Profitability of onions (sole) (Rs. /acre)

S. No.	Operations/Inputs	Cost /Acre	Percent
A	**Variable Costs**	**68379**	**79.2**
a)	Land preparation	11511	13.3
b)	Seed cost	5137	5.9
c)	Nursery raising and transplanting cost	4533	5.2
d)	Farmyard manure	5500	6.4
e)	Fertilizers	10767	12.5
f)	Weeding and hoeing	5800	6.7
g)	Plant protection (insecticides/pesticides)	2200	1.9
h)	Irrigation	10511	12.2
i)	Harvesting and curing	6750	7.8
j)	Transportation and marketing cost	5670	6.6
B	**Fixed Costs**	**18000**	**20.8**
a)	Rental value of land (for 6 months)	18000	20.8
C	**Total Cost of Production (C= A+B)**	**86379**	**100**
D	Yield (kgs/acre)	6075	-
E	Sale price (Rs./kg)	19.5	-
F	Gross revenue (Rs./acre) **(D*E)**	118463	-
G	**Gross Margin** (Rs./acre) **(F-A)**	**50083**	-
H	**Net Returns** (Rs./acre) **(F-C)**	**32083**	-
I	**Benefit-Cost Ratio** (Rs./acre) **(F/C)**	**1.37**	-

Source: Field survey data, 2019-20

enterprise for the farmers. The percentage shares of variable costs and fixed costs of production were 89.5 percent and 10.5 percent of the total cost of production, respectively. The variable costs include land preparation (7.4 %), seeds (10.5 %), nursery raising and transplanting costs (2.8 %), manures (2.6 %), fertilizers (7.4 %), weeding and hoeing (5.0 %), insecticides and pesticides (3.9 %), irrigation (6.1 %), harvesting and curing (13.7 %), and transportation and marketing costs (28.2 %) of total production costs. Among the various items of cost, transportation and marketing cost, harvesting and curing cost, and seed cost were the major items of cultivation cost in tomato (sole) production. Based on the presented data, cultivating tomatoes as a sole crop appears to be a profitable endeavor. Farmers should conduct a thorough analysis of their specific circumstances and consider other relevant factors before making decisions about crop selection and cultivation practices.

3.20. Profitability Comparison of Intercropping and Monocropping

Intercropping involves growing two or more crops together on the same piece of land, while monocropping involves cultivating only one crop. According to Table 11, the total cost of intercropping amounted to Rs. 182756/acre, which was higher than the cost of producing onions (Rs. 86379/acre) and tomatoes (Rs. 170921/acre) as sole crops. The net revenue generated from intercropping was higher than that of individual crops. Intercropping exhibited the highest benefit-cost ratio of 1.59 compared to sole crops of tomatoes (1.48) and onions (1.37), indicating that intercropping is less costly than sole cropping. Other studies conducted in various countries, including China, Egypt, and Ethiopia. [Wu *et al*., 2016; Abdel-Baset, 2020; Nigussie *et al*., 2017], have demonstrated that intercropping leads to a significant increase in crop yield per unit area compared to single-crop farming. Additionally, intercropping has been found to reduce the costs of inputs associated with farming.

3.21. Land Equivalent Ratio (LER) of Intercropping

The land equivalent ratio is a concept in agriculture that describes the relative land area required under

Table 10. Profitability of tomatoes (sole) (Rs. /acre)

S. No.	Operations/Inputs	Cost /Acre	Percent
A	**Variable Costs**	**152921**	**89.5**
a)	Land preparation	12690	7.4
b)	Seed cost	17984	10.5
c)	Nursery raising & transplanting cost	4862	2.8
d)	Farmyard manure	4405	2.6
e)	Fertilizers	12673	7.4
f)	Weeding and hoeing	8500	5.0
g)	Plant protection (insecticides/pesticides)	9610	3.9
h)	Irrigation	10503	6.1
i)	Harvesting and curing	23500	13.7
j)	Transportation and marketing cost	48195	28.2
B	**Fixed Costs**	**18000**	**10.5**
a)	Rental value of land (for 6 months)	18000	10.5
C	**Total Cost of Production (C = A+B)**	**170921**	**100**
D	Yield (kgs/acre)	16050	-
E	Sale price (Rs./kg)	15.8	-
F	Gross revenue (Rs./acre) **(D*E)**	253590	-
G	**Gross Margin** (Rs./acre) **(F-A)**	**100669**	-
H	**Net Return** (Rs./acre) **(F-C)**	**82669**	-
I	**Benefit-Cost Ratio** (Rs./acre) **(F/C)**	**1.48**	-

Source: Field survey data, 2019-20

Table 11. Profitability comparison of intercropping and sole cropping (Rs. /acre)

S. No.	Cropping System	Total Cost (Rs.)	Gross Revenue (Rs.)	Net Revenue (Rs.)	BCR	Ranking
1	Sole tomato	170921	253590	82669	1.48	**II**
2	Tomato and onion	182756	289668	106912	1.59	**I**
3	Sole onion	86379	118463	32083	1.37	**III**

Source: Field survey data, 2019-20

sole cropping (monoculture) to produce the same yield as under intercropping [49]. The results showed that the land equivalent ratio (LER) was greater than one, which implies that intercropping was more productive than sole cropping. Specifically, the total LER found was 1.31, indicating that the intercropping system produced 31 % more yield than the same area of land planted in sole crops. This suggests that intercropping is a more efficient way of utilizing land, as it enables higher yields without the need for additional land. Various studies on intercropping have shown that planting tomatoes and onions together can lead to a higher land equivalent ratio (LER). The findings presented here are in line with previous research conducted by [Soniya *et al.*, 2021; Yildirim and Guvenc, 2005; Lamlom and Ahmed, 2021], indicating that intercropping, coupled with appropriate nutrient management, can result in more efficient land utilization and higher crop yields. However, a contrasting study conducted by [Ahmed et al., 2023] discovered that intercropping tomatoes and onions had a negative effect on yield, and the resulting LER was less than one.

3.22. Production Constraints

To assess production issues, eight factors are considered, and the two most important production issues mentioned by sampled farmers were seed costs and disease and pest management, which represented 97.8 percent and 93.3 percent, respectively. Similarly, the lowest level was the access to quality water by sampled farmers in the study area (Table 13). The information presented underscores the importance of addressing key production challenges to enhance agricultural productivity and the economic well-being of farmers. Strategies aimed at reducing seed costs, improving disease and pest management practices, and sustaining access to quality water resources could contribute to a more sustainable and prosperous agricultural sector.

3.23. Marketing Constraints

Eight major marketing issues for producers have been identified and presented in Table 14. The three major marketing issues identified by the sample farmers were low prices, perishability of the product, and high travel costs representing 93.3 %, 91.1 %, and 77.8 % respectively. Similarly, the unavailability of packing materials was the lowest level for sampled farmers in the study area. The information provided highlights the need for targeted interventions and support mechanisms to address the identified marketing challenges, thereby improving market access and financial outcomes for agricultural producers.

4. CONCLUSION AND RECOMMENDATIONS

The study concluded that intercropping onions with tomatoes provides farmers with additional income and helps them meet their household needs. Compared to purchasing crops, intercropping is a more cost-effective option for farmers. It is a traditional farming practice in the study area, with a majority of farmers intercropping onions

Table 12. Land equivalent ratio (LER) of intercropping system

Cropping System	Intercrop Yield (YI)	Sole Crop Yield (YS)	Partial LER (YI_i/ YS_i)	Total LER $\sum$ (YI_i/ YS_i)
Tomato	16032	16050	1.00	**1.31**
Onion	1865	6075	0.31	

Source: Field survey data, 2019-20

Table 13. Production constraints faced by farmers

Production Constraints	Respondents (N=45)		
	Number	Percentage	Rank
Seed cost	44	97.8	I
Disease and pest management	42	93.3	II
Technical training	17	37.8	III
Quality seed	16	35.6	IV
Late sowing	11	24.4	V
Availability of labor	07	15.6	VI
Availability of water	06	13.3	VII
Availability of quality water	02	6.7	VIII

Source: Field survey data, 2019-20

Table 14. Onion marketing constraints faced by sampled farmers

Marketing Constraints	Respondents (N=45)		
	Number	Percentage	Rank
Low price	42	93.3	I
Perishability	41	91.1	II
High charges for transportation	35	77.8	III
Costly packing materials	34	75.6	IV
Lack of markets	34	75.6	V
Lack of market information	25	55.6	VI
Exploitation by Brokers and Middlemen	19	42.2	VII
Unavailability of packing material	12	26.7	VIII

Source: Field survey data, 2019-20

with tomatoes, especially for domestic use. The economic indicators, including gross margin, net return, benefit-cost ratio, and land equivalent ratio, showed promising results for intercropping over sole cropping. Intercropping had the highest benefit-cost ratio of 1.59, which was higher than the ratios observed in sole cropping of tomatoes (1.48) and onions (1.37), highlighting its cost-effectiveness. The LER value was greater than one (1.31), supporting the benefits of intercropping and indicating that farmers can increase their profits by growing onion crops at different densities. However, unpredictable weather conditions, market volatility, rising input costs, and low planting densities for onions are factors that can hinder profitability. To optimize land use and increase crop yields, farmers can benefit from adopting intercropping along with appropriate management techniques. Adopting intercropping practices can help farmers overcome challenges and increase profitability, contributing to a sustainable and resilient agriculture system.

Based on the findings of the study, the following recommendations can be made:

- To boost intercropping productivity and profitability for onions and tomatoes, farmers should prioritize the use of high-quality seeds.
- Increasing onion planting density can improve the total return of onion-tomato intercropping by optimizing resource utilization and enhancing yields.
- Governments can alleviate local market instability by intervening with market information systems, price stabilization measures, and support programs.

5. ACKNOWLEDGEMENTS

The authors express their gratitude to ACIAR and CABI-Pakistan for their assistance in conducting the baseline survey for this study.

6. DECLARATION

The study findings are exclusive to this publication and have not been published or considered for publication elsewhere. In case of acceptance for publication, the copyright of the article will be transferred to the Pakistan Academy of Sciences.

7. CONFLICT OF INTEREST

There is no conflict of interest among the authors.

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Proceedings of the Pakistan Academy of Sciences: B
Life and Environmental Sciences 60(3): 431-441 (2023)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(60-3)842

Pakistan Academy of Sciences

Research Article

Influence of Basal Salts, Sucrose and Plant Growth Regulator Levels on Nucellar Embryogenesis and Plantlet Regeneration in Monoembryonic Mango Varieties

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Abstract: Current study described stage-wise protocols for in vitro propagation of commercially important varieties of mango. Induction of somatic embryos (SE) and plantlet regeneration was obtained using nucellar explants of three superior monoembryonic mango vars.'Saroli', 'Langra' and 'Chaunsa' cultivated in Khairpur, Pakistan. The immature fruits (2.5-4.0 cm long) were surface disinfected using 30 % sodium hypochlorite (NaOCl) solution. Results revealed that significantly highest direct somatic embryogenesis (93 %) was obtained in var. 'Chaunsa' under full dark on culture medium comprising of 2.0 mg L^{-1} N6 2-isopentenyl adenine (2iP), 0.5 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D). Medium consisted of 2iP 4.0 mg L^{-1}, 2,4-D 1.0 mg L^{-1} induced significantly highest embryogenic callus (91 %) using nucellar explants in var.'Chaunsa'. Significantly highest germination (95 %) of SE was achieved in var. 'Chaunsa' on the medium comprising of microsalts of MS, macrosalts of B5, 2iP 0.1 mg L^{-1}, Kinetin (Kin) 0.5 mg L^{-1}. Highest shoot length (5.1 cm) and root length (4 cm) were obtained in var. 'Langra' on the medium consisted of microsalts of MS, macrosalts of B5, 30 g L^{-1} sucrose, 200 mg L^{-1} activated charcoal (AC), 0.1 mg L^{-1} naphthalene acetic acid (NAA), 0.2 mg L^{-1} benzyl adenine (BA). Stage-wise protocols established for the regeneration of plantlets can be useful to micropropagation of the other mango varieties of the world.

Keywords: Cuture media, *Mangifera indica* L., Micropropagation, Monoembryonic, Regeneration, Somatic Embryogenesis

1. INTRODUCTION

Mango is tropical, arborescent, evergreen tree with a life span ranging from 70 to 100 years [1, 2]. The mango propagation can be done through sexual (seed propagation) and asexual methods (grafting) [3]. The mango seeds can be categorized into monoembryonic (contain single zygotic embryo) and polyembryonic (contain one zygotic and 4-5 nucellar embryos) types [4]. The seedlings obtained from polyembryonic seeds are always true-to-type except the one that develops from the zygotic embryo [4, 5]. The monoembryonic seeds produce single zygotic seedling that is always different genotypically from the mother tree [3, 6, 7]. The grafting is traditional vegetative propagation method to grow true-to-type plants of the commercially important varieties. The in vitro propagation through vegetative tissues is an alternative method to grafting [8, 9]. Different reports [5, 10-12] utilized immature ovular halves comprising nucellus tissue as initial explant for in vitro propagation of elite mango varieties. Previously, the nucellar explants obtained from immature polyembryonic seeds [5, 10-17] and monoembryonic seeds [6, 7, 10, 18-20] have been used in somatic embryogenesis. In vitro propagation of mango was done before at different times [6, 10, 19, 21], but still there

Received: January 2023; Revised: March 2023; Accepted: June 2023
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is need to improve the elongation and rooting stages [2]. Moreover, there is no report available for direct somatic embryogenesis in mango using nucellus tissue. Pakistan is one of the major mango producers and exporters, cultivating some of the leading exporting varieties like 'Saroli', 'Langra', 'Chaunsa', 'Sindhri', 'Fajri', 'Sonaro', 'Totapuri', 'Lal Badshah', 'Anwar Ratol'. Hence the mango propagation by in vitro method using nucellus tissue is a suitable method to produce true-to-type plantlets. In vitro cultures of mango facing severe browning due to phenolic compounds occur in tissues which was tackled by addition of antioxidants, AC, quick shifting of cultures to the fresh media, incubation of cultures under full darkness. Effects of Plant Growth Regulators (PGRs) levels, sucrose, salts, AC on direct and indirect somatic embryogenesis using nucellar explants, proliferation and germination, shoot elongation and rooting were focused in present study. The results obtained in this study will apply to other mango varieties worldwide.

2. MATERIALS AND METHODS

2.1. Plant Material

Fruits (2.5-4 cm long) obtained at different intervals from 20 years old trees, 30-40 days after pollination from vars. 'Saroli', 'Langra', 'Chaunsa' (early, mid, late season varieties respectively) (Figure 1a, b). Fifty fruits were collected from each variety and brought to the laboratory for further processing. Initially fruits were washed using tap water for twenty min followed by washing with commercial liquid Lemon Max (Colgate Palmolive, Pakistan) dissolved 5 ml in 500 ml of water to clean dust particles attached to surface of fruits. Final sterilization of fruits was carried out on laminar airflow cabinet using 30 % NaOCl solution with 5 ml Tween-20 (Scharlau, Spain) continuously up to 20 min followed by final washing with distilled autoclaved water. Fruits were divided into two halves and intact immature ovular halves comprising nucellus tissue were used as initial explants. The

Fig. 1. (a) Immature fruits on the tree during 15th April ready for picking, (b) size and morphology of the fruits of 'Saroli', 'Langra', 'Chaunsa' used for in vitro culture.

remaining fruit flesh (mesocarp with epicarp) were discarded. Each isolated ovule was preserved in filtered solution of citric acid (100 mg L^{-1}) for 2-3 min. Later, ovules dissected longitudinally from the middle into two halves (Figure 2a), and each half lacking zygotic embryo was used as an initial explant.

2.1.1. Effect of 2,4-D and 2iP on Proembryogenic Callus Induction, Maturation, and Direct Somatic Embryogenesis under Full Dark Conditions

Medium consisted of (1) 2,4-D 0.0, 0.5, 1.0 or 2.0 mg L^{-1} and 2iP 0.0, 0.5, 1.0 or 2.0 mg L^{-1}. (2) 2,4-D 0.0, 0.5, 1.0 or 2.0 mg L^{-1} and 2iP 1.0, 1.5, 2.0 or 4.0 mg L^{-1}, microsalts of MS (Merck, Germany), macrosalts of B5 (Merck, Germany), agar 2.2 g L^{-1} (Oxoid, United Kingdom), gelrite 1.4 g L^{-1} (Gellan Gum, Caisson Laboratories, USA), sucrose 60 g L^{-1}, glutamine 400 mg L^{-1}, 200 mg L^{-1} AC. pH of medium fixed at 5.8 prior to autoclaving. 20 ml solution poured into each culture vessel and sterilized in autoclave at 15 psi pressure at 121 °C up to 20 min. Cultures put in complete dark at 24 °C. Transfer of cultures onto the fresh media was done at 2-3 weeks intervals.

2.1.2. Effect of PGRs on SE Germination and Plantlet Formation under Light (16h Photoperiod) consisting of cool white fluorescent light (40-60 µmol m^{-2} s^{-1})

Medium comprising of 2iP 0.1 mg L^{-1}, Kin 0.5 mg L^{-1} used for germination and plantlet formation. Shoot elongation and rooting medium was comprised of NAA 0.1 mg L^{-1}, BA 0.2 mg L^{-1}. Microsalts of MS, macrosalts of B5, sucrose 30 g L^{-1}, agar 2.2 g L^{-1}, gelrite 1.4 g L^{-1}, AC 200 mg L^{-1}, glutamine 400 mg L^{-1} used in both types of media used to multiply somatic embryos induced either directly from nucellus or indirectly via callus.

2.1.3. Effect of Basal Salts and AC on SE Multiplication and Germination

Media used for multiplication and germination of embryos was consisted of (1) micro and macrosalts of MS, 200 mg L^{-1} AC (2) micro and macrosalts of MS without AC (3) micro and macrosalts of B5, AC 200 mg L^{-1}, (4) micro and macrosalts of B5 without AC.

2.1.4. Effect of Basal Salts, AC, and Sucrose on Shoot Elongation and Rooting under White Fluorescent Light (160 µmol m^{-2} s^{-1})

Medium consisting of (1) micro and macrosalts of MS, 200 mg L^{-1} AC (2) micro and macrosalts of MS without AC (3) micro and macrosalts of B5, AC 200 mg L^{-1}, (4) micro and macrosalts of B5 without AC. (5) microsalts of MS, macrosalts of B5, sucrose 30 g L^{-1}, (6) microsalts of MS, macrosalts of B5, sucrose 40 g L^{-1}, (7) microsalts of MS, macrosalts of B5, sucrose 50 g L^{-1}, (8) microsalts of MS, macrosalts of B5, sucrose 60 g L^{-1}.

2.2. Data Analysis

Three varieties were used in the study. Four different treatments were tested from induction of proembryogenic callus up to proliferation of SE (Table 1-4) and eight treamtnets were tested for shoot and root development (Table 5-6). Three replicates were selected for each treatment and each culture vessel contained single explant. Completely Randomized Design (CRD) was used. Data were recorded after every month and analyzed as two-way ANOVA and the difference between all mean values identified by LSD ($p<0.05$) by XLSTAT.

3. RESULTS AND DISCUSSION

3.1. Effect of Auxins and Cytokinins on Induction, Proliferation and Maintenance of Embryogenic Callus from Nucelli under Dark

The callus formation from nucellus tissue depends upon the type of PGRs used in media. Auxin 2,4-D mainly induce callus in primary explants was added in the media for induction of proembryogenic callus. Results of two-way ANOVA exhibited significant effect of variety (< 0.001), treatment (< 0.002) and combined effect of variety and treatment (0.004). Data in Table 1 show that highest embryogenic callus induction (Figure 2b) was obtained in vars. 'Chaunsa' (88 %), 'Langra' (80 %) and 'Saroli' (70 %) from nucellar explants (Figure 2a) on medium comprising of 2,4-D 0.5 mg L^{-1} and 2iP 4.0 mg L^{-1} within two months of initiation stage under dark. Medium comprising of 2,4-D 0.5 mg L^{-1} and 2iP 2.0 mg L^{-1} also induced callus from nucellar explants in vars. 'Chaunsa' (81 %), 'Langra'

Table 1. Induction of proembryogenic callus in initial nucellar explants of three mango varieties under full dark conditions.

PGRs (2iP+2,4-D mg L^{-1})	Embryogenic callus induction (%)		
	Saroli	**Langra**	**Chaunsa**
1 + 0.5	55 ± 1.2^{c}	65 ± 0.8^{c}	61 ± 1.6^{c}
1.5 + 0.5	61 ± 3.2^{b}	63 ± 0.5^{c}	70 ± 2.2^{ab}
2.0 + 0.5	67 ± 2.5^{a}	77 ± 2.9^{ab}	81 ± 1.4^{a}
4.0 + 0.5	70 ± 2.6^{a}	80 ± 2.2^{a}	88 ± 2.1^{a}
Variety (mean)	63.3	71.3	75.0
Source of variability			
Treatment	< 0.002		
Variety	< 0.001		
Treatment × Variety	0.004		

Mean values in columns with standard error denoted with different superscript letters show significance level at $p \leq 0.05$.

(77 %) and 'Saroli' (67 %). On the contrary, the significantly lowest induction of embryogenic callus was noted from nucellar explants in vars. 'Saroli' (55 %),'Chaunsa' (61 %) and 'Langra' (65 %) on medium comprising of 2,4-D 0.5 mg L^{-1}, 2iP 1.0 mg L^{-1}.

Embryogenic calli induced in nucellar explants was compact, proliferated rapidly under full dark (Figure 2b). Ara *et al.* [22] observed similar results regarding formation of embryogenic calli in nucellar explants of monoembryonic mango vars. 'Amrapali' and 'Chaunsa' on medium comprising of 1.0 mg L^{-1} 2,4-D. Nower [12] noted that B5 medium comprising of 2,4-D 1.0 mg L^{-1} induced compact callus in cv. Zebda. Al-Busaidi et al. [6] described the medium comprising of 2,4-D 2.0 mg L^{-1} and BAP 0.5 mg L^{-1} to induce embryogenic callus using nucellar explants. Successful *in vitro* propagation of mango was reported previously by several workers using nucellar explants [7, 10, 14, 15]. Nucellar explants have been exploited as an initial explants in the current study since plantlets obtained using nucellus tissue are usually free from viruses due to lack of a vascular link between maternal tissue and nucellus.

Krishna and Singh [23] described that formation of embryogenic calli in mango depends on morphogenic capability of nucellus. Litz *et al.* [14] reported the formation of embryogenic callus in nucellus tissue of some polyembryonic mango varieties in 1-2 months obtained from 40-60 days old fruits. Consequently, appropriate circumstances for the formation of embryogenic calli in nucellar explants observed in monoembryonic mango varieties [10]. Young fruits (3.5 to 5.0 cm) of var. Baramasi obtained after 30-40 days of pollination for *in vitro* propagation [6, 24]. Ara *et al.* [25] utilized immature fruits of vars. 'Amrapali' (2 to 3.5 cm) and 'Chaunsa' (1.5 to 2.5 cm) for *in vitro* propagation. Malabadi *et al.* [26] induced embryogenic callus in nucellar explants obtained from 3-4 cm long fruits. Similarly, in this study excellent results were obtained using nucellar explants obtained from 2-4 cm long fruits.

Embryogenic callus (Figure 2b) was maintained on initiation medium under full darkness for 1-2 months until the formation of proembryos. Abul-Soad *et al.* [7] maintained embryogenic callus cultures on callus inducing medium comprising of 2,4-D 1.0 mg L^{-1}, 2iP 4.0 mg L^{-1}, 400 mg L^{-1} glutamine, microsalts of MS, macrosalts of B5 under full dark. The combined effect of auxin, cytokinin, and full dark was the key factor to maintain the embryogenic callus and formation of proembryos into mature SE [27, 28]. The cytokinins are important to stimulate and organize the growth of the apical meristem at the maturation stage [23].

3.2. Effect of 2,4-D + 2iP on SE Induction from Proembryogenic Callus and Maturation

Early cotyledonary stage embryos induced under dark in embryogenic callus developed into mature germinating embryos under light. Two-way ANOVA exhibited significant effect of variety (<

0.001), treatment (< 0.001) and combined effect of variety and treatment (< 0.001). Comparing the four PGR treatments within three studied varieties ('Saroli', 'Langra', 'Chaunsa'), significantly highest percentage (91 %) of SE (Figure 2d) was noted in var. 'Chaunsa' on medium comprising of 2iP 4.0 mg L^{-1}, 2,4-D 1.0 mg L^{-1} followed by 85.6 % of SE induced in same variety on medium comprising of 2iP 2.0 mg L^{-1}, 2,4-D 0.5 mg L^{-1} (Table 2). Similarly, SE induced in var. 'Langra' (82 %) and in var. 'Saroli' (76 %) on medium comprising of 2iP 4.0 mg L^{-1}, 2,4-D 1.0 mg L^{-1}. Whereas, the least SE induction percentage (44.3 %) was achieved in var. 'Saroli' on medium comprising of 2iP (1.5 mg L^{-1}). Pateña *et al.* [11] devised a reliable procedure in mango for induction of SE and formation of plantlets on medium consisted of B5 macrosalts, MS microsalts, vitamins of MS, 10-20 % of coconut water, Fe-EDTA, glutamine 0.4 mg L^{-1}, sucrose 2-6 %, 2,4-D 0.5-2.0 mg L^{-1} and gelrite 2.5 g L^{-1}.

Different reports [1, 23] observed that occurrence of 2,4-D in medium for long periods retarded the formation of SE. Occurrence of 2iP and reduced level of 2,4-D observed necessary to develop SE in date palm [29]. Nevertheless, 2,4-D is utilized largely in plant tissue culture for callus induction, however, exclusion of 2,4-D from a medium at a particular stage is also important for maturation of embryos. Later, globular stage embryos were induced in the embryogenic calli, converted into heart and torpedo stage embryos and finally developed into cotyledonary stage SE (Figure 2d). Cotyledonary stage embryos were utilized to produce mature dicotyledonous embryos

Table 2. Impact of PGR concentrations on SE formation from proembryogenic callus in three mango varieties under dark.

PGRs (2iP + 2,4-D mg L^{-1})	SE induction (%)		
	Saroli	**Langra**	**Chaunsa**
1.5 + 0.0	44.3 ± 0.2^{i}	66.0 ± 1.2^{h}	73.0 ± 0.6^{efg}
2.0 + 0.0	71.6 ± 0.4^{g}	74.6 ± 1.4^{efg}	76.3 ± 1.3^{de}
2.0 + 0.5	72.6 ± 0.2^{fg}	78.3 ± 0.5^{d}	85.6 ± 1.2^{b}
4.0 + 1.0	76.0 ± 1.2^{def}	82.0 ± 0.2^{c}	91.0 ± 1.1^{a}
Variety (mean)	66.1 ± 0.6^{c}	75.2 ± 0.3^{b}	81.5 ± 0.5^{a}
Source of variability			
Treatment	< 0.001		
Variety	< 0.001		
Treatment × Variety	< 0.001		

Mean values in columns with standard error denoted with different superscript letters show significance level at $p \leq 0.05$.

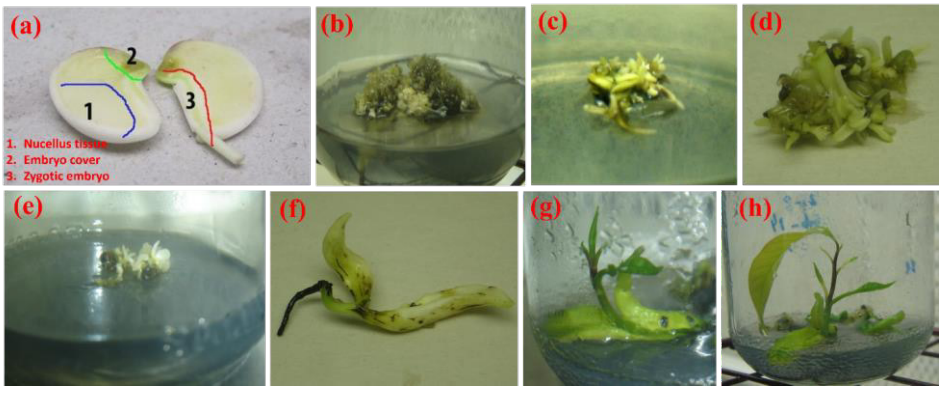

Fig. 2. (a) Immature ovular halves of variety 'Chaunsa' containing nucellus tissue, (b) embryogenic callus, (c) formation of SE from embryogenic callus, (d) maturation of SE, (e) direct somatic embryogenesis, (f) root formation in SE, (g) shoot formation in SE, (h) shoot elongation and rooting.

followed by formation of quick plantlets under light. In this way, further development of SE (ready to produce true leaves) took place under light via subsequent subcultures (Figure 2f-g).

3.3. Effect of 2,4-D + 2iP on Direct Somatic Embryogenesis from Nucellus Tissue under Full Dark

Results of two-way ANOVA revealed the significant effect of variety (< 0.003), treatment (< 0.001) while combined effect of variety and treatment (< 0.07) remained nonsignificant. The data presented in Table 3 indicate that the initiation media comprising of several treatments of 2,4-D and 2iP induced direct SE in nucellar explants of 2-4 cm fruits under full dark within two weeks, which were shifted quickly under light for further proliferation and germination (Figure 2e). Significantly highest induction of direct induction of SE recorded in nucellar explants obtained from var. 'Chaunsa' (86 %) followed by var. Langra (75 %) and Saroli (71 %) on medium comprising of 2iP 2.0 mg L^{-1}, 2,4-D 0.5 mg L^{-1}, 200 mg L^{-1} AC, ascorbic acid 100 mg L^{-1}, glutamine 400 mg L^{-1}, microsalts of MS, macrosalts of B5 (Table 3). On the contrary, significantly lowest percentage of direct somatic embryogenesis (51 %) was obtained in var. Saroli followed by var. Langra (55 %) from nucellar explants obtained from 2.5 cm fruits on medium comprising of 1.5 mg L^{-1} 2iP. In var. 'Saroli' direct SE (71 %) also induced in nucellar explants on medium comprising of 2,4-D 0.5 mg L^{-1}, 2iP 2.0 mg L^{-1}, 200 mg L^{-1} AC, 100 mg L^{-1} ascorbic acid, 400 mg L^{-1} glutamine, microsalts of MS, macrosalts of B5. SE are also induced directly in var. 'Chaunsa' (70 %) from nucellar explants of 3.5 cm long fruits on medium comprising of 2 mg L^{-1} 2iP. Furthermore, medium comprising of 2,4-D and 2iP induced significantly highest direct somatic embryogenesis compared to the media containing only 2iP, the embryos were observed to be multiplying rapidly while transferred under light. Several studies [15, 23, 30, 31] utilized the medium comprising of B5 macrosalts, MS microsalts, 4.52 to 9.04 µM 2,4-D, for somatic embryogenesis in mango using nucellus tissue. SE induced directly on nucellar explants (Figure 2e) shifted quickly from dark to light conditions for further proliferation and maturation (Figure 2d). The germination (Figure 2f-g), plantlet formation and rooting (Figure 2h) were also obtained under light.

3.4. Effect of Basal salts of MS and B5, AC on Proliferation of SE under Light (16h Photoperiod with cool white fluorescent light (40-60 µmol m^{-2} s^{-1})

Two-way ANOVA show significant effect of variety (< 0.001), treatment (< 0.001) and combined effect of variety and treatment (< 0.001) on multiplication of SE onto the media consisting of different basal salts with and without AC, 2iP (0.1 mg L^{-1}), Kin (0.5 mg L^{-1}) (Table 4). Data in Table 4 show that highest proliferation of SE obtained in var. 'Chaunsa' (95 %) followed by var. 'Langra' (88 %) and var. 'Saroli' (77 %) on medium comprising of micro and macrosalts of MS, AC 200 mg L^{-1} (Figure 3a). Medium consisted of micro and macrosalts of MS without AC exhibited significant reduction in

Table 3. Effect of different treatments of PGRs on direct induction of SE from nucellus tissue in three mango varieties under full dark.

PGRs	**Direct somatic embryogenesis (%)**		
(2iP + 2,4-D mg L^{-1})	**Saroli**	**Langra**	**Chaunsa**
1.5 + 0.0	51±0.57^{c}	55±0.43^{c}	62±0.27^{c}
4.2 + 1.0	69±2.88^{ab}	71±3.15^{ab}	80±2.66^{b}
2.2 + 0.5	71±2.15^{a}	75±1.23^{a}	86±2.11^{a}
2.2 + 0.0	55±1.73^{c}	64±2.74^{c}	70±1.22^{c}
Variety (mean)	61.5	66.3	74.5
Source of variability			
Variety	< 0.003		
Treatment	< 0.001		
Variety × Treatment	< 0.07		

Mean values in columns with standard error denoted with different superscript letters show significance level at $p \leq 0.05$.

Table 4. Impact of basal salts and AC on proliferation of SE in three mango varieties under light.

	Proliferation of SE (%)		
Treatment	**Saroli**	**Langra**	**Chaunsa**
Micro and macrosalts of MS, AC 200 mg L^{-1}	77.0 ± 1.9^{c}	88.0 ± 1.4^{b}	95.0 ± 0.3
Micro and macrosalts of MS	45.0 ± 1.5^{h}	51.0 ± 1.2^{fg}	49.0 ± 0.6^{gh}
Micro and macrosalts of B5, AC 200 mg L^{-1}	70.0 ± 0.6^{d}	92.0 ± 1.5^{ab}	94.0 ± 0.2^{a}
Micro and macrosalts of B5	58.0 ± 1.3^{e}	51.0 ± 0.4^{fg}	55.0 ± 1.9^{ef}
Variety (mean)	62.5	70.5	73.3
Source of variability			
Variety	< 0.001		
Treatment	< 0.001		
Variety × Treatment	< 0.001		

Mean values in columns with standard error denoted with different superscript letters show significance level at $p \leq 0.05$.

proliferation of embryos in vars. 'Saroli' (45 %), 'Langra' (51 %) and 'Chaunsa' (49 %) (Figure 3b). Medium comprising of micro and macrosalts of B5, 200 mg L^{-1} AC revealed significant proliferation of SE in var. 'Chaunsa' (94 %) followed by var. 'Langra' (92 %) and var. 'Saroli' (70 %) (Figure 3c). In contrast, the significantly lowest proliferation was noted in vars. 'Saroli' (58 %), 'Langra' (51 %) and 'Chaunsa' (55 %) on medium consisted of micro and macrosalts of B5 but without AC (Figure 3d). Different reports recommended the medium solidified with gelrite and phytagel for proliferation of embryos [15, 32, 33]. Chaturvedi *et al.* [34] used liquid medium for somatic embryo

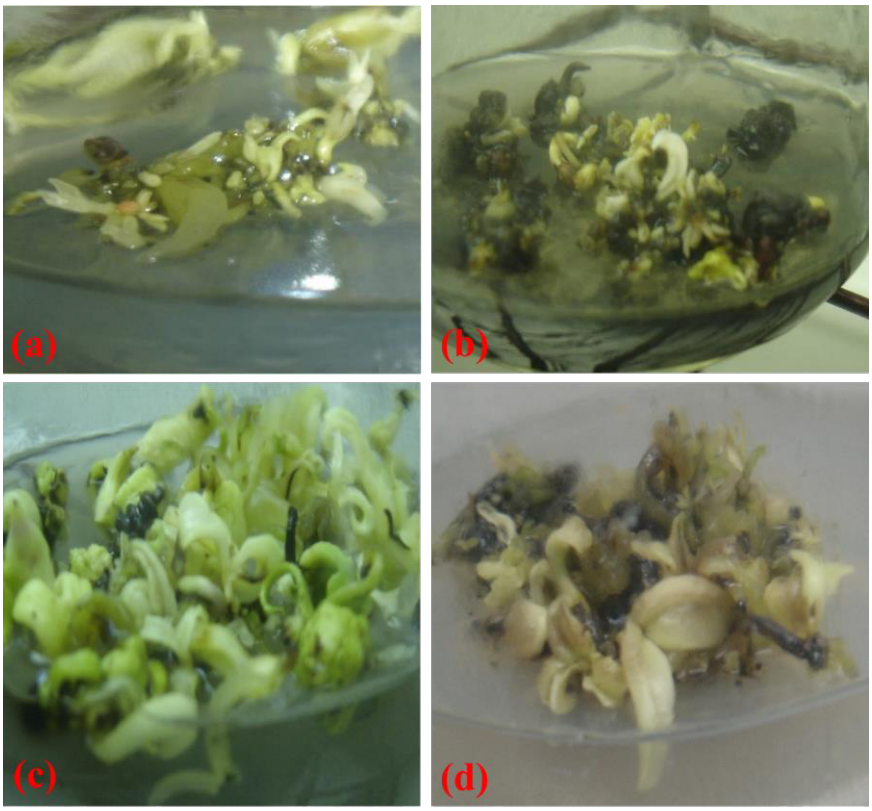

Fig. 3. (a) proliferation of SE on medium containing MS microsalts, B5 macrosalts, 0.1 mg L^{-1} 2iP, 0.5 mg L^{-1} Kin (b) proliferation of SE on medium consisted of micro and macrosalts of MS, 0.1 mg L^{-1} 2iP, 0.5 mg L^{-1} Kin (c) multiplication of SE on medium consisted of 200 mg L^{-1} AC, 2iP 0.1 mg L^{-1}, Kin 0.5 mg L^{-1} (d) multiplication of SE on medium without AC, 2iP 0.1 mg L^{-1}, Kin 0.5 mg L^{-1}.

maturation, proliferation and plantlets formation. Xiao *et al.* [35] used 23 µ M Kin in medium for proliferation and germination of SE. Laxmi *et al.* [32] suggested addition of GA_3, N6-benzyl amino purine, MS microsalts, B5 macrosalts and additives for proliferation and germination of embryos.

3.5. Effect of Basal Salts, AC and Sucrose on Shoot Elongation under Light (16h Photoperiod with cool white fluorescent light (160 µmol m^{-2} s^{-1})

Two-way-ANOVA for shoot length revealed impact of treatment as significant ($p<0.001$) while effect of variety and interaction between variety and treatment were found significant, but at lesser significant levels as $p<0.008$ and $p<0.025$, respectively (Table 5). The shoot elongation medium comprising of micro and macrosalts of MS with 200 mg L^{-1} AC revealed significantly highest shoot length in var. 'Langra' (5.1 cm) followed by vars. 'Chaunsa' (4.2 cm) and 'Saroli' (4.0 cm). Medium comprising of similar salts but without AC showed poor shoot length in var. 'Saroli' (2.0 cm), var. 'Chaunsa' (2.2 cm) and var. 'Langra' (2.3 cm). Medium comprising of micro and macrosalts of

Table 5. Impact of different treatments of MS and B5 salts, AC and sucrose on *in vitro* shoot length of three mango varieties under light.

Treatment	Saroli	Langra	Chaunsa
Micro and macrosalts of MS, AC 200 mg L^{-1}	4.0 ± 0.2^{c}	5.1 ± 0.4^{a}	4.2 ± 0.6^{bc}
Micro and macrosalts of MS	2.0 ± 0.5^{efgh}	2.3 ± 0.3^{de}	2.2 ± 0.4^{def}
Micro and macrosalts of B5, AC 200 mg L^{-1}	4.7 ± 1.2^{ab}	4.3 ± 0.7^{bc}	4.6 ± 0.3^{ab}
Micro and macrosalts of B5	1.5 ± 0.5^{hi}	2.1 ± 1.6^{efg}	1.3 ± 0.2^{i}
Microsalts-MS, macrosalts-B5, Sucrose 30 g L^{-1}	4.3 ± 1.3^{bc}	4.9 ± 0.8^{a}	4.3 ± 0.2^{bc}
Microsalts-MS, macrosalts-B5, Sucrose 40 g L^{-1}	2.7 ± 0.6^{d}	2.3 ± 0.4^{de}	2.2 ± 0.2^{def}
Microsalts-MS, macrosalts-B5, Sucrose 50 g L^{-1}	1.7 ± 0.6^{fghi}	1.6 ± 0.2^{ghi}	1.7 ± 0.3^{fghi}
Microsalts-MS, macrosalts-B5, Sucrose 60 g L^{-1}	1.5 ± 0.2^{hi}	1.7 ± 0.2^{fghi}	1.3 ± 0.9^{i}
Variety (mean)	3.0 ± 0.0^{a}	2.8 ± 0.1^{b}	2.7 ± 0.1^{b}
Source of variability			
Variety	0.008		
Treatment	< 0.001		
Variety × Treatment	0.025		

Mean values in columns with standard error denoted with different superscript letters show significance level at $p \leq 0.05$.

Fig. 4. Impact sucrose treatments, (a) 30 g L^{-1}, (b) 40 g L^{-1}, (c) 50 g L^{-1}, and (d) 60 g L^{-1} on *in vitro* shoot elongation and rooting in vars. 'Saroli', 'Langra', 'Chaunsa' under light.

B5, 200 mg L^{-1} AC enhanced shoot length in vars. 'Saroli' (4.7 cm), 'Chaunsa' (4.6 cm) and 'Langra' (4.3 cm), whereas the plantlets cultured on the medium with similar salts but lacking AC showed poor growth of plantlets. Medium comprising of 30 g L^{-1} sucrose, NAA (0.1 mg L^{-1}), BA (0.2 mg L^{-1}) induced significantly highest shoot length in vars. 'Langra' (4.9 cm), 'Saroli' (4.3 cm) and 'Chaunsa' (4.3 cm) (Figure 4a) than rest of the sucrose treatments (40, 50, 60 g L^{-1}) (Figure 4b-d). Al-Busaidi *et al*. [6] utilized B5 macrosalts and MS microsalts throughout all *in vitro* growth stages of mango including shoot regeneration. Litz [27] and Laxmi *et al.* [32] observed that 20 g L^{-1} sugar was important for plantlet formation in mango. Hemphill *et al.* [36] observed elongation of cotton (*G. hirsutism*) shoots cultured on medium comprising of 3 g L^{-1} AC. Current protocols also described positive impact of 200 mg L^{-1} AC on mango shoot-root development under light.

3.6. Effect of Basal Salts, AC and Sucrose on Root Elongation under Light (16h Photoperiod)

Treatment effect (p<0.001) significantly influenced the root elongation, and effect of variety found significant but on the least level (p<0.046), whereas the interaction effect of both failed to express any significance in root elongation (Table 6). Data in Table 6 show that highest root length was obtained on medium comprising of micro and macrosalts of MS, 200 mg L^{-1} AC in var. 'Langra' (4.0 cm) followed by 'Chaunsa' (3.7 cm) and 'Saroli' (3.2 cm). On the contrary, the root length was decreased significantly on medium comprising of micro and macrosalts of MS but without AC. Medium consisted of micro and macrosalts of B5 with 200 mg L^{-1} AC improved root length in vars. 'Saroli' (3.2 cm), 'Chaunsa' (3.1 cm), 'Langra' (3 cm), whereas the medium with similar salts but without AC caused significant reduction in root length. Simultaneously, the significantly highest root length was obtained on the medium comprising of 30 g L^{-1} sucrose, microsalts of MS, macrosalts of B5. Out of four concentrations of sucrose (30, 40, 50 and 60 g L^{-1}), with microsalts of MS and macrosalts of B5, only 30 g L^{-1} sucrose produced highest root length in var. 'Langra' (3.8 cm) (Figure 4a), var. 'Saroli' (3.6 cm) and var.'Chaunsa' (3 cm). Laxmi *et al.* [32] recommended lower sucrose quantity in addition to B5 macrosalts, MS microsalts for germination SE of mango, led to the plantlet formation. In the current study noted that 30 g L^{-1} sucrose was effective in better growth of roots. Obtained results are in agreement with Abul-Soad *et al.* [7] regarding vigorous growth of roots in mango shoots on medium comprising of 30 g L^{-1} sucrose in addition to 200 mg L^{-1} AC, B5 macrosalts, MS microsalts. Ara *et al.* [37] suggested a procedure for rooting in mango plantlets obtained from nucellar SE and described that IBA was most responsive in rooting. NAA (0.1 mg L^{-1}) is also

Table 6. Impact of different treatments of MS and B5 salts, AC and sucrose on *in vitro* root length of three mango varieties under light.

Treatment	Saroli	Langra	Chaunsa
Micro and macrosalts of MS, AC 200 mg L^{-1}	3.2 ± 0.7^{bcd}	4.0 ± 0.6^{a}	3.7 ± 0.7^{ab}
Micro and macrosalts of MS	1.6 ± 0.4^{efgh}	1.5 ± 0.8^{efghi}	1.8 ± 0.4^{ef}
Micro and macrosalts of B5, AC 200 mg L^{-1}	3.2 ± 0.5^{bcd}	3.0 ± 1.6^{d}	3.1 ± 0.5^{cd}
Micro and macrosalts of B5	1.4 ± 0.7^{fghi}	1.7 ± 1.2^{efg}	1.0 ± 0.3^{i}
Microsalts-MS, macrosalts-B5, Sucrose 30 g L^{-1}	3.6 ± 1.3^{abc}	3.8 ± 0.2^{a}	3.0 ± 0.2^{d}
Microsalts-MS, macrosalts-B5, Sucrose 40 g L^{-1}	1.6 ± 0.6^{efgh}	2.0 ± 0.3^{e}	1.5 ± 0.7^{efghi}
Microsalts-MS, macrosalts-B5, Sucrose 50 g L^{-1}	1.3 ± 0.3^{fghi}	1.2 ± 0.8^{ghi}	1.0 ± 0.3^{i}
Microsalts-MS, macrosalts-B5, Sucrose 60 g L^{-1}	1.1 ± 0.3^{hi}	1.0 ± 0.6^{i}	1.0 ± 0.4^{i}
Variety (mean)	2.1	2.3	2.0
Source of variability			
Variety	0.046		
Treatment	< 0.001		
Variety × Treatment	0.137		

Mean values in columns with standard error denoted with different superscript letters show significance level at $p \leq 0.05$.

a extensively utilized PGR in rooting media for growth of different plant species including date palm [38].

4. CONCLUSION

Successful somatic embryogenesis and plantlet regeneration, shoot elongation and rooting was obtained using nucellar explants obtained from immature fruits of vars.'Saroli', 'Langra' and 'Chaunsa' through direct and indirect somatic embryogenesis. Surface sterilization of immature fruits resulted in maximum survival of initial nucellar explants. PGRs were observed effective for callogenesis, direct and indirect somatic embryogenesis. Browning was reduced using ascorbic acid, AC, and culture conditions (i.e full dark). 2,4-D and 2iP combinations induced embryogenic callus or direct somatic embryogenesis. High proliferation of proembryogenic callus in dark, somatic embryogenesis and plantlet regeneration accomplished under light conditions. Different experiments were conducted at shoot elongation and rooting stages improved the shoot and root growth and elongation. Sucrose 30 g L^{-1}, microsalts of MS and macrosalts of B5 were better for healthy growth of plantlets. The current *in vitro* protocols of the superior monoembryonic mango varieties will be helpful to propagate other superior monoembryonic and polyembryonic varieties grown in the area and worldwide. Current study described the protocols induced direct somatic embryogenesis will support to obtain true-to-type plantlets of the elite varieties.

5. CONFLICT OF INTEREST

There is no competing interest among the authors.

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Proceedings of the Pakistan Academy of Sciences: B
Life and Environmental Sciences 60(3): 443-454 (2023)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(60-3)840

Pakistan Academy of Sciences

Research Article

Assessment of Antidiabetic and Cyto-Regenerative Activity of *Ficus carica* through Gene Expression Analysis in Diabetic Rat Model

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Abstract: Diabetes mellitus is a metabolic disease of the endocrine system, characterized by chronic hyperglycemia resulting from insulin resistance or defective insulin production. Among the complementary and alternative medicines, diet-based approaches are gaining popularity worldwide for the management of it. *Ficus carica*, one of the oldest plants cultivated on the earth, is rich in phytochemicals including anthocyanins, phenolics, flavonoids, and organic acids. The present study was designed to analyze the therapeutic potential of dried fig and extract for their potential against hyperglycemia and related complication in the diabetic rat model. Diabetes was induced by using alloxan monohydrate and divided into five groups including Negative-, Positive-, standard drug- group, treated-I (given extract), and treated-II (given 10 % dried figs). Fig extract was administered through the intragastric tube, and fig paste was mixed in the feed of the experimental group, and then rats were decapitated after 6 weeks to collect the blood and serum. At the end of the study, biochemical analysis such as fasting blood glucose (FBG), serum glucose, and insulin was performed. Histopathological study of the pancreas showed cell deformation in the positive control group whereas damage was reversed in treated groups. The pancreas was also saved for gene expression analysis. The results revealed that the positive control group has lower expression of INS-1, INS-2, Pdx-1, amylin, and GLUT-2 genes. Results revealed that serum glucose and FBG started to normalize after the administration of treatment (Glibenclamide, dry fig, and fig fruit extract), and insulin concentration also started to improve. 10 % dried fig was more effective to control hyperglycemic conditions, which might be due to the presence of fiber. However, the gene expression was more modulated in the group treated with fig extract. The findings of current research suggested the utilization of fig and fig-based products because of their potential to reverse the damage induced by the alloxan or stressors of daily life.

Keywords: Diabetes Mellitus, Pancreases, Regeneration, *Ficus carica*, Genes, Extract, Histopathology

1. INTRODUCTION

Diabetes Mellitus (DM) has become a pandemic in the last few years, if it is not controlled over a long period, it may lead to the development of serious health ailments including retinopathy, blindness, cardiovascular diseases, nephropathy, and neuropathy. However, if proper care is given to patients, these problems can be prevented and delayed [1]. According to the National Diabetes Survey of Pakistan 2016-17 (NDSP), about 26.3 % of individuals are diabetics, of which 7.1 % have been recently diagnosed [2]. Urban people (28.3 %) have more incidence than rural (25.3 %) ones. Unfortunately, nearly 6 % (4.6 million) of people are not aware of their disease. The major risk factors related to DM are aging, obesity, family history, hypertension, and dyslipidemia [2]. According to a recent estimate by IDF, Pakistan has attained the 3rd position, with 33 million people, and is predicted to reach 62.2 million by 2045 [3]. The management of DM and its complications without any marked side effects is still a major challenge. Numerous conventional

Received: January 2023; Revised: May 2023; Accepted: June 2023
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methods such as allopathic medicines, and herbal and medical plants are also in use. The acceptability of complementary and alternative medicines (CAM) has increased many folds in the last few decades. Many surveys have reflected that about 48.5 % of people use at least one or more forms of CAM in Australia and the US including herbal medicine, medicinal plants, and supplements [4]. Globally, almost 30 % of diabetic patients are using CAM, however, in Pakistan, approximately 50 % of diabetic patients rely on these types of medicines, based on personal knowledge and practices. Probiotics**,** vitamins, minerals, and herbs are the major products in these categories. These products are prepared and extensively promoted as dietary supplements. Likewise, numerous plant extracts, pulp, seeds, stems, roots, and sometimes whole fruits, vegetables, and herbs are also used by consumers to treat various diseases. These are famous for being harmless and cost-effective [5].

Ficus carica Linn. belongs to angiosperms and comprises almost 800 species of plants. One of the most consumed and famous fruit and is known by various names such as *Tian* (Arabic), *Anjir* (Urdu), and *Figari* (Hindi),. A few fruits are mentioned in Holy Quran and Ahadith, the fig is one of them. In the Holy Quran, the first verse of Surat At-Tin illustrates the benefits of this fruit as it says ***(I swear), by the fig and the olive*** (Quran, 95:1). Fig fruit is popular for its sweet, delicious taste having nutritional as well as therapeutic benefits. It is consumed by humans as well as by animals in dried and fresh forms. In the Mediterranean region, the fig is known as poor man's food and is extensively consumed in the dried and fresh form [6]. Figs are energy-dense fruit with appreciable amounts of minerals and fiber. It is cholesterol and sodium-free. The fresh fig fruit has nearly 80 % water, 17.3 % carbohydrate, 1.7 % fiber, 1.2 % protein, 0.6 % ash, and 0.3 % fat along with 76 Kcal per 100 g [7]. Fig is rich in hydrocarbons, aliphatic alcohols, volatile compounds, fatty acids, and some other plant metabolites such as coumarins, flavones, triterpenoids, and steroids, [8]. Numerous bioactive components have been isolated in different parts of fig. Phenolic compounds composition is affected by climate conditions, cultivars, processing method, production location, ripening stage, and storage method. These are also popular for their antioxidant potential. Anthocyanins are mainly present in fig varieties having blue, pink, and violet-colored pulp or skin. These bioactive compounds, help to regenerate β-cells and alleviate the symptom of DM [9].

In traditional medicines including Siddha, Unani, and Ayurveda, figs are used extensively for the cure and prevention of numerous health ailments. It has been utilized for the treatment of disorders related to the cardiovascular system, endocrine system, gastrointestinal tract (anorexia, colic, diarrhea, indigestion, vomiting, and ulcer), infectious diseases (gonorrhea, scabies, and skin disease), inflammation, liver, reproductive system (menstruation), respiratory system (asthma, bronchial problems, cough, sore throats) and spleen [10, 11]. Fig is also used in a blend with various medicinal herbs, milk, and honey. The fig fruit is used in both dried and fresh forms. Depending on the variety and type, dried figs can be commercialized for diverse uses, such as table consumption or to prepare other commercial products like canned figs and fig paste. Mission variety is used as dried fruit and to make juices and paste, however, Adriatic and Kadota varieties are specifically utilized for paste production. In California, a larger amount of produced fig is used to make energy bars and cookies. Fig is also used in baked products like pastries, pie, and cooked dishes. Low-quality dried figs are used mostly to flavor coffee and to prepare concentrated juices [12]. Fresh unpeeled and peeled figs are used in several ways in bakery products including cakes, fig pies, and puddings. Numerous products like fig ice cream, fig jams, fig marmalade, fig Newtons, fig paste, and fig rolls are commercially available. The addition or filling of fig's paste in wheat and corn flour, along with other ingredients such as oil, syrup, resulting in the production of delicious bakery products. Moreover, sugar syrup from the whole fig is also prepared at the household level [13].

In various efficacy studies, the therapeutic and nutritional potential of fig has been proven. Flavonoids have insulin-mimetic and insulin-secretagogue action, which depends upon the type, quantity, and structure of bioactive compounds. Luteolin, quercetin, and rutin are the prominent flavonoids found in fig and its various components. The bioactive compounds enriched extract of different parts of fig has been evaluated for various

mechanisms such as antioxidant potential and anti-hyperlipidemia. The phenolic-rich extract of fig was evaluated for antioxidant potential and anti-hyperlipidemia in the streptozotocin-induced diabetic rats' model. Indigenous antioxidant enzymes in the heart liver, and kidney has improved after intake of the extract [14]. The c-Jun N-terminal kinase (JNK), Janus Kinase / Signal Transducer and activator of transcription (JAK-STAT), and FOXO1 are the established cell stress and insulin signaling pathways in DM. About 0.2 % dietary intake of cyanidin 3-glucoside helped in the improvement of insulin sensitivity and fasting glucose in high-fat and obese mice. Moreover, the concentration of mRNA of inflammatory cytokines including TNFα and interleukin-6 was also reduced along with repressed infiltration of macrophage in adipose tissue. Insulin signaling transcriptional factors such as FOXO1 are a chief modulator of insulin signaling in β-cells, hepatocytes, and adipocytes. It regulates gluconeogenic enzymes in the fasting state whereas in the fed state reduces gluconeogenesis through insulin-mediated phosphorylation of FOXO1. Cyanidin 3-glucoside down-regulates the enzymes like Glucose-6-Phosphatase and Phosphoenolpyruvate carboxy kinase in the liver and adipose tissues. Moreover, it down-regulates the JNK activation and promoted the phosphorylation of FOXO1 [15]. Keeping this in view, the current experiment is designed as an attempt to explore the antidiabetic and cyto-regenerative ability of fig and fig extract in β-cells and underlying cellular mechanisms.

2. MATERIAL AND METHODS

2.1. Procurement of Materials

Sun-dried Afghani fig required for research was procured from Faisalabad (Pakistan) local market. Alloxan monohydrate and all reagents for biochemical evaluation were procured from Sigma-Aldrich (Sigma Aldrich, Tokyo, Japan) and Merck (Merck KGaA, Darmstadt, Germany). Glibenclamide tablets 5mg (Daonil®) were purchased from Sanofi-Aventis (Pvt.) Ltd., Pakistan. PCR Optical 8-Tube Strip was acquired from the Applied Biosystems™ MicroAmp™ Thermo Fisher Scientific, USA, SYBR® Green qPCR super mixes were procured from BIO-RAD, USA, and TRIzol was bought from Thermo-Fisher Scientific, Massachusetts, USA. Male Sprague Dawley rats were obtained from the National Institute of Health (NIH), Islamabad, Pakistan for bio-efficacy trials.

2.2. Preparation of Fig Fruit Extracts

The dried fig was freeze-dried with liquid nitrogen and was converted into a fine powder with the motor pestle after. Subsequently, fig fruit extract was obtained through a solvent extraction method by following the modified method of Bucić-Kojić *et al*. [16]. The fig fruit powder (25g), was extracted with 500 mL of ethanol (70 % (v/v)) at 80ºC for 45 mins in the ultrasonic-assisted water bath. Afterward, the sample was placed on the orbital shaker for 3 hrs. at 280 rpm. The obtained mixture was centrifuged at 5000 resolution per minute for 15 minutes, and then polyamide Chromafil disposable filter AO-45/25 was used to filter the supernatant. The rotary evaporator was used to concentrate the acquired solution and freeze-dried. Obtain dried extract was stored in glass vials and stored at -40ºC.

2.3. Bio-efficacy Study

Healthy young albino rats (50), approximately 180-200g weight, were housed in an Experimental Animal House of the Faculty of Food, Nutrition, and Home Science, University of Agriculture, Faisalabad (UAF). The research was performed by observing the Institutional Biosafety Committee (IBC) guidelines, UAF (D. No.: 8856/ORIC, Dated: 28 November 2019). The animals were acclimatized one week earlier to the experimentation under standard conditions. The temperature (23±2°C) and humidity (50±5 %) were provided throughout the period with 12 hrs. light-dark cycle. Rats were given normal feed and water *ad libitum*. The blood glucose level was checked before inducing diabetes in the rats. Afterward, diabetes was induced using a single intraperitoneal injection of alloxan monohydrate (150mg alloxan dissolved in normal saline per kg body weight). The rats were served with dextrose solution (10 %) within the 12 to 24 hrs. of alloxan administration to avoid mortality. Fasting blood glucose level was measured using a glucometer (Vivachek™ Ino BGMS, Biotech Co., Ltd Hangzhou, China) by taking a blood sample from the tail vein after 3, 7, and 10 days for confirmation of diabetes. The rats exhibiting ≥300 mg/dL of fasting blood glucose (FBG), were

divided into the following groups (5 rats per group) for interventional trial:

G_0: Negative control group, rats were fed on the normal diet

G_1: Positive control group, Alloxanized diabetic rats were fed on the normal diet

G_2: Glibenclamide, Alloxanized diabetic rats treated with Glibenclamide (0.6 mg/kg body weight) and fed on the normal diet

G_3: Treated-I, Alloxanized diabetic rats treated with an extract of fig (400mg/kg body weight) and fed on the normal diet

G_4: Treated-II, Alloxanized diabetic rats treated with 10 % dried figs mixed in the normal diet

2.3.1. Biochemical analysis

The rats (3 from each group) were decapitated (after 4 weeks) at the termination of the efficacy trial, to collect blood samples and centrifuged at 4000 rpm for 10-15 mins for the separation of plasma and serum. The sample was stored at -20˚C to use later for biochemical tests using respective methods. With the help of the glucometer, the FBG was measured from the tail vein. A commercially available kit (Bioclin® Glucose Monoreagent diagnostic kit, BioClin Therapeutics, Brazil) was used to measure serum glucose. However, the insulin was determined by using the kit (Insulin ELISA®, Calbiotech Inc, USA).

2.3.2. Histopathological examination

The pancreases of each decapitated rat were preserved in 10 % formalin solution for histopathology whereas, for mRNA extraction, pancreas tissue was stored in Trizol. Histopathology of the pancreas was performed following the methods of Nurdiana *et al*. [17].

2.3.3. Gene expression analysis

RNA isolation was performed from the pancreas by using the TRIzol method (Johnson, 2018), and isolated RNA samples were quantified by using nanodrop. Afterward, isolated RNA was subjected to cDNA synthesis [18] and protein expression was quantified using real-time qPCR. As a housekeeping gene, Beta-actin was used. The expression of underlying cellular mechanisms including insulin signaling pathway (INS-1, INS-2, Pdx-1), calcium signaling pathway (Pias-2, Calm-2, Grk-2), regeneration (IGF-1, FOXA-1, KI67), hormones (amylin, leptin), and glucose transporter (GLUT-2). During PCR following protocol was followed.

Denaturation of the cDNA: for 15 sec at 95 ^{o}C
Annealing temperature: 58 °C for 30 sec
Extension time: 20 seconds at the temperature of 72 ^{o}C for 39 cycles

Afterward, the 2*(- ΔΔct) method was used to analyze the qRT-PCR data.

2.3.4. Statistical Analysis

The recorded results were evaluated statistically by using analysis of variance (ANOVA) and Tukey's HDS test was performed to determine significant differences among the groups as described by Montgomery [19]. Results are presented in Mean values ± standard deviation.

3. RESULTS

3.1. Biochemical Analysis

Mean squares showed significant variation in the FBG, serum glucose, and serum insulin among the different experimental groups (Table 1). The highest FBG (345.74±12.77 mg/dL) was observed in the positive control group (G_1), followed by the group treated with Glibenclamide (G_2) (284.42±10.41 mg/dL). The rat groups treated with dried fig fruit extract (G_3) and 10 % dried fig fruit supplemented diet (G_4) showed significant downregulation in FBG levels.

The recorded values for FBG in G_3 and G_4 were 261.23±11.19, and 210.91±7.71 mg/dL, respectively. The highest serum glucose (278.85±10.30 mg/dL) was observed in the positive control group (G_1) followed by the group treated with Glibenclamide (G_2) (227.67±8.02 mg/dL). The groups treated with fig-based interventions showed a significant reduction in serum glucose levels. The group G_3 and G_4 reported 210.69±9.03 and 169.91±5.95 mg/dL glucose levels, respectively. The highest insulin (24.80±0.45 µIU/mL) was observed in the negative control group (G_0),

Table 1. Effect of treatments on fasting blood glucose, serum glucose, and serum insulin in the experimental rats

Experimental groups	Parameters		
	Fasting Blood Glucose (mg/dL)	**Serum Glucose (mg/dL)**	**Serum Insulin (µIU/mL)**
G_0	96.32±8.27^d	85.79±4.99^d	24.80±0.45^a
G_1	345.74±12.77^a	278.85±10.30^a	15.28±0.79^c
G_2	284.42±10.41^b	227.67±8.02^b	22.69±0.88^b
G_3	261.23±11.19^b	210.69±9.03^b	24.33±0.79^a
G_4	210.91±7.71^c	169.91±5.95^c	22.58±0.23^b

Mean ± SD; Mean values within a column, bearing a different superscript are significant
G_0: Negative control group, **G_1:** Positive control group, **G_2:** Glibenclamide, **G_3:** Treated-I, **G_4:** Treated-II

followed by the G3 (24.33±0.79 µIU/mL), then G2 (22.69±0.88 µIU/mL), and G4 (22.58±0.23 µIU/mL). The hyperglycemic condition started to normalize after the administration of treatment (Glibenclamide, dry fig, and fig fruit extract), and insulin concentration also started improving.

3.2. Histopathological Examination

Uncontrolled inflammatory processes and hyper generation of free radical moieties make cells susceptible to damage and injuries. The histopathological examination assists in evaluating the type and degree of organ damage. In this research, a histopathological assessment of the pancreas was done to predict the efficiency of figs (extract and feed) in comparison to alloxan and Glibenclamide. The histological examination of pancreatic tissues is presented in Figure 1. In the negative control group (G_0), the acinar cells and β-cells of the pancreas were present in the normal proportion, showing complete, and regular pancreatic volume. The cytoplasm of acinar cells was stained light with prominent dark-stained nuclei which are organized in lobules. The β-cells are seen surrounded by acinar cells and by a fine capsule. Neither necrosis nor inflammatory cell infiltration nor any microscopic lesion around the islet tissues and the alveolar cells was observed in G_0. In positive control rats (G_1), administration of alloxan leads to the inflammation and destruction of the β-cells, alongside the steatosis of the acinar cells. The necrosis of the islet tissues leads to a reduction in the number and size of pancreatic islets. The

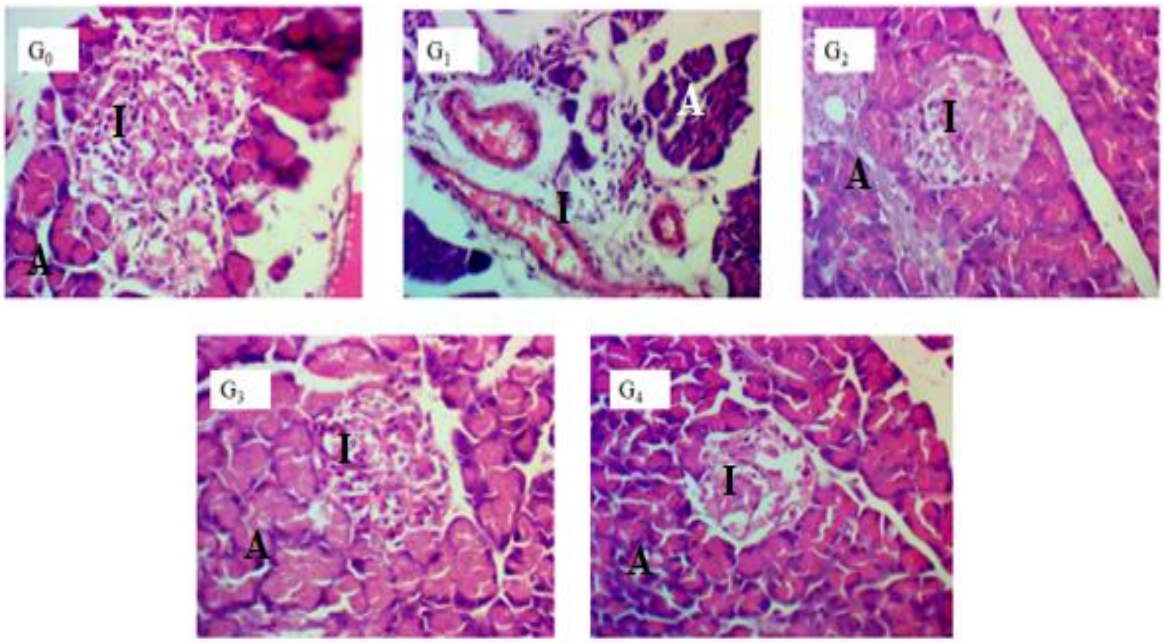

Fig. 1. Histopathological indications of pancreas tissues
A= Acinar cells I= Islet of Langerhans, H&E stain, Magnification= 40x, Sacle bar= 100 µm

acinar cells around the β-cells are arranged loosely and the nucleus occupies a large area, which does not look normal.

In glibenclamide-treated rats (G_2), the disruption of the β-cells and the acinar cells steatosis along with infiltration of inflammatory cells was observed. It also showed moderate destruction of alveolar cells. Compared with the normal group, G_2 showed improvement in pancreatic structure, volume, and the number of pancreatic cells, with compact cytoplasm, and reduced vacuolar degeneration. In the group treated with fig fruit extract (G_3), significant protection of β-cells and acinar cells has been seen. The acinar cells were seen as normal with only mild disruptions. The islets have a substantial proportion of β-cells with scanty inflammatory cell infiltration. Similarly, the group fed on a 10 % figs fruit-supplemented diet (G_4), showed normal proportions of the acinar cells and β-cells. The islet cells were surrounded by acinar cells and the fine capsule. Slight necrosis and inflammatory cell infiltration were observed. The characteristic interlobular and intralobular ducts were also seen.

3.3. Gene Expression Analysis

To probe the effect of dietary interventions on the insulin production capacity and cyto-regenerative ability of β-cell, underlying cellular mechanisms including insulin signaling pathway (INS-1, INS-2, Pdx-1), calcium signaling pathway (Pias-2, Calm-2, Grk-2), and regeneration genes (IGF-1, FOXA-1, KI67), were studied through gene expression analysis. Moreover, genes of amylin, leptin (hormones), and glucose receptor GLUT-2 were also analyzed and presented in Figure 2. The expression level of insulin signaling genes is shown in Figure 2 (a, b, c). In the G_1, the expression level of INS-1, INS-2, and Pdx-1 genes were significantly down-regulated as compared to the negative control group (G_0; 2.45±0.07, 2.67±0.02, and 2.39±0.03, respectively). Glibenclamide and fig fruit extract treated groups and the group fed on 10 % figs fruit-supplemented feed, exhibited significantly higher expression levels for INS-1, INS-2, and Pdx-1 genes in comparison to G_1. The expression levels of Pias-2, Calm-2, and Grk2 were significantly upregulated (4.31±0.14, 4.90±0.14 and 4.34±0.11 respectively) in G_1 as compared to G_0 (1.19±0.01, 1.09±0.01, and 1.23±0.00 respectively) as shown in Figure 2 (e, f, g). G_2 and fig fruit-based interventional groups exhibited significantly lower expression levels of Pias-2, Calm-2, and Grk2 genes in comparison to G_1. The result of the current study demonstrates the positive effect of fig-based interventions in regulating glucose homeostasis. The expression level of IGF-1 (3.12±0.01), FOXO-1 (2.78±0.06), and Ki-67 (5.22±0.10) were significantly upregulated in the positive control group when compared to the negative control group (G_0; 1.34±0.09, 1.49±0.01, and 1.56±0.03 respectively) as shown in Figure 2 (g, h, i). Treated groups such as G_2, G_3, and G_4 exhibited a significantly lower expression level of the IGF-1 gene in comparison to G_1. In the positive control group, the expression level of amylin and GLUT-2 was significantly downregulated (0.67± 0.01 and 0.44±0.01) as compared to G_0 (1.23± 0.04 and 1.99±0.06) respectively. However, in the positive control group, the expression level of leptin was significantly upregulated (1.78±0.06) as compared to G_0 (0.56±0.02) as shown in Figure 2 (j, k, l). The results depicted that fig extract and feed supplementation have the potential to modulate the expression of this gene.

4. DISCUSSION

4.1. Biochemical Analysis

Biochemical analysis can reveal the necessary information that is required for accurate diagnosis of diseases and effect of treatments.

Plasma glucose is a key prognostic factor for the diagnosis of DM. The results of the current study are well backed by the previous findings. In a study, the methanolic extract of fig fruit exhibited a dose-dependent decline in FBG levels at 250 and 500 mg/Kg of the extract, *i.e.,* 165 and 50 mg/dL, respectively [20]. Purified fig fruit extracts containing abscisic acid were administered at two different doses, to investigate their effect on the glycemic index and insulinemic index in humans. The higher doses of abscisic acid (1200 mg) reduced insulinemic and glycemic reactions by ~25 %. This indicates that fig fruit extract supplementation is a good intervention for glycemic response management [21]. In another study, hexane, ethyl acetate, ethanol, and aqueous extracts of fig fruit were investigated for their

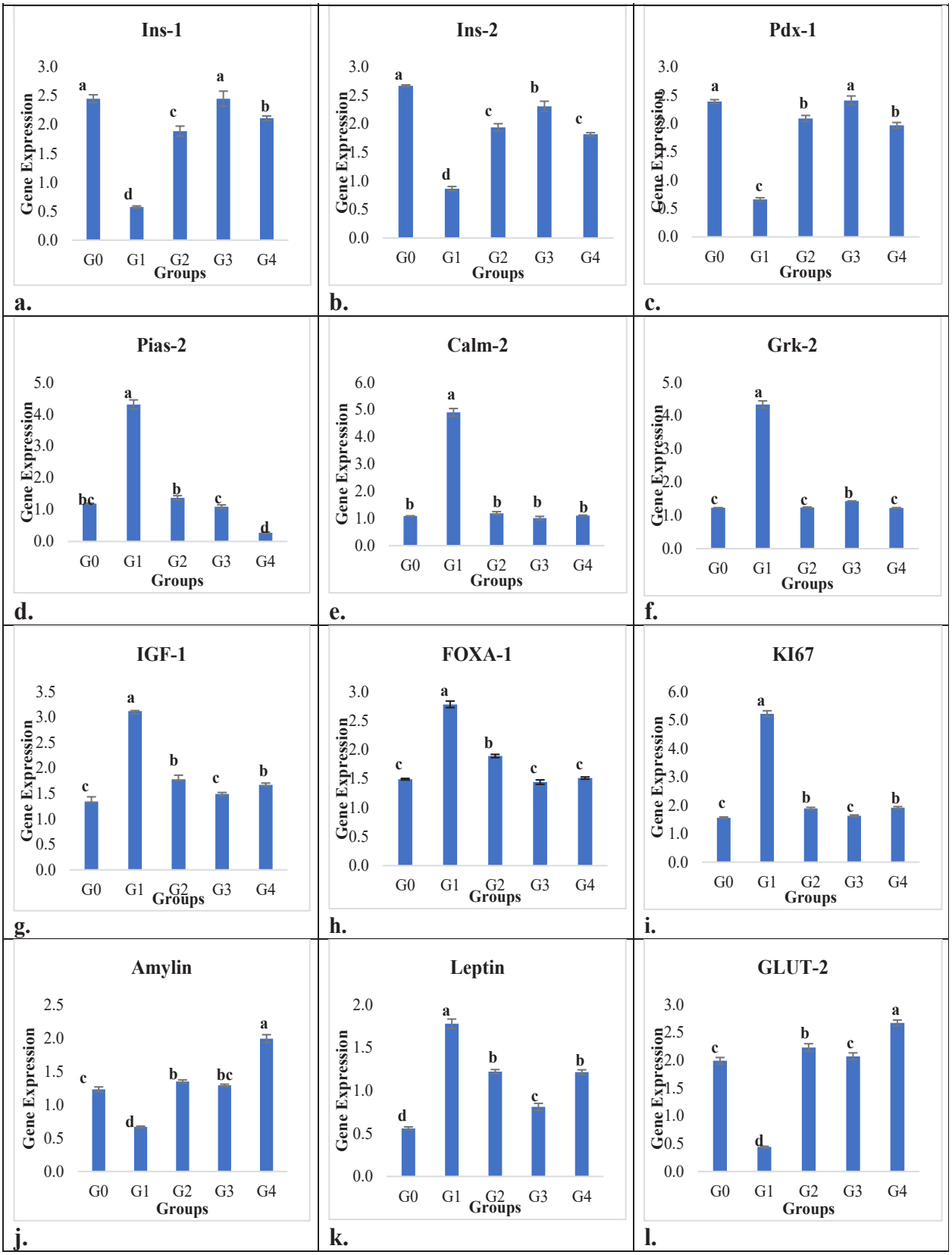

Fig. 2. The expression level of the insulin signaling pathway (a. INS-1, b. INS-2, c. Pdx-1), calcium signaling pathway (d. Pias-2, e. Calm-2, f. Grk-2), regeneration (g. IGF-1, h. FOXA-1, i. KI67), hormones (j. amylin, k. leptin), and glucose transporter (l. GLUT-2) in the pancreas of the experimental rats Mean values, bearing a different superscript are significantly different from each other ($p < 0.05$)

potential to hinder α-amylase and α-glucosidase. The IC50 values indicated the better potency of the ethanolic extract to inhibit α-amylase (315.89 mg/mL), and α-glucosidase (255.57 mg/mL) [22]. The consumption of ethyl acetate fig leaf extracts reduced the overall glucose concentration to 129.14 mg/dL which was comparable to the glucose level (131.32 mg/dL) in the group treated with Glibenclamide [23]. In another study, the effect of ficusin on serum insulin levels in diabetic rats was evaluated and the findings suggested significant improvement in the levels of insulin (22.77 μIU/mL) in a dose-dependent manner [24].

In a subsequent study, fig fruit was analyzed for its anti-hyperglycemic potential at different concentrations *i.e.,* 50, 100, and 150 mg/Kg in STZ diabetic rats over 14 days. Results showed that fig extract was able to reduce blood glucose from 379 to 87 mg/dL at the dose of 150 mg/Kg in 14 days. However, positive control remained hyperglycemic, and blood glucose increased from 361 to 505 mg/dL [25]. In the current research, the group fed on a 10 % figs fruit-supplemented diet showed better glycemic control among the interventional groups. This may be attributed to the dietary fiber content in fig fruit which hinders glucose absorption, along with regulation of the lipid levels without disturbing the gastrointestinal tract (GIT) system [26]. The reduction of hyperglycemic conditions in the experimental group fed on dried fig fruit and extract might be done to better control glucose levels in serum due to the presence of bioactive components in fig fruit that have insulin-mimetic properties [27]. Comparable results are reported by El-Shobaki *et al*. [28], that diabetic rats showed drastically higher serum glucose (228 mg/dL) compared to normal rats (94 mg/dL) and diabetic rats treated with 5, 10, and 20 % fig fruit supplemented diet (198, 198, and 177 mg/dL, respectively). 4, 6, and 8 % fig leave supplemented diet (158, 138, and 131 mg/dL, respectively).

4.2. Histopathological Examination

Exhaustion of β-cells resulted in insulin deficiency. Insulitis is commonly seen in islets containing residual β-cells [29]. In a study, the pancreas of normal rats exhibited embedded β-cells in the exocrine portions. However, the diabetic group showed pathological changes in the exocrine and endocrine parts of the pancreas along with a noticeable reduction in the β-cells [30]. The alloxan-induced diabetic rats showed necrosis of the islet tissues with the destruction of the alveolar cells [31]. Moreover, alloxan administration resulted in the infiltration of inflammatory cells in both β-cells and acinar cells [32]. STZ-induced diabetes decreased insulin-immunoreactive expression along with defects in insulin action in the peripheral tissues which resulted in hyperinsulinemia that destroyed the cell integrity and functional ability of the β-cells. However, the fig-treated diabetic rats in the study conducted by Irudayaraj *et al*. [23] showed increased insulin-immunoreactive expression indicating the cytoprotective (β-cells) role of the fig extract. Similarly, fig leaf extract showed a protected effect on β-cells in diabetic rabbits [33].

4.3. Gene Expression Analysis

INS-1 and INS-2 are genes encoded for precursor protein that undergoes proteolytic cleavage and produces insulin which is stored in secretory granules of β cells of the pancreas [34]. Pdx-1, another gene, encodes a protein that is a transcriptional activator of various genes, including glucokinase, glucose transporter type 2 (GLUT2), somatostatin, and insulin. This nuclear protein plays a key role in the glucose-dependent regulation of insulin gene expression. The defects in this gene cause pancreatic agenesis and Type-2 diabetes [34]. The mechanism involved might be the alloxan-mediated generation of free radicals *i.e.* ROS inside pancreatic β-cell leading towards the decreased expression of the PDX-1 gene, which in turn causes the suppression of other genes involved in the insulin signaling pathway including INS-1 and INS-2 [35]. Plant extracts with the ability to inhibit the enzymes are useful in DM care. Numerous studies have reported that phytocompounds such as rutin, quercetin, and cyanidin alter the digestion as well as metabolism and absorption of carbohydrates in the gut [36]. Insulin resistance is a major problem in the management of DM. Defects in insulin receptors, transport of glucose, oxidation of glucose, metabolism of fatty acid, and synthesis of glycogen lead to insulin resistance. Tissues of adipose, liver and skeletal muscle are the most resistant to insulin in Type-2 DM. GLUT4 is active in adipose tissue and muscle and regulates the uptake of glucose [37]. Augmented expression of these receptors

facilitates glucose storage as glycogen. The results of current research showed that fig extract and feed supplementation have the potential to modulate the expression of insulin signaling pathway genes. This may be attributed to the bioactive moieties of figs that reverse the damage induced by the alloxan. In a study, the treatment of insulinoma cells with resveratrol upregulated the expression of some key genes such as GLUT2, Pdx1, and INS1 through regulating SIRT1 [38].

Pias-2 gene encodes a member of the protein inhibitor of the activated STAT family, which modulates several cellular processes including cell proliferation, innate immune system, inflammation cascade, and DNA damage. It is also suspected to modulate insulin resistance pathogenesis [39]. The Calm-2 gene is a member of the calmodulin gene family. It is a calcium-binding protein that plays a role in signaling pathways, cell cycle progression, and proliferation. Calm-2 gene has been identified to be associated with a risk of Type-2 diabetes [40]. The Grk2 gene plays an essential role in regulating insulin signaling and insulin-mediated glucose homeostasis in diabetic animals and its inhibition can restore insulin sensitivity in metabolically active tissues [41]. The result of the current study demonstrates the positive effect of fig-based interventions in regulating glucose homeostasis. The protein encoded by the IGF-1 gene is like insulin in function and structure and involved in facilitating growth and development [42]. The FOXO gene family plays a vital role in the development and maintenance of the endocrine pancreas. The Ki-67 gene encodes a nuclear protein that is associated with cell proliferation [43]. In the present study, the Ki-67 was augmented in positive control as beta cell damage (due to Alloxan) increase the expression of this gene.

It is apparent from the present results that fig extract and fruit have the potential to modulate the expression of the above analyzed genes, due to the presence of some bioactive components that reverse the damage induced by the alloxan. The results of the present study are supported by previous studies where different bioactive components from plants were tested for their potential to modulate gene expressions. In a previous study, the higher concentration of ROS resulted in increases in IGF-1 mRNA expression and protein in the rats [44]. Moreover, a higher level of ROS in the G_1 was also observed in the current study which might result in the overexpression of the IGF-1 gene. It is evident from the results that fig extract and feed supplementation have the potential to restore the antioxidative potential of the body by modulating the expression of this gene. Figs have several potential bioactive components such as flavonoids, phenolics, and anthocyanins that reverse the damage induced by the alloxan. In another study, polyphenols from tomato and soy help to downregulate the expression level of IGF-1 [45]. In rat pancreatic cells, supplementation with epigallocatechin gallate for two hours increased the expression of Pdx-1 and FOXO1 resulting in augmented β-cell viability and insulin secretion [36].

Amylin plays an important role in satiety regulation by controlling (slower) gastric emptying, which controls the spikes in post-prandial glycemic load [46]. The average surge in the amylin gene expression in G_4 compared to G_0 and other groups might result in better control of blood, and serum glucose (discussed in sections 3.1. and 4.1.). Leptin has been understood to have a main role in energy balance. It plays a role in mediating energy balance and food intake suppression which leads to weight loss. It can conclude from the result that fig extract and feed supplementation have the potential to modulate the expression of this gene. It can also interpret that fig has some bioactive components that reverse the damage induced by the alloxan.

5. CONCLUSION

Recently, for managing diabetes-related complications, a growing interest has been noted in developing natural antidiabetic drugs, especially from plant sources. Recent studies have reported that the crude extracts and active compounds from various Ficus species exhibit antidiabetic properties under various in vitro and in vivo models. In conclusion, the present work has demonstrated that 10 % dried *Ficus carica* incorporated in feed and its ethanolic extract have the potential to normalize oxidative stress, induce regeneration in cells, and reverse the damage induced by alloxan. It helped to normalize the blood and serum glucose and improved insulin concentration. Moreover, it was able to modulate the expression of genes of the

insulin signaling pathway (INS-1, INS-2, Pdx-1), calcium signaling pathway (Pias-2, Calm-2, Grk-2), regeneration pathway (IGF-1, FOXA-1, KI67), hormones (amylin, leptin), and glucose transporter (GLUT-2). INS-1, INS-2, amylin, and GLUT-2 gene expression were downregulated in diabetic rat control, where these genes showed more expression in treated groups.

6. ACKNOWLEDGMENTS

The authors gratefully acknowledge the funding received from the HEC, Pakistan for awarding the scholarship.

7. CONFLICT OF INTEREST

The authors have no conflict of interest.

8. ETHICAL STATEMENT

The study was conducted in an Experimental Animal House of the Faculty of Food, Nutrition, and Home Science, University of Agriculture, Faisalabad (UAF). The research was performed by observing the Institutional Biosafety Committee (IBC) guidelines provided by the institution (UAF). The ethical approval number is D. No.: 8856/ORIC, Dated: 28 November 2019.

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10. DECLARATION

I declare that the results are original, and the same material is neither published nor under consideration elsewhere. The approval of all authors has been obtained and in case the article is accepted for publication, its copyright will be assigned to the Pakistan Academy of Science.

Proceedings of the Pakistan Academy of Sciences: B
Life and Environmental Sciences 60(3): 455-462 (2023)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(60-3)844

Pakistan Academy of Sciences

Research Article

Municipal Solid Waste Management in Skardu: Current Status, and Corrective Measures

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Abstract: Solid waste management (SWM) is a marginalized sector in Gilgit Baltistan, causing a frightening situation, especially in the municipal area of district Skardu. The total municipal area of Skardu is about 4260 sq. km with a population of approximately 112996. In line with other government departments, the Gilgit Baltistan waste management company (GBWMC) is responsible for collecting and dumping solid waste. The current study was devised to calculate, characterize, and analyze the past status, and current position of municipal solid waste (MSW) production, so that necessary management practices and corrective measures can be carried out more efficiently in the study area. A series of interviews of concerned persons as well as extensive field surveys were conducted. The method used by GBWMC to collect waste was: door-to-door collection, placement of waste bins, and collection from the arterial roads. Waste was collected manually in polythene bags, handcarts, and baskets. The findings revealed that approximately 45-50 tonnes of waste was generated per day in the winter and in the summer it increases to 50-55 tonnes. The average waste generation was 0.43 kg per capita per day and is increasing at a rate of 2.28 % annually. The main sources of waste were commercial and household waste contributing approximately 60 % and 40 % of the total waste production respectively. There was no scientific disposal system for the collected waste which is dumped on the dumping sites by utilizing available resources considering Environmental protection agency (EPA) regulations along the Indus River bank. Thus specific steps should be taken to develop a proper scientific disposal system of collected wastes by incorporating the experts' expertise and modern technologies.

Keywords: Solid Waste Management, Skardu, GBWMC, Household Waste, Commercial Waste

1. INTRODUCTION

The environmental problems and their consequences are increasing day by day with the population explosion. One of the major problems faced by the contemporary world is solid waste management (SWM) [1]. Solid Waste (SWs) are all such non-flowing constituents produced by households, commercial and institution formations and discharged from their locations for example all litter and drain cleanings, street sweepings, construction waste, animal and plant waste, etc. [2]. Municipal solid waste (MSW) mostly called trash or garbage is mostly solids or semi-solids which are produced due to domestic, commercial, or institutional formations [3]. MSW consists of durable and non-durable materials such as food scraps, bottles, papers, furniture, clothing, and

Received: January 2023; Revised: May 2023; Accepted: June 2023
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various inorganic waste, however, waste products from demolition activities, and hazardous waste products from hospitals are not included in MSW [4]. Unwanted or rejected materials produced from industrial, residential, agricultural, mining, and commercial activities contaminate our environment and create environmental problems [5]. Moreover, the huge production of SW needs collection and disposal to minimize related problems [6]. In addition, changing human activities in society produces maximum quantities of waste, and its disposal is a major problem [7].

A SW hazard is one of the most concerning problems of the modern world and its proper disposal and management have remained challenging. As the intensity of problems related to SW differs with time and location specific studies need to be carried out in respective areas to find suitable management strategies. Human generates a huge amount of waste daily which are above the capability of the environment to incorporate it into the ecosystem or reduce its harmful effects on living organisms. This has been further made worse by many materials that are not replaced by environmental forces and consequently remain together in the surroundings making SW a major issue for human beings [8]. In addition, ever-changing human activities and living standards increase the generation of waste products. A sudden increase in volume and varieties of solid and hazardous waste are a result of continuous urbanization, industrialization, and economic growth, and it is a serious concern for national and local governments. These issues can be resolved through effective and sustainable management of generated waste [9]. However, if remained ignored it can create several environmental problems especially aesthetic degradation, the production of microbes, and soil, water, and air pollution in the environment [3].

The SWs generated from anthropogenic activities from individual to household, commercial to industry level have several forms that include recyclable, combustible, hazardous, compostable, and residual waste [10]. The collection, recycling, and disposal of this waste is an important concern for those stakeholders who are responsible for developmental activities, especially in the health and environment sectors. Furthermore, SWM strategies also play a significant role to reduce the emissions of greenhouse gases (GHGs) and to recover material and energy from SWs [11].

Many developing countries like Pakistan are facing serious environmental problems. Rapid population growth due to poor family planning, high fertility, illiteracy, and reduced gross domestic production (GDP) growth have put massive pressure on the country's natural resources and have expressively enhanced environmental pollution causing a huge SW production [2]. Rapid urbanization and waste generation are directly related and it is one of the foremost problems in developing countries. In Pakistan, the migration rate is ever-increasing [12], meanwhile, resources and expertise are scarce. In addition, none of the rural and urban areas has a proper SWM system from proper collection to proper disposal. Nearly 50 % of the total waste generation passes through the process of collection to disposal. The remaining uncollected waste causes various diseases like cholera, diarrhea, typhoid, hepatitis, dysentery, etc. [13]. In addition, it also has an immense impact on soil health and can deteriorate its properties leading to food insecurity and causing a serious threat to human survival [14].

Like other major cities of Pakistan, Gilgit-Baltistan is also facing serious challenges regarding SWM. During the past several years, considerable migration happened from rural to urban areas inducing a population explosion. The population census carried out in 1998 revealed that the population of Gilgit-Baltistan was 0.884 million [15]. However, according to GBEPA [16], the population was raised to 1.1 million and 14 % of the population lived in urban centers. The population growth rate in Gilgit Baltistan is 2.56 % comparing the national average which is 1.8 % with a male-to-female rate of 52:48.

Skardu City is one of the major metropolitan cities of the country where immigrants from all parts of the country tend to settle every year. The population, buildings, and houses are increasing manifold every coming year. According to GBWMC [17], the total population of district Skardu is approximately 238644 and the population of the municipal area of Skardu is approximately 112996, which comprises 16142 households, 4672 commercial units, and 424 industrial units. Every

year thousands of national as well as international tourists visit this city to see its natural beauty and enjoy the natural weather, environment, and the peaceful spring and autumn. Therefore, a better and improved system of both the municipality and waste management seems very crucial at present to combat the challenges. The main aim of the present study was to quantify, characterize and analyze the current status and corrective measures of SW generated in the municipal area of Skardu, Gilgit Baltistan, Pakistan. This study will provide a future consensus on SW and detect the threats to the natural environment by SW and uses this data for better management of SW produced in the study area.

2. MATERIALS AND METHODS

2.1. Description of the Study Area

The present study was carried out in the municipal area of district Skardu (Figure 1). Skardu is the capital of the Baltistan division and one of the major districts in Gilgit Baltistan. It is situated along the Kohistan-Ladakh terrane (35°17′25″N 75°38′40″E), with an elevation of approximately 2,300 meters above sea level in the Northern areas of Pakistan at the confluence of the Indus and Shigar Rivers [18,19]. Due to its immense beauty and natural resources, Skardu is well-known for national and international tourists in all seasons. It is considered a tourist hub during summer. According to GBWMC (2021), the total population of the municipal area of Skardu is approximately 112996 [17]. The study was focused on commercial & household SW generation, composition, transportation, and dumping methodology in Skardu City.

2.2. Municipal Committee and Gilgit-Baltistan Waste Management Company

SWM is one of the major challenges for Pakistan due to the abrupt explosion of population and lack of awareness among citizens. Like other cities in Pakistan, Skardu is also facing severe challenges regarding SWM. In Gilgit-Baltistan before 2016, the waste management (WM) sector was being operated by Municipal Committee. The WM section of the Municipal Committee was supposed to collect only main road waste daily, whereas there was no proper mechanism to collect waste from Mohallas/ Sectors and streets due to the shortage of human resources and vehicles causing environmental, health, and sanitation-related problems, especially deteriorating the natural beauty of the area. To meet the needs of environmental challenges in Gilgit-Baltistan, Waste Management Company (GBWMC) was established in 2016 in Gilgit city. After a successful operation in Gilgit, it was also established in Skardu in December 2017 while in other districts it has been recently established. GBWMC has enough human resources and vehicles to provide door-to-door services at the municipal

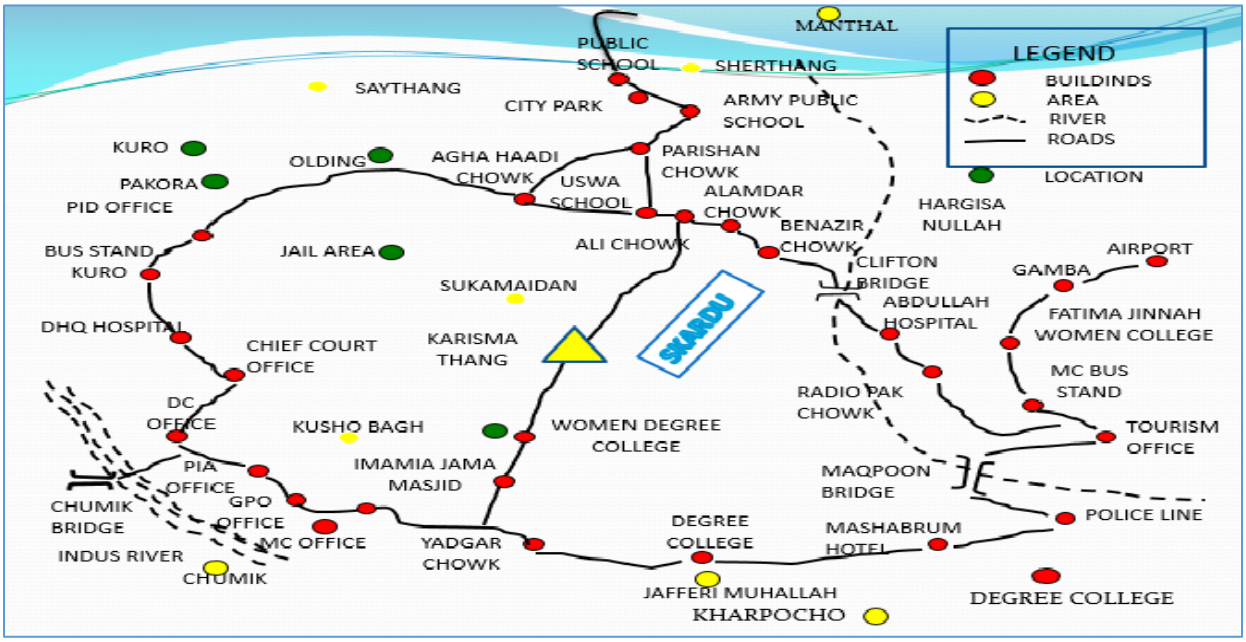

Fig. 1. Map of the Study area (Municipal area Skardu)

command area on a daily basis providing services for 24 hours (Table 1). The vehicles move from the station early morning to the destinations as well as at night for the same cause.

2.3. Research Design and Execution

The current study was based on field base observational surveys of different localities of the Skardu town area. In this study, extensive interviews were conducted with different officials from GBWMC. A series of interview sessions were also conducted with field officers, junk dealers, and scavengers to acquire data about the ratio of waste generation in the summer and winter seasons, waste quantity, waste quality, waste collection techniques, equipment used in waste collection and human resources in the GBWMC. The official data related to SWs and their management procedures were also acquired from the officials of GBWMC.
In different areas of the city field surveys on waste collection, transportation, segregation, recycling, and disposal practices were also carried out.

2.3.1. Operational Mechanism

GBWMC Skardu has the responsibility of SWM in Skardu town and is working for the collection, transportation, and dumping of SW in the town area as well as all tourist spots. To ensure the provision of efficient and well-managed sanitation services, the municipal area has been divided into 07 Sectors and Sub-Sectors based on the workload, available resources, and supervision requirements. In each sector, one supervisor, several operators, and workers have been deputed based on the population and average waste generation. GBWMC has been operating in morning, evening, and night shifts. The morning shift has the responsibility to collect the waste from Muhallas and Arterial Roads. All sectors have been operating in the morning shift. As far as the evening shift is concerned, it operates in the poultry and vegetable markets. In addition, it also responds the evening time complaints. Night shift has the mandate to clean the commercial areas through complete sweeping during the night such as waste of hotels located in commercial areas.

Table 1. Comparison of available resources and efficiency among the Municipal Committee and Gilgit-Baltistan Waste Management Company.

Resource		**MC Skardu**	**GBWMC Skardu**
Human resources	Sanitary Worker	45	58
	Sanitary Operator	09	39
	Sanitary Supervisor	03	17
	Sanitary Inspector	01	_
	District Management Officer (DMO)	-	01
Types of machinery	Trippers	-	30
	Tractors	06	09
	Dumpers	-	02
	Bucket	-	01
	Blade	-	01
	Bikes	-	15
Waste Collection	Daily	8-9 Tonnes (Approx.)	50 Tonnes (Approx.)
	Monthly	240 (Approx.)	1500 Tonnes Approx.
	Yearly	2880 Tonnes (Approx.)	18000Tonnes Approx.
Complaint Lodged on Helpline	Yearly	-	450/500
Collection Efficiency	Daily Basis	40-45 %	85-90 %

2.3.2. Transfer Station

The transfer station is a transit point for the movement of garbage to the landfill site. All the collected waste is brought to transfer stations via mini tippers. Then this waste is transferred directly into tractors and dumpers. These vehicles in turn take the garbage to the landfill site for final disposal. GBWMC has two Transfer stations currently. One is located at Ali Haider Chowk Oldling while the other one is located in the Maqponser.

2.3.3. Gilgit Baltistan Waste Management Company Skardu Organizational Structure

GBWMC has the responsibility of SWM in the municipal area of Skardu and is performing the responsibility of the collection, transportation, and dumping of SWs in the municipal area three times a day on a daily basis. The following flow diagram shows the organizational structure of the GBWMC (Figure 2).

2.3.4. Budget of Gilgit Baltistan Waste Manage ment Company Skardu

The current annual budget of GBWMC is approximately 50 million Rupees for possible expenses including salaries of employees, and fuel charges for multi-dumper, tractor, dumper, and bikes. It also includes repairing and maintenance of vehicles and sanitary items like a mask, gloves, other admin office charges, and expenditure of awareness seminars.

2.4. Waste Collection Methods

2.4.1. Door-to-Door Collection

GBWMC provides door-to-door services in the municipal area via Mini Trippers. It has introduced a packed-waste system to manage waste. The general citizens were sensitized by the door-to-door awareness campaigns and distributed leaflets and broachers about packing waste and their social responsibilities; keeping their waste packed in plastic bags at their doorstep in the morning or waiting for the trippers. Special horns are installed on mini trippers that call the local community to bring their waste outside. Shopkeepers have wastebin/containers at their shops. In this way, waste is collected from residential and commercial areas smoothly.

2.4.2. Placement of Waste-Bin

Waste bins are placed where trippers/vehicles are not accessible. The residents of certain areas bring their waste and put it into the Wastebins in the morning and the operators clean these bins regularly.

2.4.3. Collection from Main /Arterial Roads

Workers collect waste from main roads on a daily basis by using wheel-borrows. The waste materials and garbage mostly include wrappers, papers, plastic cans, etc. However, waste from arterial roads is collected twice or thrice a week.

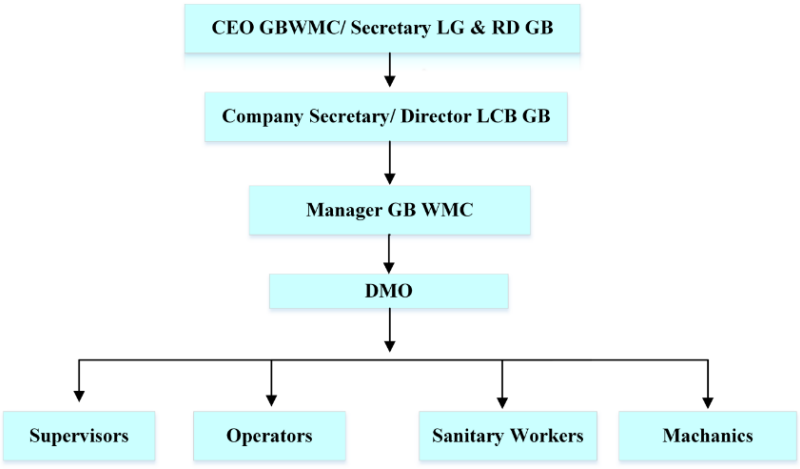

Fig. 2. Organizational structure of Gilgit Baltistan Waste Management Company Skardu

2.4.4. Manual Sweeping

GBWMC deployed 10 teams for manual sweeping in commercial areas i.e., Hussaini Chowk to Boys Degree College daily. Manual sweeping is done during the night shift in a proper uniform with health and safety gadgets by workers. Necessary tools, brooms, and waste pickers/wheel-borrow are also provided to the workers.

2.5. Waste Transportation

To transport the waste available vehicles and equipment include nine open-body tractor trolleys, thirty Trippers, two Dumper, a Bucket, and Blade. Trippers are being used to collect and dispose of waste from different sites in the town area with the help of 2-3 workers allocated to each vehicle [17]. These vehicles make 2-3 trips per working day. The collected waste is then transported to the dumping sites.

2.6. Dumping Sites

GBWMC Skardu is managing its landfill site since 2018 by burying waste. Burying waste is an easy and inexpensive method of waste management. Although, it is not a proper solution to dispose off waste in landfills however it minimizes the hazards and environmental damages like avoiding air pollution and annoying odor, etc. that is generated by the decomposition of this waste. The landfill sites are situated in the New Hussainabad area which is 5 km away from Skardu city.

3. RESULTS AND DISCUSSION

3.1. Waste Generation Overview

The volume of SW production within the municipal area of Skardu varied from season to season. During winter and autumn seasons, waste generation was usually get reduced largely as the citizens prefer to store the waste for harsh and frosty winters as well as during this season a large number of families migrate to other cities for winter which reduces waste generation. The produced waste comprised municipal/ kitchen/domestic waste, commercial waste, and other scraps, etc. The study revealed that in Skardu City, total waste generated during the winter season was approximately 45-50 tonnes per day with a generation rate of 0.4-0.66 kg/ capita/day while in the summer season, the ratio of waste production was comparatively increased due to overflow of tourists [20]. For the year 2020, the total waste collected by GBWMC [17] was approximately 13,128 tonnes. The maximum waste of approximately 1281 tonnes was collected in May and the minimum amount of waste was collected during January, which was approximately 882 tonnes.

For the year 2021, the total waste collected by GBWMC [was approximately 17782 tonnes. The maximum waste of 1640 tonnes was collected in June and the minimum waste was collected during January which was 1262 tonnes. It was observed that the trends of the waste collection varied and diverted from normal trends during the different months of the year 2020 due to the Juglot Skardu Road (JSR) blockage and fuel shortages for vehicles [21] in Skardu causing inefficient waste collection during peak season. However, the waste collection in the city during the year 2021 reflects the actual waste generation trends (Figure 3).

3.2. Nature of Generated Waste

The generated waste was composed of kitchen waste, fabrics, confectionery, snacks wrapper, rocks, spray cans, glass, polythene (shopping) bags, milk pack boxes, plastic bottles, clothes, wood pieces, slaughter and poultry waste, old tires, cutting hairs, papers, old shoes, electronic waste, medical waste, glass bottles, electrical items, blades, agricultural inputs packaging's, vegetable and fruits, etc. Similar results were obtained by Hussain et al. [22] who studied Quantification, Composition, and Disposal Methods of Municipal Solid Waste at Konodas Gilgit.

3.3. Solid Waste from Commercial Units and Household Units

According to the statistical data provided by GBWMC [17], the total population of the municipal area of Skardu is approximately 112996. Results revealed that the average waste generated in the municipal area of Skardu is approximately 50 tonnes/day, which comprises household waste and commercial waste. The average waste generation in the municipal area was approximately

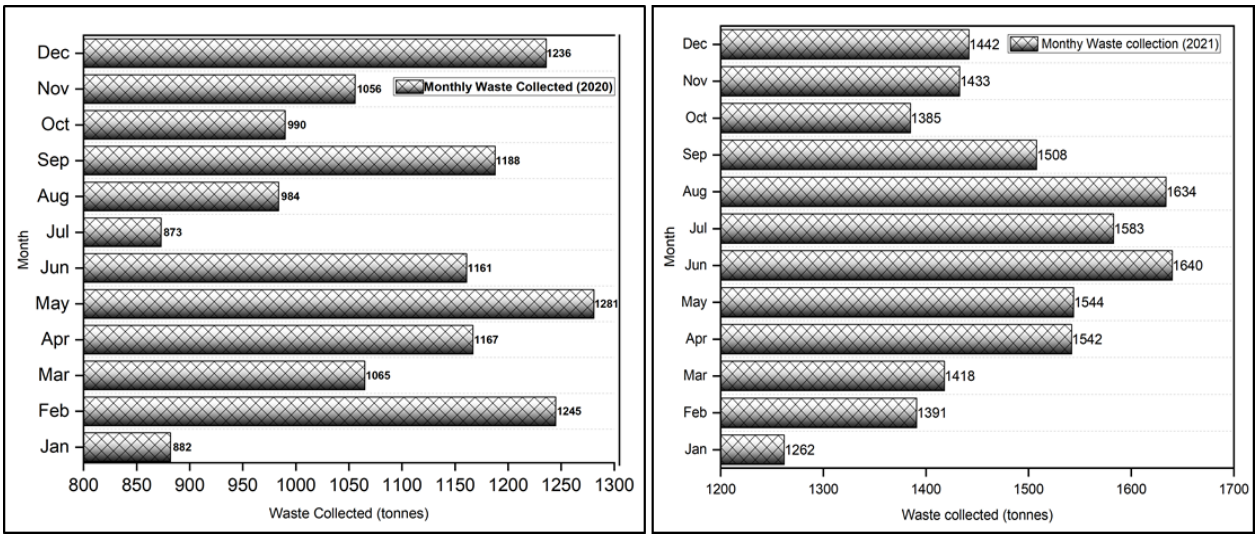

Fig. 3. Total waste collection (a) January to December 2020 and (b) January to December 2021

0.43 kg per capita per day whereas, in Gilgit city, it was 0.16 kg/day during 2016-17 reported by Ali *et al*. [3]. In the municipal area, approximately 20 tonnes of solid waste including organic and inorganic waste were being generated per day from residential areas (Table 2). Every month, the waste collected by GBWMC was approximately 600 tonnes from households while the annual collected waste was approximately 7200 tonnes. The total waste generated per day in commercial areas was approximately 30 tonnes and the waste generation growth rate was approximately 2.28 % annually. These results are in line with the findings of Ali *et al*. [3] that the daily collected waste from commercial and residential areas were 31.5 and 11 tonnes respectively. According to the GBWMC [17], the budget required to collect a tonne of waste is approximately 2976.6 rupees so the total amount required to collect waste in the municipal area of Skardu is approximately 148830 rupees/day.

Table 2. Daily waste generation in the study area.

Parameters	Total/day (Tonnes)
Total solid waste from a commercial area	30
Total solid waste from a household	20
Total solid waste from Skardu Municipal	50

4. CONCLUSION & RECOMMENDATIONS

SWM is a challenging task for GBWMC due to insufficient budget and human resources, lack of awareness among the masses, and ineffective policies. The ratio of waste generation in Skardu is increasing day by day thus its sustainable management is needed urgently. The local government and Non-government organizations (NGOs) should pay attention to providing proper strategies to resolve this major issue. It is necessary to develop a mechanism to remove SWs from non-designated spots and must define a proper strategy to collect waste from residential and domestic areas and should opt for an eco-friendly disposal system like incineration and landfills.

Based on this study the company needs to plan to combat the impending challenges related to waste materials. The following recommendations and suggestions are set forth for the organizations to be seriously considered and taken into account in the future. The organization requires arranging a maximum awareness campaign in all the districts as well as also needs to cooperate with the local government in designing the map of the city for waste and municipal garbage and sewage activities. In addition, it is suggested to work on advancing the source of income of the organization through local and national markets and organizations to minimize the financial burden of the organization. Furthermore, the organization requires to work on a plan to protect natural resources such as water, green landscapes, lakes, and the Indus River from obnoxious waste.

5. ACKNOWLEDGEMENTS

The authors acknowledge all contributors who give their

input during the research period especially GBWMC, and WAPDA.

6. CONFLICT OF INTEREST

There is no conflict of interest among the authors.

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Proceedings of the Pakistan Academy of Sciences: B
Life and Environmental Sciences 60(3): 463-475 (2023)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(60-3)848

Pakistan Academy of Sciences

Research Article

Assessment of Karachi as an Urban Heat Island Threat through Remote Sensing and GIS Techniques

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Abstract: The present study aimed to assess the threat of transformation of Karachi into an Urban Heat Island, so, ambit was having calculated temperature, buildup areas, and normalized difference vegetation index through remote sensing and GIS techniques. The Landsat satellite data was used to differentiate the temperature in different years. These images were processed through Envi 4.7, Erdas Imagine, and ArcGIS 10.3.1. The results revealed that the maximum temperature was found up to 30.52, 35.25, 33.60, 46.73 °C; the buildup area was 23, 34, 26, 45 %; the NDVI results showed ranging from 0.224-1, 0.07-0.43, 0.201-1, 0.29-0.7 during this years. The average spatial land use temperature and buildup area increased by 1.03 and 1.9 times from 1990 to 2019. The maximum NDVI was observed during 2019, because of heavy rainfall as a result which supports promoting more greenery. With an increase in the buildup area, a significant change in the temperature of the territory was simultaneously observed. Therefore, this indicates a major task for urban developers extenuating the subsequent urban heat island occurrence. That is, for the first time it is scientifically substantiated and confirmed by the results that when creating a city development plan, it is extremely important to exclude the possibility of the urban heat island occurrence through preliminary studies. The practical value of the study lies in sound recommendations, one of which is the need for future urban development to emphasize urban plantings, including vertical forests to prevent UHI occurrence in the area of Karachi city.

Keywords: Build-up areas; land use/land cover; emissivity; satellite monitoring; temperature increase; NDVI

1. INTRODUCTION

Urbanization has significantly altered the existing scenario of land use/land cover (LU/LC), which may subsequently enhance the land surface temperature (LST) in many regions of the world [1-3]. The trend of urban development growth going to decrease the ordinary land, and vegetation covers and substitute it with new buildup landscapes like buildings, industrial hubs, streets, commercial infrastructures, etc. The urban heat island (UHI) concept is realized by various research, which confirms that it has a role in the climate change impact most likely in cities [4, 5]. It is one of the significant phenomena it requires

Received: February 2023; Revised: April 2023, Accepted: June 2023
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more investigation to address the environmental degradation impact and climate change impact [6, 7]. The vegetated area is mostly roofed with a green cover. As it is observed that ripened trees have covered the canopy region near 50 m^2. Thus, about 50 m^2 canopy-covered area has high impending to decrease the intrusion of sun rays these green cover types absorb and expose energy in the form of long radiation waves [8, 9].

As the metropolis urban buildup observes warmer than countryside areas near about 5 to 10 degrees centigrade in surroundings [10]. The average difference in daylight temperature between urban and countryside areas was 5 to 15 centigrade as compared to night time 5 to 10 centigrade at night time considering the daytime differences in temperature [11]. Due to this UHI phenomenon is considered a model for climate change impact research. Moreover, the climate alters more due to UHI's impact on an urban buildup in the last decades of the century. Generally, UHI concentration varies hourly and season vise and it is influenced by the local topography, city size, city population density, geometry, cloud cover incoming solar radiations, development of commercial and industrial, land utilization, and land concealment (LU/LC) physiognomies and countryside regions other factors are airstream swiftness and vegetation profusion [12, 13]. The plants and exposed areas naturally control the land to a maximum rural extent. However, exposed land characteristically controls the landscape in most urban areas [14]. Throughout daylight time the buildup area in an urban hub fascinates and stocks two-fold quantity of heat than its countryside [15], buildup structural materials such as stepping-stone and steel consume complex temperature dimensions in an urban environment as compared to rural materials like dry soil and sand. All the materials, including construction materials such as plaster, wood, and glass obey the same laws of nature concerning heat transformation. In current ages, the influence of climate on gender fitness has observed a problem of an enhanced consequence, particularly since the latent influences of global heating and an augmented. UHI consequence because of urbanization [16]. In current years, the influence of weather on living beings has become a matter of enhanced consequence, particularly considering the possible influences of universal heating and an enhanced UHI influence, because of urbanization [17].

The UHI has developed one of the prime problems associated with the urban population and the automation of human civilization, as the augmented hotness related to the UHI inclines to worsen the pressures on people and might cause health hazards due to thermal stress [18]. The most common impacts during nighttime are breathing problems, overall uneasiness, temperature pains, and fatigue, a temperature-associated morality overall increase in human health problems [19]. In Pakistan, Karachi is densely populated and its urbanization is increasing rapidly. The urban heat island effect phenomenon will be bulging in Karachi. According to Pakistan statistics, it is the extremely rising city in Pakistan. Karachi is the most vulnerable to this urban heat island effect. There is a rapid rise of mass migration concerning vegetation loss and an increase in buildup area. UHI effects perspective can be understood by using RS & GIS spatial analysis methods and using the Landsat 5 and Landsat 8 images. Anthropogenic activities play a vital role to boost Urban Heat Island (UHI) and resulting cause to decrease in the vegetation cover and increases land surface temperature (LST) during the last 29 years in the regions of Karachi. Furthermore, Karachi is a megacity of Sindh province, which is the location in the southern part of Sindh, and due to the huge and multi-culture population, it was chosen in this investigation to assess the difference in surface heat, built-up areas, and greenery. The temperature of urban heat islands might be affected by different anthropogenic activities including manufacturing, transportation, economic and domestic activities. It was hypothesized that an increase in the built-up area of Karachi has been increasing the land surface temperature in different years. The aim of the present study was to assess the threat of transformation of Karachi into an urban heat island (UHI), for this, ambit we have calculated temperature, buildup areas, and normalized difference vegetation index (NDVI) in Karachi during the years 1990, 2000, 2010, and 2019 via remote sensing and GIS techniques.

2. MATERIALS AND METHODS

Urban heat Island effects can be estimated with environmental and meteorological properties viz.,

air temperature, humidity, and greenhouse gas emission. The map site of the study area is indicated in (Figure 1). The source of data was the USGS online website, and we downloaded the following satellite imaginaries data sets. 1990 LANDSAT 5; 2000 LANDSAT 5; 2010 LANDSAT 5 and 2019 LANDSAT 8 and processed the imaginary data sets through various important software such as Arc GIS 10.3.1, ERDAS Imagine, and ENVI 4.7. In the initial process, Landsat 5 and 8 satellite imagery requires preprocessing such as layer stacking to show the natural color of the imagery and geo-referencing according to our region.

In the first step Satellite image of Landsat 5 and 8 of two slots of time from 1990, 2000, 2010, and 2019 are geo-referenced using Envi 4.7 in the UTM/WGS84/42N coordinate system. In the second step, all the images of the following years (1990, 2000, 2010, and 2019) are stacked using the Envi 4.7 software layer Stack tool. While in the third step, the satellite image was subset using the subset tool of the Arc GIS10.3.1 software. In the fourth step, we will calculate the Normalized Difference vegetation index of subset images by using Envi 4.7. It calculates the wholesome vegetation cover of the area. It uses the ratio between the (NIR) near-infrared (Band 4) besides the red band (Band

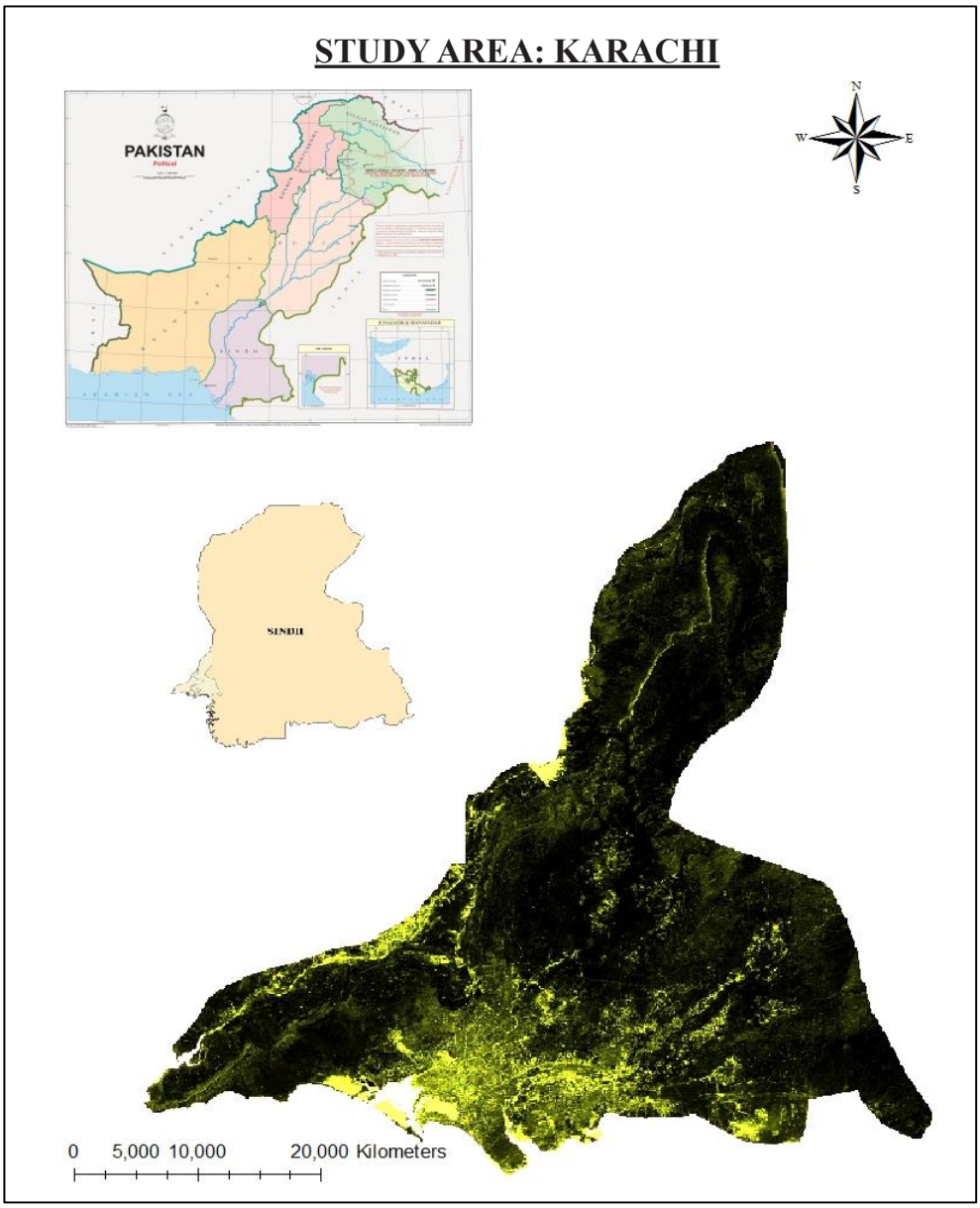

Fig. 1. Map of the study area

3). Both these bands contain information on the vegetation index. In the final step, radiance values are calculated using the Erdas imagine 10.2 DN value tool, further the DN value for transformed into the inverse of the Planck which gives out the temperature value. Additionally, the radiance value converted into the Kelvin represents the precise spatial distribution of temperature. The flow diagram of the study area is indicated in (Figure 2).

2.1. Extraction of Satellite Imaginary Data

In order to obtain the LST, LULC, and NDVI of the study area Landsat ETM 5 and 8 Images are transferred from the United States Geological Survey website of the years 1990, 2000, 2010, and 2019.

2.2. Pre-processing of Satellite Images

All Landsat 5 and 8 images were downloaded under a criteria set of 0 to 10 % cloud cover. No atmospheric correction was employed on these images. The USGS has already reprocessed for geometric distortions and radiometric correction. All the images are geometrically modified and geo-referenced through the USGS under Universal Transverse Mercator (UTM) zone 42 N to organize the system and the WGS1984 datum.

2.3. Pre-processing for Land Use Classification

The shape file of Karachi city was downloaded from open map tiles that depict the Karachi city area. The vector file is already rectified under the WGS 1984 datum, UTM zone 42. The shape file was used to extract the studied area from the satellite image by using the spatial analyst tool extracted by mask (Figure 2).

In a second step, the extracted image was processed for the supervised classification method by using the maximum likelihood technique in Arc GIS10.3.1. The signature file was generated with a spatial analyst that is utilized for the further image processing step (Table 1). The method for a composite band for land use classification is indicated in (Figure 3).

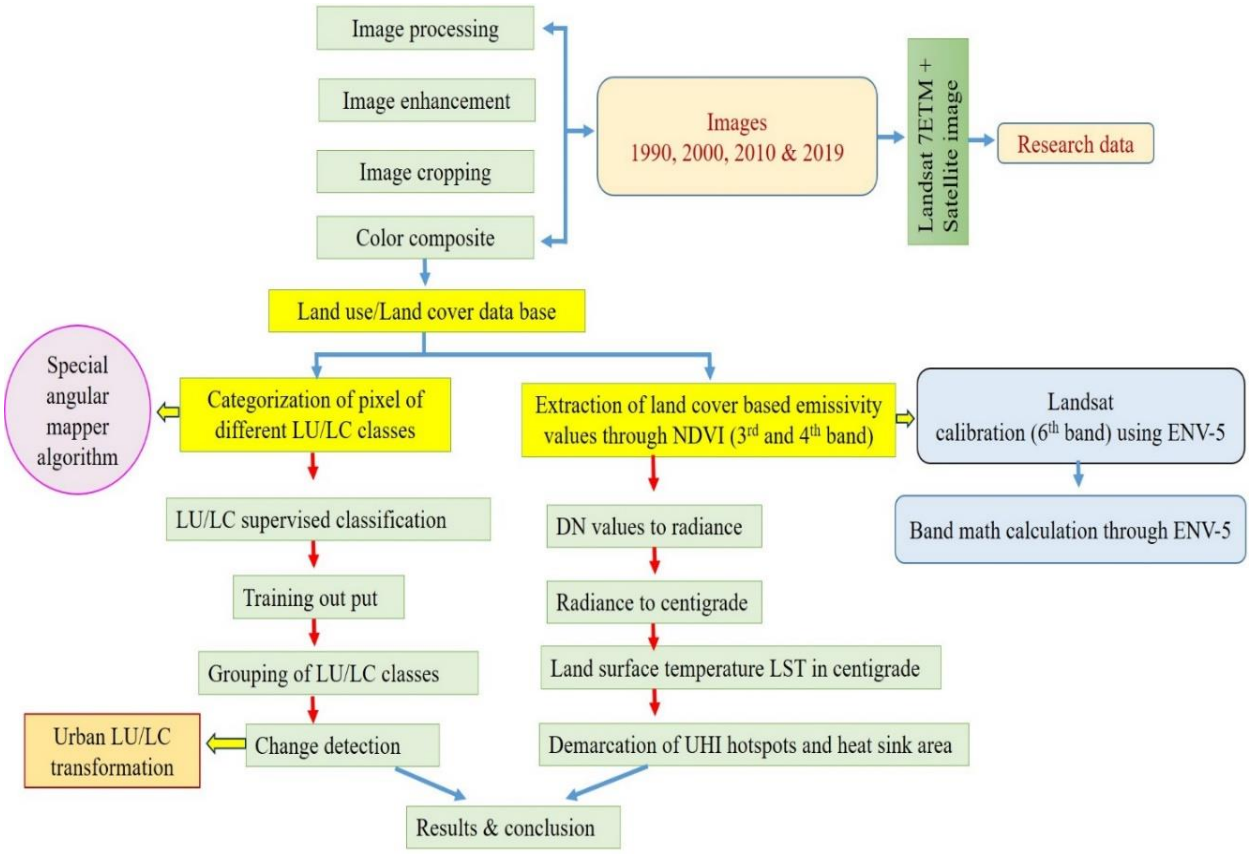

Fig. 2. Research methodology

Table 1. Shows the distribution of classes according to visual-spatial analysis

The classes color	Representation of color as
Yellow	Open surface or Barren ground.
Green	Vegetation Sparse or dense vegetation and Parks.
Blue	Water bodies ditch ponds and stagnant water, river Dam water.
Dark Yellow	Build up areas, buildings, roads houses, and other concrete structures.

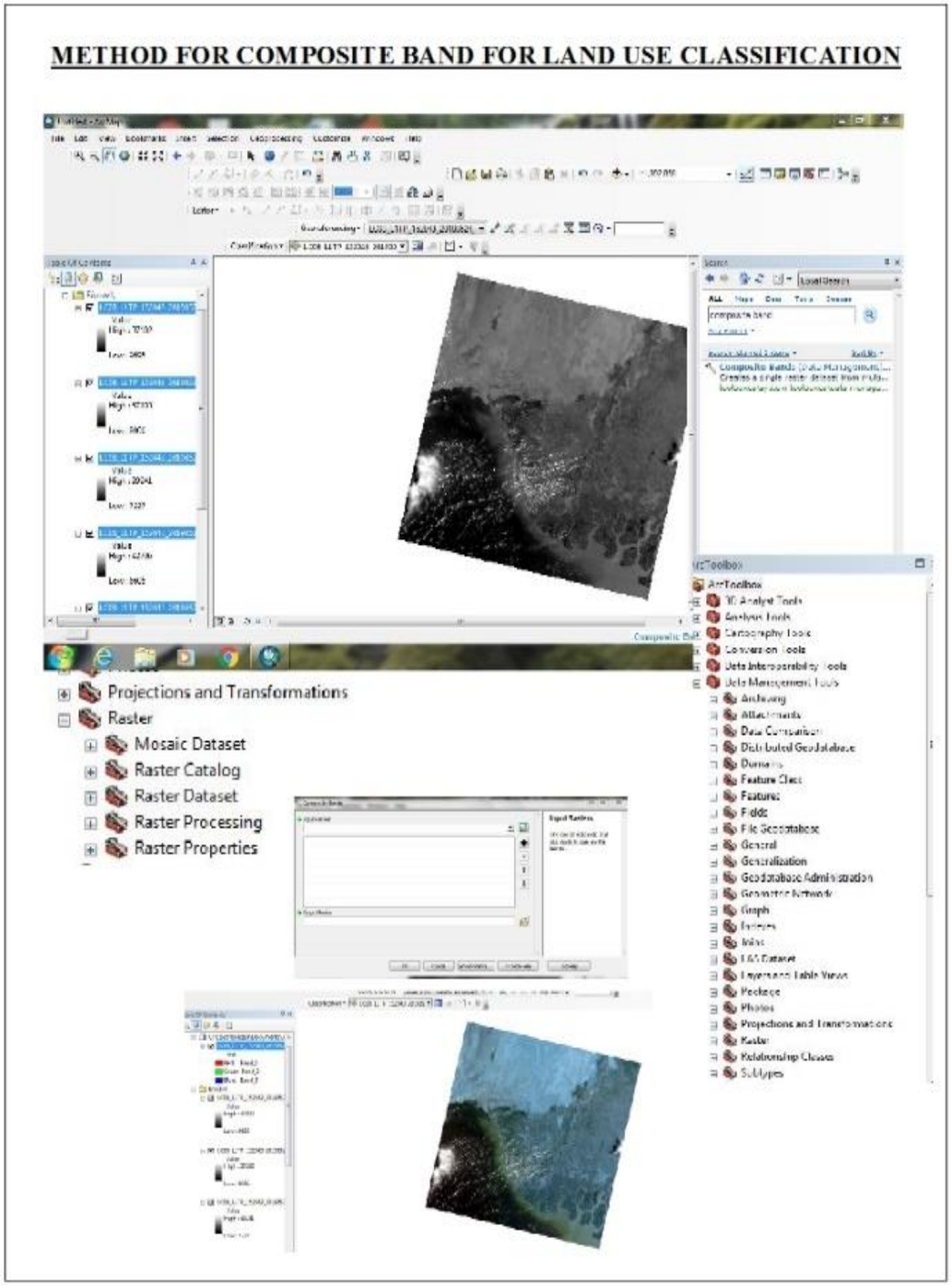

Fig. 3. Method for the composite band regarding land use classification

2.4. Data processing for land surface temperature, derivation of LST from the satellite image

The thermal band (10.4-12.5 μm) of the May Landsat 5ETM+ sensor with 30 m resolution was used to extract the LSTs over the selected area of Karachi (1990, 2000, 2010, and 2019).

2.5. DN means digital number converted into SR (Spectral Radiance)

By using Equation (1), the DNs of a thermal band from the Landsat 5 ETM+ image captured in May were first converted to spectral radiance (1990, 2000, 2010, and 2019).

$$L= (Lmax-Lmin)/(Qcalmax-Qcalmin)* *(Qcal-Qcalmin) +Lmin \quad (1)$$

Where L is the Spectral radiance of band 6 ETM Landsat 5; Lmax – Radiance maximum of band 6 ETM Landsat5; Lmin – Radiance minimum of Band 6 ETM Landsat5; Qcal max – QUANTIZE calculated maximum reflectance; Qcalmin – QUANTIZE calculated minimum reflectance; Qcal =L – Calculated radiance of band6 ETM Landsat 7.

2.6. Conversion of SR into RST (Radiance Surface Temperature)

Equation (1) is charity to adapt the spectral radiance value of the thermal band into radiant superficial heat. The conversion is carried out with the statement of Uniform emissivity by using the pre-launch calibration constant. The Uniform emissivity means that all the calculated radiant surface temperature was referenced with a blackbody A black body is a surface that absorbs all the incidents of electromagnetic radiation. It neither transmits nor reflects the radiation. The following equation is used.

$$TB= K2/ (\ln (K1/ L\lambda+1) \quad (2)$$

Where,

K1 – calibration constant 1 (666.09 $Wm^{-2}sr^{-1}$ μm^{-1}); K2 – calibration constant 2 (1282.71 K); L_λ – the spectral radiance at the sensor in $Wm^{-2}sr^{-1}$ μm^{-1}; T_B: radiant surface temperature (in Kelvin) brightness temperature at the satellite.

Conversion of kelvin radiant surface temperature into the centigrade

$$C=K-273.15 \quad (3)$$

The above equation is commonly used to convert the radiant surface temperature in Kelvin to Centigrade.

C – Centigrade Temperature; K: Kelvin Temperature calculated from radiance 273.15 – Constant for converting Kelvin into the Centigrade temperature. Calculation of the Normalized Difference Vegetation Index. NDVI was cumulated followed by reflectance value for the near-infrared and red band

$$NDVI = (\rho_{nir} - \rho_{red})/ (\rho_{nir}+\rho_{red}) \quad (4)$$

Chlorophyll contains green, highly absorbs blue (0.4 μm-0.5 μm) and red (0.6 μm-0.7 μm) spectrum, and reflects green (0.5 μm-0.6 μm). Highly reflectance NIR (Near-infrared) (0.7 μm-1.3 μm).

The formula for NDVI Calculation in Landsat 5 and Landsat 7 is the same:

NDVI=(Band4-Band3)/(Band4+Band3).

The formula for NDVI Calculation Landsat 8 is:

NDVI=(Band5-Band4)/ (Band5+Band 4)

3. RESULTS

3.1. Land Surface Temperature

The data in Figure 4a showed that the minimum spatial land use temperature of the year 1990 was observed at 22.8 °C in northern areas of Karachi, while the maximum spatial land use temperature was observed at 30.6 °C in the west-south parts of Karachi city. As shown in (Figure 4b), the minimum spatial land use temperature of the year 2000 was found up to 30.6 °C in the Northeast, Northwest, and Southeast parts of China, but the maximum spatial land use temperature of the year 2000 was noted by 26 °C in the West south parts of the Karachi. As shown in (Figure 4c) the minimum spatial land

use temperature during the year 2010 was observed as 33.7 °C in the Northeast, Northwest, and East southern parts of Karachi, whereas the maximum spatial land use temperature during the year 2010 was noted as 35.2 °C in the West southern parts of Karachi. Furthermore, the lowest spatial land use temperature during the year 2019 was found nearly 25.7 °C in the Eastern, Northwest, and southeastern regions of Karachi, whereas the highest spatial land use temperature during the year 2019 was found about 46.8 °C in the Northwest and west southern parts of Karachi (Figure 4d). Overall, it is assumed that the average highest spatial land use temperature significantly increased 1.03 times from 1990-2019.

3.2. Build-up Areas Satellite Images

The data in Figure 5a indicated that the water

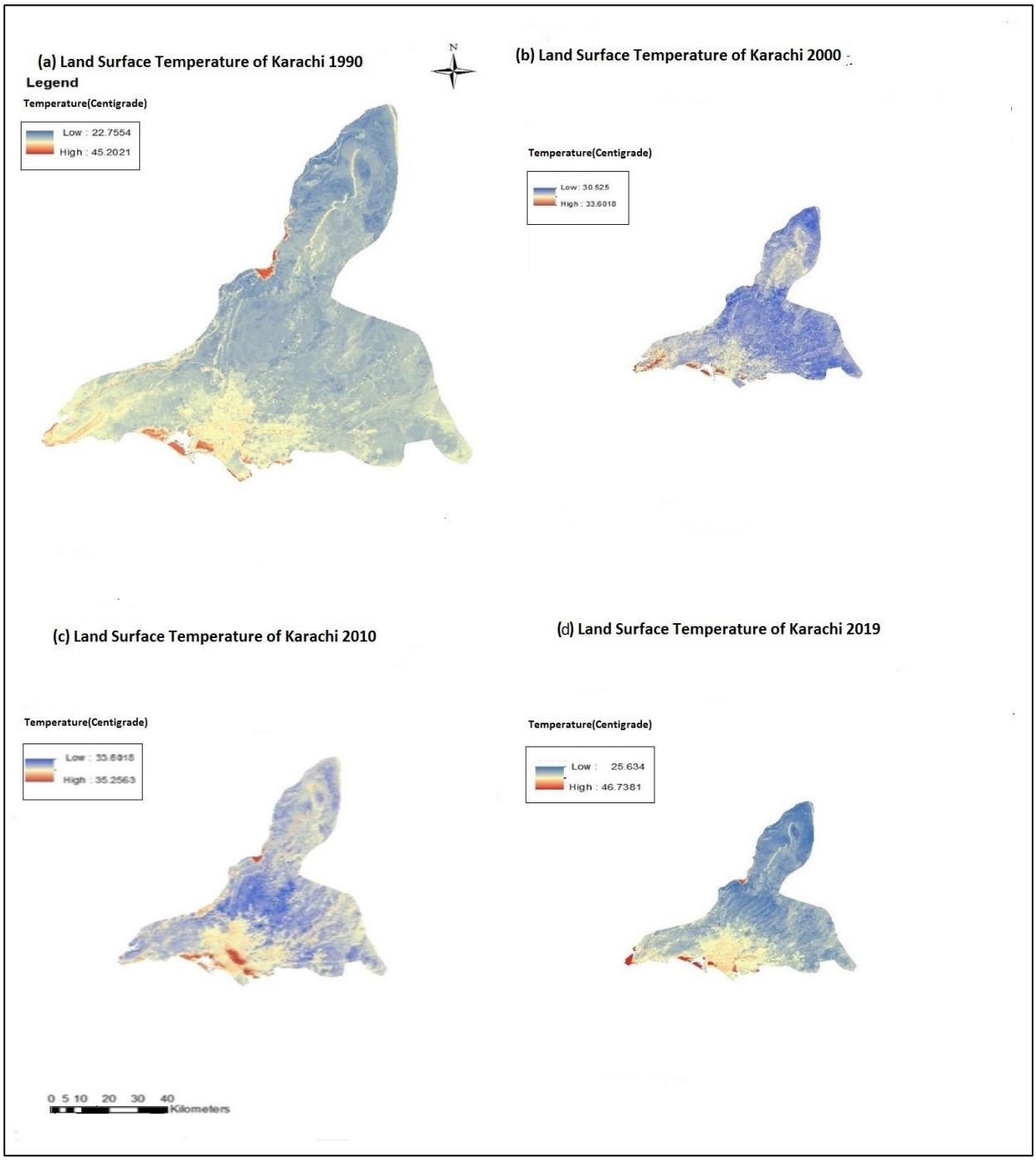

Fig. 4. Spatial distribution of land surface temperature in Karachi in the years (a) 1990, (b) 2000, (c) 2010 and (d) 2019 classification

content in this study area was observed by 10 % in the northwest and southern parts of Karachi during the year 1990. While the maximum constructed build-up areas were observed by 23 % in the Southern parts of Karachi city during the year 1990. Furthermore, the open land was 29 % in most parts of the study areas during the year 1990. Besides, the dominant rugged terrain area was observed by 38 % in most areas of Karachi, while the greatest rugged terrain area was found in the Northeast and central areas of Karachi during the year 1990. The data in Figure 5b indicated that the average water content in the study area was 10 %, while minimum water content was observed in the western part and maximum water contents were noted in east-west parts of Karachi during the year 2000. In addition, the total buildup areas during the year 2000 accounted for up to 34 % in the west southern parts of the megacity Karachi. Besides, the rugged terrain areas observed 35 % in the northeast and

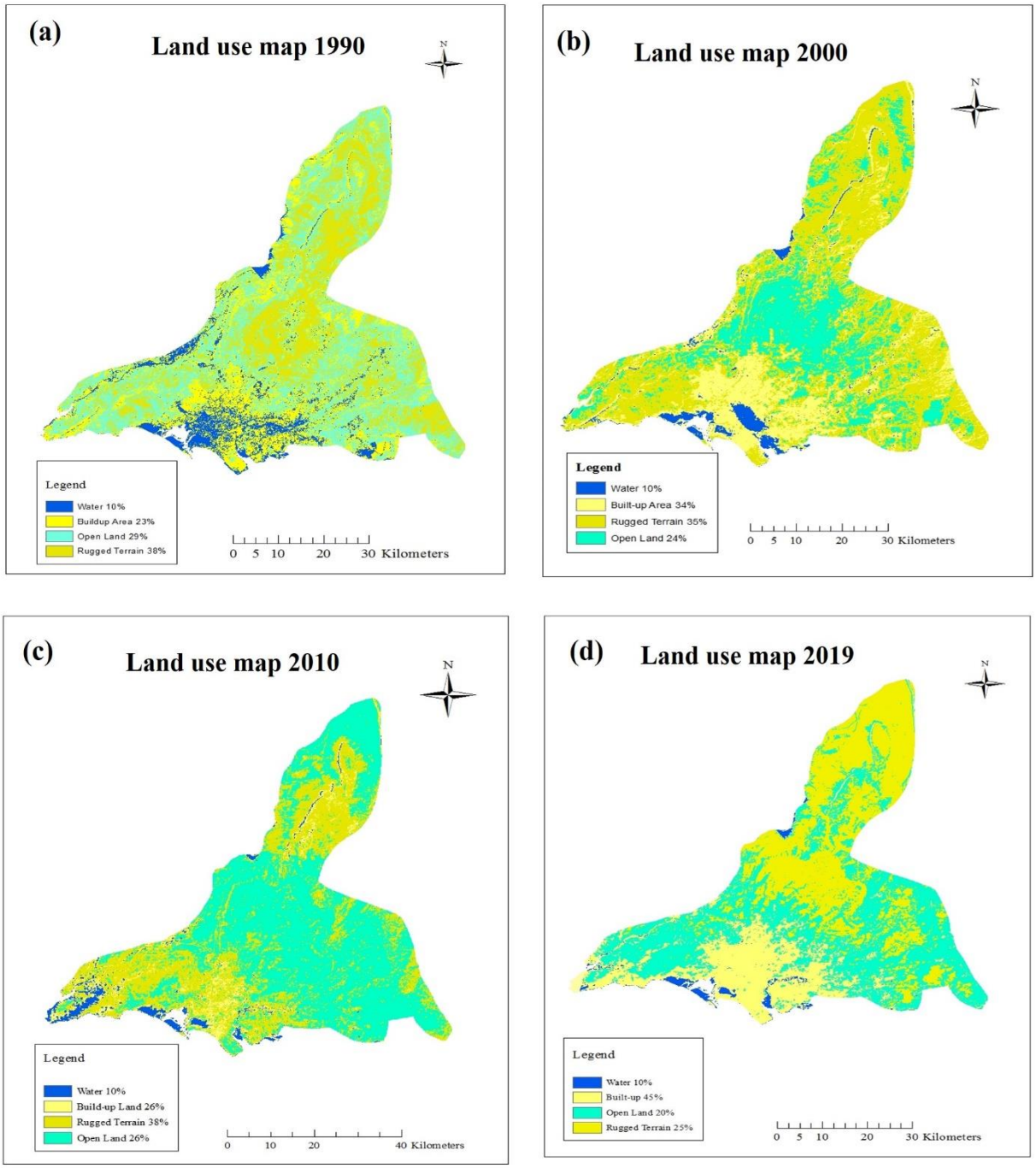

Fig. 5. Spatial distribution of land-use area of Karachi in the years (a) 1990, (b) 2000, (c) 2010, and (d) 2019

west northern parts of Karachi city during the year 2000. The maximum open land was observed by 24 % in the central parts of Karachi during the year 2000. The water content in the western and southern parts of the study area was 10 % during the year 2010. Furthermore, the average buildup area in the year 2010 was found up to 26 % in the west southern areas of Karachi. Furthermore, the average rugged terrain areas were 38 % in the eastern and northern parts of Karachi during 2010. Besides, the maximum open land areas were covered by 26 % in the central areas of Karachi during the year 2010 Figure 5c. The data in Figure 5d represented that the average proportion of water was observed by 10 % in the study area of Karachi during the year 2019. In the case of the buildup area, the maximum area was noted by 45 % in the southern parts of the megacity Karachi during the year 2019. Likewise, the open land was found to be 20 % in the southern parts of Karachi during the year 2019. Meanwhile, the rugged terrain areas were noted by to 25 % in the northern parts of Karachi during the year 2019. The above results indicated that the buildup area increased significantly by 1.9 times, respectively, from the year 1990 to 2019.

3.3. NDVI Imagery Interpretation

The data in Figure 6a indicated the spatial distribution of NDVI of Karachi during the year 1990. Hence, the barren rocks and land were observed in the western and southern parts of Karachi due to low rainfall and air and semi-arid climatic conditions. Furthermore, less NDVI in the study area was observed ranging from 0.076-0.224 in the western and southern parts of Karachi. While very, less dense vegetation in the study areas was observed ranging from 0.224-1 in southern parts of Karachi during the year 2019. As shown in Figure 6b revealed that the barren rocks and land contents in the study areas were observed in the eastern and northwestern parts of Karachi during the year 2000. While the low NDVI population was observed ranging from -0.02-0.07 in the east and southern parts of Karachi, the high NDVI population was noted ranging from 0.07-0.43 in the southern parts of Karachi during the year 2000.

The NDVI data in Figure 6c showed that the barren rock and land were noticed in most parts of the study area. Besides, the low vegetation including shrubs and grasslands was observed ranging from 0.051-0.201 in the central and southern parts of Karachi during 2010. However, the high vegetation including shrubs and grasslands was observed ranging from 0.201-1 in the western and southern parts of Karachi during the year 2010. The NDVI results in Figure 6d highlighted that the water contents in this area were observed ranging from -1-0.01 in the western and northern parts of Karachi during the year 2019 might be due to rainfall. The low vegetation index such as shrubs and grassland in the southern parts of the study area seemed to range from 0.19-0.29 in Karachi city, but the high vegetation index was ranging from 0.29-0.7 in the east, west, and northern parts of Karachi during 2019. The above NDVI data indicated that the lower NDVI in the selected years were observed during 1990, 2000, and 2010 as compared to 2019.

4. DISCUSSION

In Karachi city, there was an expansion in yearly least temperature during the time of 1990-2019 (especially in the last piece of the period 2010-2019 contrasted with the periods 1990-2000). The yearly least temperature pattern increases by 0.2 °C per year. The consequences of the recent work indicated that the maximum variation in spatial land surface temperature distribution in Karachi was observed by 30, 33.7, 35.2, and 46.8 °C from 1990, 2000, 2010, and 2019. On the other hand, the average proportion of buildup areas gradually increases to 23, 26, 31, and 45 % in the years 1990, 2000, 2010, and 2019. The results indicated that the spatial LST distribution and the average proportion of buildup areas increased significantly due to anthropogenic activities.

The highest air temperature in the city compared to the rural areas was obvious, because of the emissivity estimation of each LU/LC, backward connections between vegetation plenitude and LST were seen all in the examination region which is consistent with the results of a similar study conducted in China [14]. It was obvious from the visual translation of NDVI and LST that the LU/LC type vegetative stops and houses with frontal nurseries show a virus spot on the picture because of the bounty of foliage. In any case, the mechanical zone and the household's lack of gardens; no vegetation, and the development of high-rise

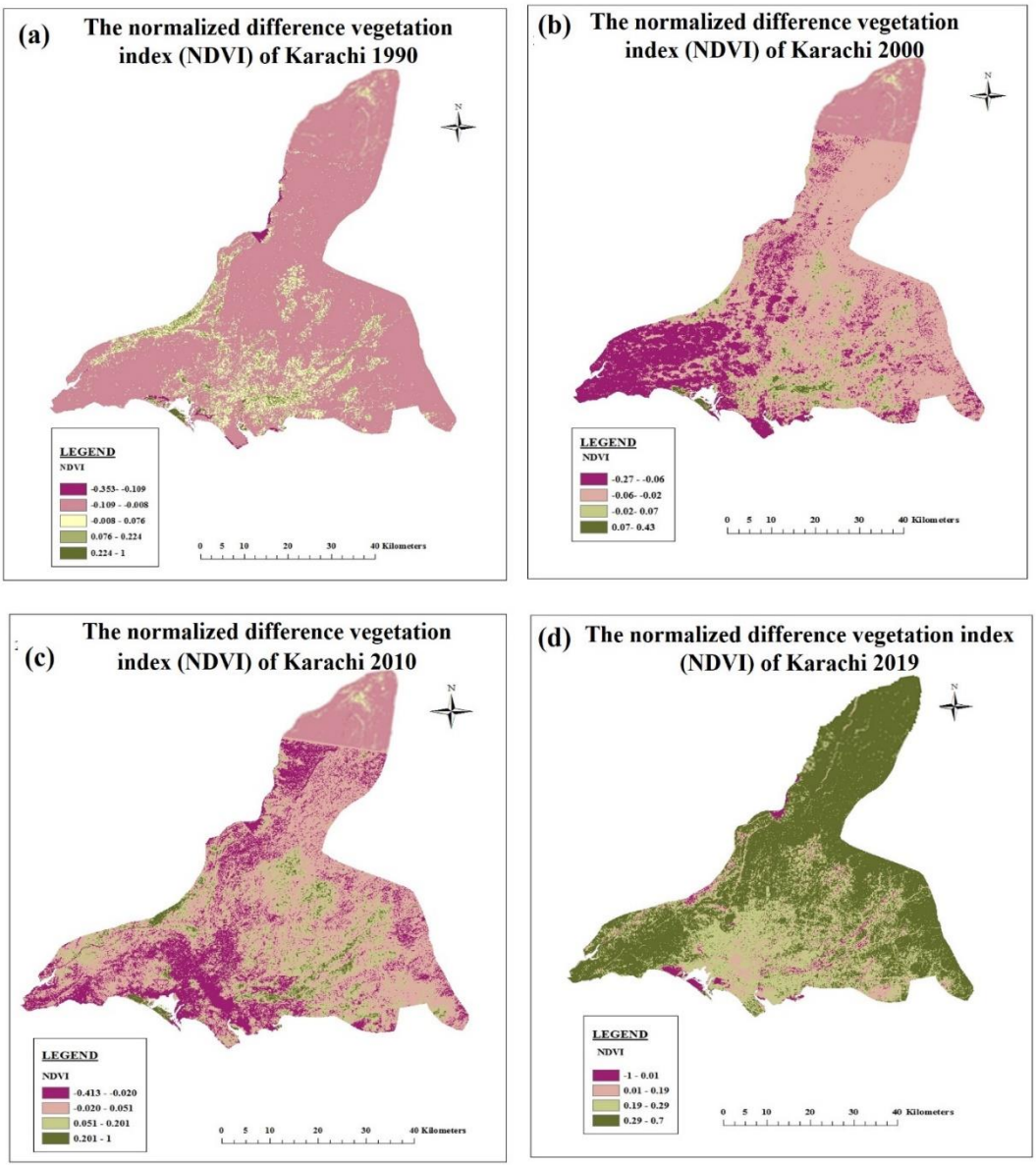

Fig. 6. Spatial distribution of normalized difference vegetation index of Karachi in the years (a)1990, (b) 2000, (c) 2010 and (d) 2019

buildings are directly or indirectly responsible for rising temperatures in Karachi city. In addition, the less vegetation these territories contain and the current of no nursery homes may influence the surface temperature. As private LU/LC now involves huge parts of the city the warm attributes of such LU/LC could probably considerably affect Karachi's warm climate. It is along these lines vital for strategy producers to decide which kinds of private houses could be generally useful to be fabricated. The area of the mechanical region ought to be taken into thought in the event that we need to diminish the general warm impression of the city. The warm reflection decrease may either be by suggesting arrangements for a broad ranch in the zone or move it out of the city [20]. The association among the spatial range of UHI and built-up features, like city size, progress area, water quantity and mean NDVI [21]. The periodic difference in the development of UHI with the assistance of Landsat TM data is observed by [22, 23]. Although, Chen *et al*. [24] reported that Landsat TM and ETM+ images from 1990 to 2000 in the PRD were designated to recover the intensity of temperatures and type of land use and /or land cover. Li *et al*. [25] observed the seasonal outline of the urban heat island for Shanghai about surface temperature conditions and the fractional vegetation index.

Considering the importance of the analysis of urban heat island and NDVI, with veneration to its effect in defining the microclimate and health in built-up areas, it comes to be an imperative part of the research. Thus, the current study goals at investigating LU/LC changes, their impact on UHIs patterns, and their spatial relationship with respective LSTs in Karachi. In imperative to understand the UHI problem in Karachi. The most common and useful technique of remote sensing has been utilized in this study and results of the study have similar conclusions to studies by other methods [8, 26]. The satellite images are processed for the spatial analysis techniques by using the RS (Remote Sensing) and GIS software [27]. It is assumed that the maximum creation of hot spots was identified in constructed buildup regions, where the urban heat island was formed, while the vegetation cover shows the minimum temperature. For this study, Envi4.7, ERDAS imagine 10.2, and Arc GIS 10.3.1 have been used in this investigation. Satellite images might be processed for the (NDVI) Normalized Difference Vegetation Index for the constructed area Index aimed at understanding the urbanization of Karachi further; Spectral radiance might be estimated by using the thermal band of the satellite image for the temperature calibration of the region, for the UHI impact. This analysis will elaborate on the effects of UHI and assess the influences of the UHI. Moreover, UHI is responsible for producing environmental impacts Such as increases in the consumption of energy, elevated emission of greenhouse gases, and adverse effect on human health like heat stroke.

5. CONCLUSION

It was concluded that the LST and land use changes are very important as both facilitate crucial information for urban planners and country policymakers for natural resources. The recent study was carried out with a detailed interpretation of LST and LU/LC changes in the Karachi region. We elaborate on the influence of UHI on Karachi areas from 1990, 2000, 2010, and 2019 by using RS and GIS practices. The anthropogenic activities play a vital role to boost UHI and to cause to decrease the vegetation cover and increases LST during the last 3 decades in the region of Karachi. There are different kinds of modes of transfer of heat through various materials like conduction, convection, and radiation or we can say infrared. Radiation is the initial mode of conduction and convection takes place. All the materials, including construction materials such as plaster, wood, and glass obey the same laws of nature concerning heat transformation. This study has been deciphered through spatial analysis with the help of Land use classification, meteorological data interpretation, and land surface temperature. After the detailed review and interpretation of the above data, it shows that temperature and urban buildup are reciprocal to each other as buildup increases also temperature increases. The obtained data revealed that the temperature has gradually increased with the construction of a high-raised building in the years 1990, 2000, 2010, and 2019. The changes in NDVI results were observed in the studies years 1990, 2000, 2010, and 2019, but the maximum greenery was observed during 2019 as compared to other studied years. It could be due to heavy monsoon rain in 2019. The overall results will help the urban planner and policymakers to well plan the city with minimum utilization of natural resources to overcome these land-use changes, LST, and UHI influences in the future.

6. RECOMMENDATIONS

The substitution of vegetation and open land by metropolitan scenes land use changes and anthropogenic action will in general be the primary elements liable for the identified variations in the atmosphere examples and hotness increment of Karachi City. The investigation incorporated the clarification of the land use changes, consequences for the neighborhood atmosphere of Karachi City dependent on satellite images. Future studies should be focused on districtwide distribution temperature, build-up area, and vegetation. In addition, low-absorbent construction material must be used in high-rise buildings and a new vertical forest system should be adopted in urban areas. It was assumed that the vertical forest has some demerits like the breeding of mosquitoes, insects, pests, and deep root destruction of buildings, so these factors should be taken into account to design a green roof/ vertical forest plan. Future studies must be carried out to investigate the urban heat island in Karachi based on district-wise and NDMI aiming to reduce the temperature of a densely polluted area by using RS and GIS techniques.

7. CONFLICT OF INTEREST

The authors have no relevant financial or non-financial interests to disclose.

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Proceedings of the Pakistan Academy of Sciences: B
Life and Environmental Sciences 60(3): 477-487 (2023)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(60-3)854

Pakistan Academy of Sciences

Research Article

Zinc Oxide Nanoparticles Mitigate Toxic Effects of Cadmium Heavy Metal in Chilli (*Capsicum annuum* L.)

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Abstract: Heavy metals contaminated soils and water sources are one of the major global causes of inhibition of plant growth and productivity. Different strategies are being employed to overcome the challenging issue to increase plant yield requirements to fulfil the needs of future generations. The objective of the present study was to observe the effects of spray (foliar) of green synthesized ZnO nanoparticles (100 ppm), alone and its interaction in conjugation with Cd (Cd+ZnO-NPs) 100 ppm of both on the growth and biochemical activities of the target plant, *i.e.,* two chilli varieties. After two weeks of transplant, treatments *viz.,* Control (T1), ZnO nanoparticles 100 ppm (T2), Cd 100 ppm (T3), and ZnO nanoparticles 100 ppm + Cd 100 ppm (T4) were given for six weeks and different parameters of growth and biochemical analysis were made. Results have shown that 100 ppm foliar spray of ZnO-NPs has significantly increasing effects on root and shoot growth of chilli plants in alone (ZnO nanoparticles) and Combined (ZnO nanoparticles +Cd heavy metal) treatments mitigating toxic effects of Cd stress. A similar increase in values of total carbohydrates, soluble proteins, free amino acids, and photosynthetic pigments were observed mostly in a combination of Cd+ZnO-NPs treatment showing remediation properties of ZnO nanoparticles against Cd stress in chilli plant. In conclusion, it may be suggested that 100 ppm ZnO-NPs foliar spray can have an increasing effect on the growth parameters of the plants under stressful conditions of Cd heavy metal.

Keywords: Cd, Chilli, Interactions, Mitigation, ZnO-NPs

1. INTRODUCTION

The development of tolerance against several abiotic stresses including heavy metals, salinity, high temperature, drought, etc. is the worldwide need to improve the productivity of food with limited available resources. For this purpose, the use of minimum active mineral fertilizer can be helpful to overcome the toxic effect of heavy metals on plants. Different agricultural approaches have been employed to reshape modern agriculture [1].

Heavy metal toxicity in soil and water is correlated to a significant increase in the production of reactive oxygen species (ROS) such as superoxide, free radicals (O-), hydroxyl free radicals (OH-), and singlet oxygen (O_2). Which can cause oxidative stress within plants cell [2-4]. In the soil, heavy metals are classified into two types. The first category includes vital micro-nutrients which are required for proper plant growth, such as Fe, Mn, Zn, Cu, and Mg, while the second includes non-essential elements with uncertain biological and physiological roles, such as Cd, Cr, Pb, As, Co, and Hg, etc. [5-8]. Heavy metal deposition is of significant concern for environmental and nutritional purposes [9, 10].

Cadmium (Cd) is supposed to be one of the highly contagious non-essential trace metals due to its rapid transportation [11], water solubility, prompt absorption into the crop roots system, and transportation to the shoots [12]. Furthermore, it can translocate through the food chains and causes serious threats to humans, animals, and plants [13]. As a result, reducing Cd absorption and retention in crops is crucial in ensuring food safety [14, 15].

Nanotechnology is one of the most arising multi-disciplinary technologies with an

Received: February 2023; Revised: May 2023; Accepted June 2023
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encouraging agriculture method in the present era. Recent advances in nanotechnology have impacted agriculture and renewable energy [16]. Term nanotechnology has been used for the materials, systems, and processes which function at a hundred nanometer (nm) or less scale. Nanoparticles (NPs) consist of particles with very tiny dimensions particles and a comparatively large surface area due to distinctive characteristics such as a high ratio of surface area to volume, which advances their reactivity and putative biological activity [17]. Because of their unique properties and novel features, Nanoparticles have been extensively utilized in many areas of daily routine such as energy production, cosmetics, drug carriers in catalysts, and environmental energy [18].

The rapid development of nanotechnology, and encouraging studies depicted that metal oxide NPs could have significant potential to inhibit various heavy metal (Pb, Cu, and Cd, etc.) uptake and their accumulation in crops [19, 20]. Among this effect of zinc oxide NPs, titanium oxides NPs, Fe_3O_4 NPs, SiO_2 NPs, and Ag NPs, etc. has been studied by different workers [21-26]. Most of the workers have studied the impact of nanoparticles on heavy metals generally focusing on root application, whereas foliar applications studies are very little. For an instant, it is reported that foliar amendment of zinc oxide nanoparticles has inhibitory effect on Cd uptake and reduction of toxicity in maize. Furthermore, having the potential to improve plants' growth parameters, antioxidant, biochemical, and photosynthetic pigments activities found in the growth of plants under heavy metal Cd stress [27].

The red Chilli (*Capsicum annuum* L.) is a valuable cash crop of the family Solanaceae with worldwide distribution and consumption. We hypothesized that zinc oxide (ZnO) NPs application may have amelioration potential to protect the plant from Cd toxicity, and may improve growth, biochemical and physiological attributes of the plants. The aims of the present study were to explore the responses of plants with ZnO nanoparticles and Cd heavy metal in alone form and their interaction of zinc oxide NPs with Cd heavy metal in combined form simultaneously in two varieties of chilli. The results may be explored to overcome Cd toxicity in the target plant. Keeping in view, the combined exposure of zinc oxide NPs and Cd was studied with a novel strategy to reduce Cd accumulation and mitigation its toxicity in the chilli varieties.

2. MATERIALS AND METHODS

2.1. Characterization and Synthesis of Zn Oxide NPs

Zn oxide NPs were synthesized by green synthesis method as described by Lee [28] with slight modifications, using leaves extract of green tea (*Camellia sinensis*). 25 gm fine powder of washed and air-dried green tea leaves was added to 500 ml of de-ionized water and was heated at 80 °C for 120 minutes using the water bath. The dark green coloured extract obtained was twice time filtered twice through filter paper Whatman No. 2. Green tea extract (60 ml) was mixed with 140 ml of 0.2 M solution of Zinc acetate dihydrate (Sigma).

The reacted solution of green tea extract and Zn acetate dihydrate was dried at 60 °C for overnight in an oven. Zn oxide nanoparticles were finally calcinated at 100 °C for 60 minutes. The resultant powdery Zn Oxide nanoparticles were depicted by XRD (X-Ray Diffraction), FT-IR (Fourier Transform Infra-Red Spectrophotometer), SEM (Scanning Electron Microscopy) and (EDX) Energy Dispersive X-Ray analysis.

2.2. Experimental Setup of Chilli Varieties

Experiments were conducted in botanical garden of the University of Gujrat (UOG), Pakistan. There are 2 varieties of chilli (Var. 1 hot pepper upward and Var. 2 hot pepper downward) were selected for the current study. Seeds of both varieties were collected from the local market and sown in labelled 2 × 2' sized beds containing well-dried loam soil mixed with animal manure. Seedlings of uniform size were transplanted into pots when plants were 6 inches in length. After two weeks of transplantation, four treatments were given such as Control (T1), ZnO nanoparticles 100 ppm (T2), Cd 100 ppm (T3), and Cd 100 ppm + ZnO 100 ppm (T4) having five replicates of each treatment. ZnO nanoparticles were utilized to plants by foliar spray whereas heavy metal treatments were given through root solution. After six weeks of treatments, physiological and biochemical attributes were studied. One plant of each replicate was harvested

for growth parameters (dry and fresh weight of root and shoot) measurements.

2.3. Chlorophyll Contents

Chlorophyll contents (Chlorophyll 'a', Chlorophyll 'b', and Chlorophyll 'Total') were calculated by the method described by Witham *et al.* [29]. Fresh leaf (0.5 g) material was ground in 20 ml of 80 % acetone for the preparation of plant samples for chlorophyll content estimation. The sample absorbance was observed at 645, 653, and 663 nm wavelength by using a spectrophotometer (UV 1100). The chlorophyll contents were calculated using the following formulas.

Chl. a (mg/g) =
[12.7(D 663) – 2.69 (D 645) × V/1000 × 1/W]

Chl. b (mg/g) =
[20.9(D 645) – 4.68 (D 663) × V/1000 × 1/W]

Ch. Total (mg/g) =
[20.2(D 645) – 8.02(D645) × V/1000 × 1/W]

2.4. Estimation of Soluble Protein

Soluble proteins were analyzed by following the Bradford dye method as described by Bradford. [30]. Fresh leaves (0.2 g) were standardized in 4 ml of 50 mM phosphate buffer to maintain the pH of the buffer around about 7.5. The extract samples were prepared according to the protocol applied by Bradford and BSA (Bovin serum albumin) was treated for standard curve formation. Bradford reagent (dye) was used for color formation in the samples. Absorbance of the samples was calculated at the level of 595 nm by using a spectrophotometer (UV 1100). TSP (Total soluble proteins) was detected by using the given formula.

TSP (mg/g FW (Fresh Weight) =
Reading of sample × Volume of Sample × Dilution factor / Weight of fresh tissue × 1000

2.5. Estimation of Free Amino Acids

Free amino acids of the samples were estimated by the method followed by Lowry [31]. Plant samples of 1 ml were extracted for estimation of soluble proteins and kept in a test tube and put in 1 ml of ninhydrin solution 2 % and 10 % pyridine and were mixed into all test tubes and the mixture was heated for 30 minutes in a water bath. The absorbance of the coloured solutions was observed at 570 nm by using the spectrophotometer (UV-1100). However, free amino acids were determined with the help of the given formula.

TFAC (mg/g FW (Fresh Weight) =
Reading of sample × Volume of Sample × Dilution factor / Weight of fresh tissue × 1000

2.6. Total Carbohydrates

Total carbohydrates were estimated as described by Ashwell [32]. A well-ground dried sample of 0.2 g was taken in a test tube mixed with 10 ml of 6 N HCl and was kept overnight using an electric shaker for complete digestion of the sample. Anthrone solution of 10 ml was kept in a test tube and added 1 ml of the sample solution in a test tube and was well shaken. Each test tube was heated for 12 minutes in a water bath. The samples were cooled down, and absorbance was estimated at 625 nm wavelength by using a spectrophotometer (UV-1100). Total carbohydrates of the samples were calculated with the help of following the given formula.

Total Carbohydrate (mg/g of plant dry weight) =
Conc. of glucose solution/absorbance of glucose × absorbance of the sample

2.7. Statistical Analysis

The data of the present study was analyzed and subjected to ANOVA based on experimental design followed by Gomez and Gomez [33]. The follow-up of the Analysis of Variance included the LSD 0.05. DMRT tests were also applied to compare the means of the treatment.

3. RESULTS AND DISCUSSION

3.1. Characterization of Nanoparticles

The nanoparticles of zinc oxide were characterized to confirm the biocompatibility, solubility, and suitability of NPs for application in biological science. XRD, SEM, FT-IR, and EDX analysis were performed for the characterization of NPs (Fig. 1).

3.2. XRD Analysis

The size of ZnO-NPs was confirmed by XRD spectroscopy. For this XRD spectroscopy. Zn-Ka radiation at a wavelength of 26 °A and a current of 30 mA was used. Power of 40 KV was used for this analysis. Characterization peaks of NPs were according to the Miller indices. For the calculation of the size of nanoparticles, the Debye Scherrer formula was used.

$$D = \frac{0.9\lambda}{\beta cos\theta}$$

Where λ= wavelength of X-ray, β= full-width half maximum in radiance, θ= Bragg's diffraction angle. The value of D was calculated at 16 nm.

The calculated average size of Zn oxide NPs was 15 to 40 nm and as shown in Figure 1. It shows spectra of XRD analysis of ZnO nanoparticles after preparation and calcination of ZnO at 100° C for complete water removal and attaining higher crystallinity of the nanoparticles. Diffraction planes (100), (101), (102), (103), (110), and (112) with corresponding peaks are obtained. The hexagonal structure of ZnO NPs is confirmed by this spectroscopy.

3.3. FT-IR Spectrophotometry

Fourier Transform Infra-Red Spectrophotometry of powdered ZnO-NPs sample was performed to analyze the interfaces for the purpose of knowing the availability of various functional groups as shown in Table 1.

In the IR spectrum obtained from green tea, the band at 3394 cm^{-1} shows stretching vibration of the hydroxyl (OH) group present in alcohols, water, and phenols. Other bands obtained at 2926, 2864, 1627, 1741 and 1037 cm^{-1} show different types of stretches confirming the presence of a variety of functional groups present in amino acids, polyphenols, proteins, polysaccharides, and other biomolecules as shown in Table 1. The reduction of these biomolecules and their stabilization role is clear in the ZnO-NPs from the absorption bands of such biomolecules.

ZnO-NPs showing two new peaks at 457 cm^{-1} and 682 cm^{-1} wavelengths may be concluded as characteristic peaks of Zinc oxide NPs. A higher percentage of phenolic groups are responsible for reduction ability and higher amino acids; amide linkages in proteins may have a crucial role in the stabilization process of ZnO-NPs (Figure 1).

3.4. Growth Attributes

Results in Figure 2 have shown a significant difference effect in both chilli varieties. An increase in fresh shoot and root weights of both chilli varieties

Table 1. Identification of functional groups present on the surface of Nanoparticles (ZnO) with the help of FTIR analysis.

Wavenumber (cm^{-1})	Structural Formula	Functional Groups Name
900-665	N-H wag	1°, 2° amino
1300-1150	C-H wag, (-Ch-X)	Alkyl halides
1320-1000	C-O stretch	Alcohols, carboxylic acids, esters, ether
1470-1450	C-H bend	Alkane
1500-1400	C-C stretch in-ring form	Aromatics
1600-1585	C-C stretch in-ring form	Aromatics
1710-1665	C=O stretch	α, β-unsaturated esters aldehydes, ketones
1760-1690	C=O stretch	Carboxylic acids
1760-1690	C=O stretch	Carbonyls
3100-3000	C-H stretch	Aromatics
3330-3270	-C≡C-H: C-H	Alkynes (terminal)
3300-2500	O-H stretch	Carboxylic acids
3400-3250	N-H stretch	1°, 2° amino
3500-3200	O-H stretch, H-bonded	Alcohols, Phenols

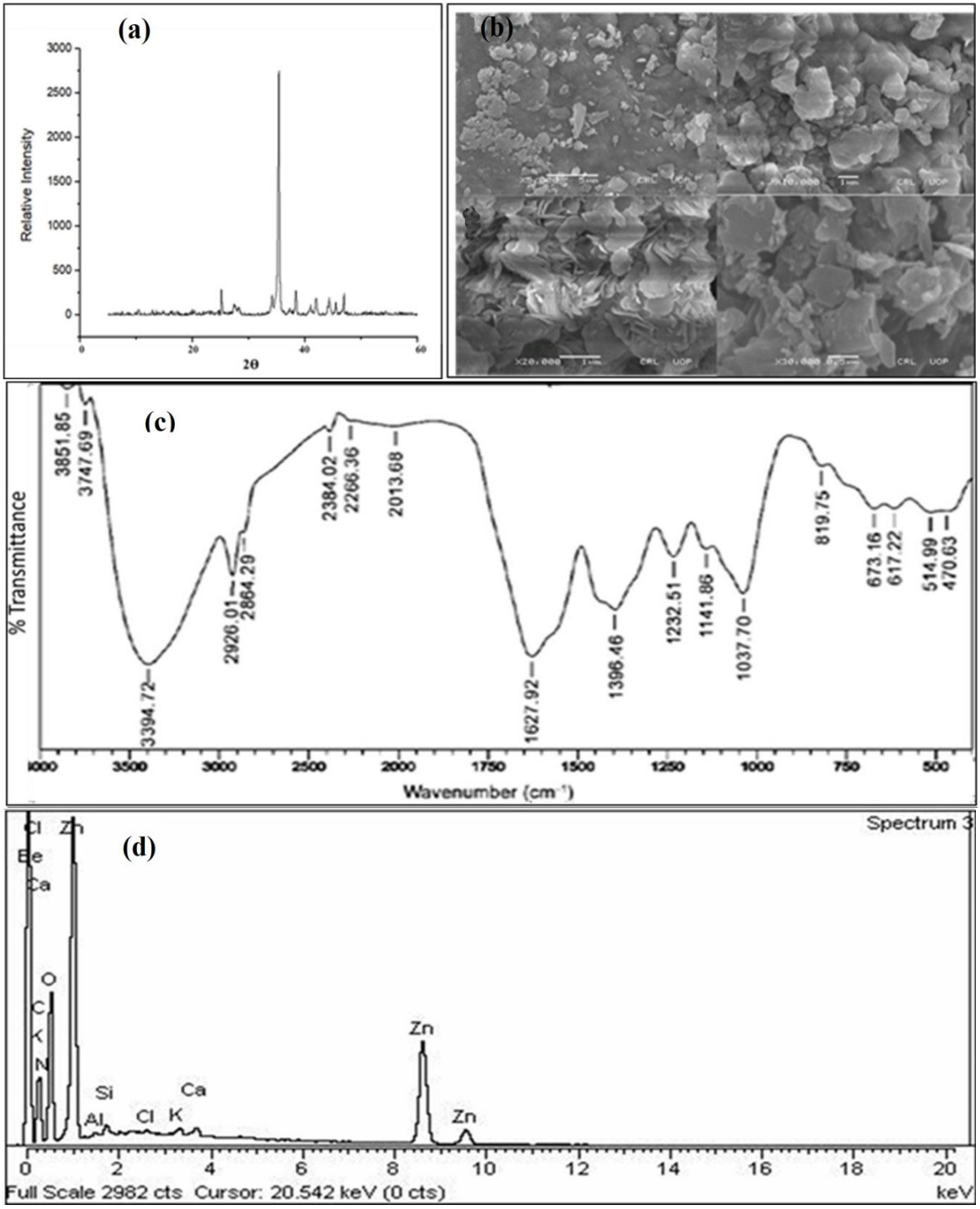

Fig. 1. Representation of vertical farming system (Source: [5])

was seen in treatment having a combination of 100 ppm Cd+ZnO NPs as compared to all other treatments. Shoot dry biomass production was significantly decreased in chilli variety 1 in the treatment of alone ZnO-NPs and alone Cd whereas both varieties were successful in maintaining it in the combined treatment of ZnO + Cd. An increase in root dry biomass production was calculated in two varieties in all treatments as compared to control but only significant in variety 1. Results of dry biomass production of root and shoot in chilli. Chilli varieties have shown that foliar amendment

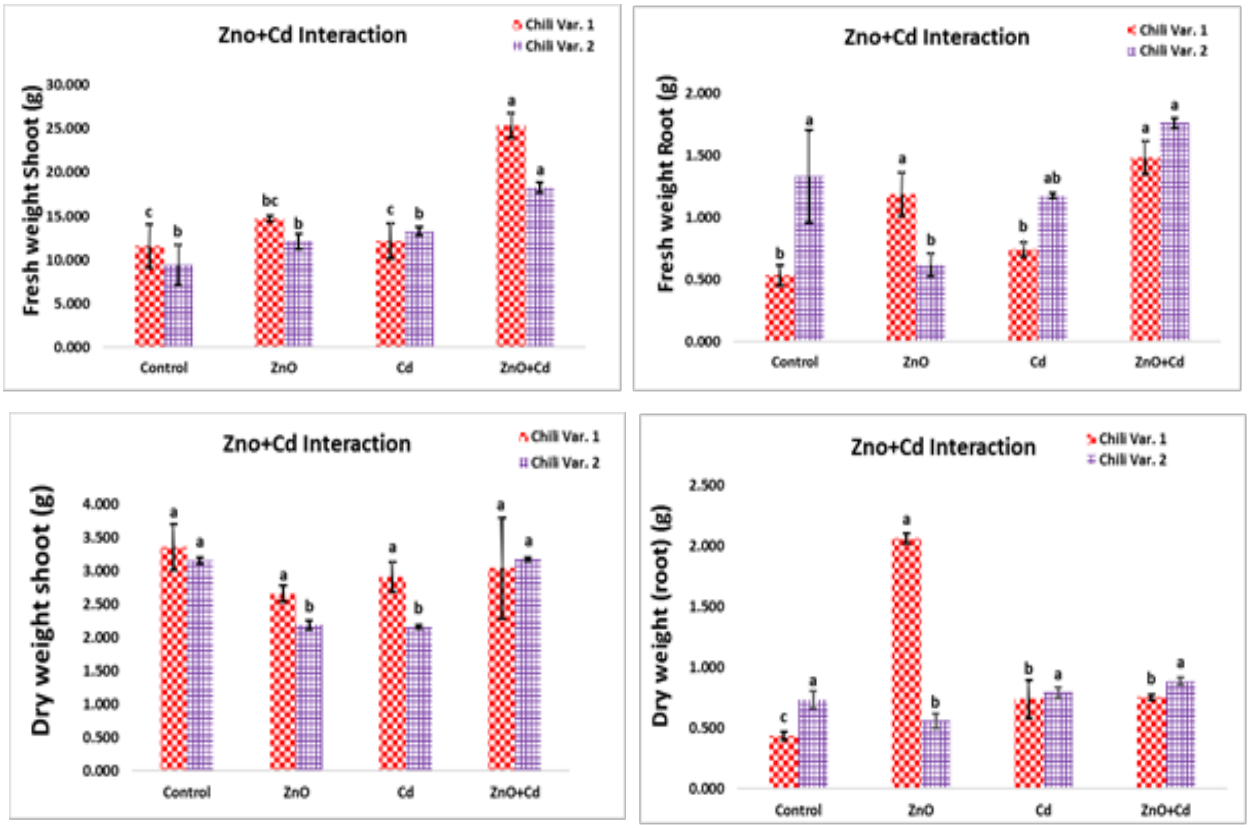

Fig. 2. Fresh and dry, shoot and root weights of chilli varieties in response to different treatments.

of 100 ppm of ZnO-NPs in combination with 100 ppm of Cd heavy metal treatment found a maximum effect in both varieties of chilli. Such increased dry biomass may be due to the potential of ZnO-NPs to alleviate the toxic effects of Cd heavy metal on the growth of chilli plants. These findings confirm the results of earlier workers who documented the positive role of ZnO nanoparticles in the mitigation of Cd heavy metal toxicity and enhancement of growth [34-38].

Data of photosynthetic pigments of chilli varieties has shown an increase in chlorophyll a, b, and total values. This improvement of photosynthetic pigments may be the result of inhibition of Cd uptake in plants. Zinc (ZnO-NPs) being an important constituent of different enzymes and precursor metabolites involved in the biosynthetic machinery of photosynthetic pigments. This improvement is matching with the findings [39-42].

3.5. Biochemical Attributes

Results for chlorophyll contents are shown in Figure 3. It is clear that foliar spray of ZnO nanoparticles has a considerably decreasing effect on chlorophyll 'a', chlorophyll 'b', and chlorophyll 'Total' in chilli variety 1. Whereas both chilli varieties maintained their all-photosynthetic pigments with treatments of Cd alone and a combination of Cd+ZnO-NPs like that of control.

3.6. Total Carbohydrates of Root and Shoot

Results for carbohydrates of shoot and root are represented in Figure 4. It has revealed that ZnO nanoparticles and Cd alone treatments have no significant effect on root and shoot carbohydrates contents of both varieties as compared to control, except ZnO + Cd combination treatment in which significant increase of shoot and root carbohydrates content was observed. Data of photosynthetic pigments of chilli varieties has shown an increase in chlorophyll a, b, and total values. This improvement of photosynthetic pigments may be the result of inhibition of ROS production due to a reduction in Cd in plants. Zinc (ZnO-NPs) being an important constituent of different enzymes and precursor metabolites involved in the biosynthetic machinery

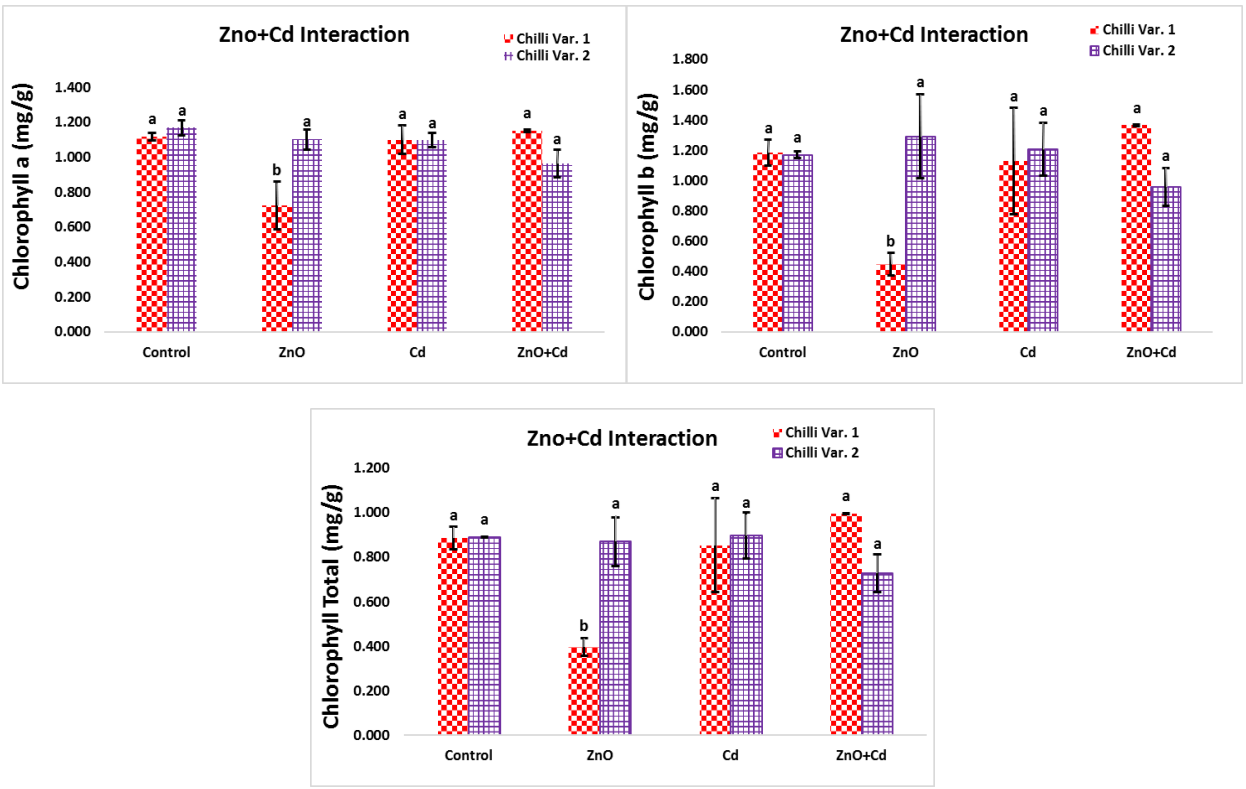

Fig. 3. Chlorophyll contents of chilli varieties in response to different treatments.

of photosynthetic pigments. This improvement is matching with the findings [39-42].

3.7. Soluble Proteins and Free Amino Acids

The soluble proteins and free amino acids of two varieties of chilli are shown in Figure 5. Results have shown that 100 ppm of Cd treatment has a significantly decreasing effect on soluble proteins of chilli variety 2 and free amino acids of both varieties, whereas the increased value of soluble proteins was observed in chilli variety 1 under the same treatment. A significant increase in soluble proteins was observed with ZnO + Cd combined treatment whereas the increased value of free amino acids was noted both in ZnO alone and ZnO + Cd combined treatments.

Data for analysis of variance (ANOVA) for growth and biochemical attributes are shown in Table 2. In growth attributes, almost all parameters including shoot and root, fresh and dry weight showed highly significant differences in both varieties among parameters except the dry and fresh

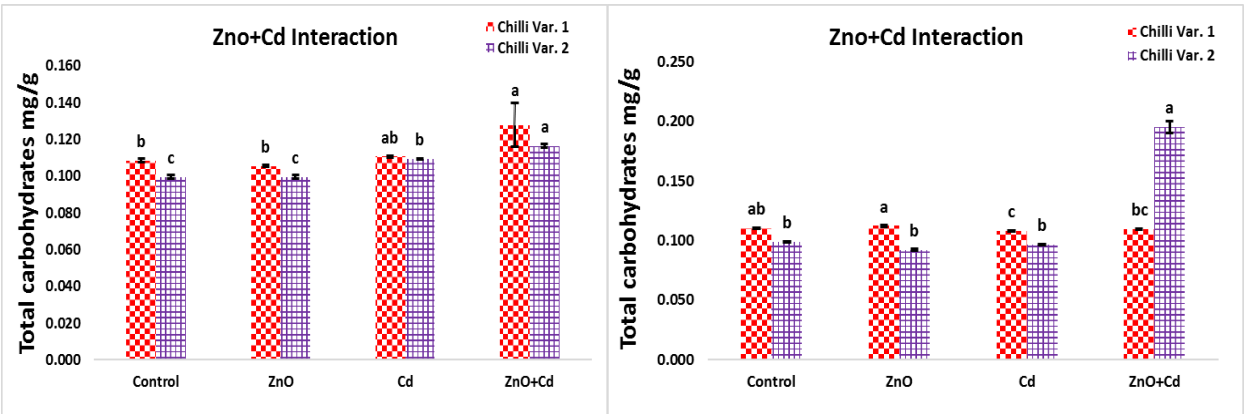

Fig. 4. Total carbohydrate contents of chilli varieties in response to different treatments

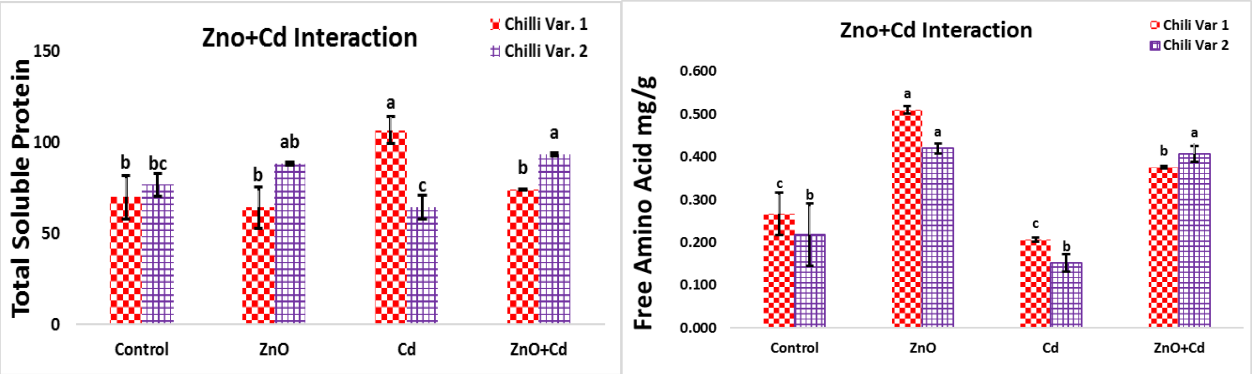

Fig. 5. Soluble protein and Free Amino Acid of chilli varieties in response to different treatments.

Table 2. F-values derived from ANOVA for growth and biochemical attributes.

Parameters	Varieties	F-ratio	P-Values	$LSD_{0.05}$
		Growth Attributes		
	Var. 1	0.442	0.7256ns	1.305
Dry Weight Shoot	Var. 2	154.18	0.0000***	0.143
	Var. 1	20.469	0.0000***	0.179
Dry Weight Root	Var. 2	6.583	0.0041**	0.159
	Var. 1	0.442	0.725ns	0.247
Fresh Weight Shoot	Var. 2	123.583	0.0001***	0.129
	Var. 1	12.51	0.0001***	0.362
Fresh Weight Root	Var. 2	6.022	0.0060**	0.577
		Biochemical Attributes		
	Var. 1	6.147	0.179*	0.263
Chlorophyll ‘a’	Var. 2	0.823	0.516 ns	0.283
	Var. 1	4.798	0.0338*	0.599
Chlorophyll ‘b’	Var. 2	0.668	0.5948ns	0.571
	Var. 1	5.717	0.0217*	0.36
Chlorophyll ‘Total’	Var. 2	0.328	0.5142ns	0.283
	Var. 1	2.993	0.0767ns	0.018
Carbohydrate (Shoot)	Var. 2	129.503	0.0000***	0.002
	Var. 1	11.135	0.0008***	0.001
Carbohydrate (Root)	Var. 2	405.566	0.0000***	0.007
	Var. 1	26.878	0.0000***	0.079
Free Amino Acid	Var. 2	11.67	0.0007***	0.121
	Var. 1	4.469	0.025*	27.855
Soluble Protein	Var. 2	8.629	0.0025**	13.589

*F= F-ratios were obtained from ANOVA tables, LSD=Least significant difference at P=0.05, NS=Non significance; *, **, ***, significant at 0.05, 0.01 and 0.001, respectively.*

weight of shoot showed non-significant differences in variety 1 among parameters. However, the results of biochemical parameters including Chlorophyll, a, b, Total, Carbohydrate shoot, and root, free amino acids, and soluble protein found significant differences among all biochemical parameters except chlorophyll a, b, Total in variety 1 and shoot carbohydrates and chlorophylls of variety 2 showed non-significant differences.

Data of results of free amino acids and soluble proteins has shown increased values for both parameters, but this increase is shown only in alone ZnO NPs and combined Cd+ZnO NPs treatments whereas decreased values of both attributes are depicted in alone Cd heavy metal treatment. Adjustment of different osmolytes in plant cells under stressful conditions may be the result of the production of various organic solutes including free amino acids, protein soluble, and carbohydrates, not necessarily all solutes but each plant may have a free choice of selection of any of osmolytes under stress. The increase in free amino acids and soluble proteins in our trial may be in accordance with the findings of Shallan *et al.* [45], who noted an increase in free amino acids and soluble protein values due to the activity of NPs in cotton plants under stress. Similar positive role of ZnO nanoparticles was given by Hussain *et al.* [46], who reported that ZnO-NPs increases the growth of Wheat plant by reducing electrolyte leakage, providing osmotic adjustment to the plants growing under Cd stress.

4. CONCLUSION

The present study concluded that increased uptake of Cd from the soil has an inhibitory effect on the root and shoot biomass production of plants resulting in decreased growth. Similar decreased values of total carbohydrates, chlorophyll contents, proteins soluble, and free amino acids were also observed showing toxicity of Cd heavy metal. Exogenous application of Zinc in the form of a spray (foliar) of ZnO nanoparticles has enhanced the growth of chilli plants. Same enhancements were also observed in values of total carbohydrates, chlorophyll contents, proteins soluble, and free amino acids both in alone and combination with Cd treatments. It may be established that ZnO-NPs may have the potential to increase the organic solutes of the plants by increasing free amino acids and proteins soluble providing osmotic adjustment in plants resulting in inhibition of Cd uptake. This defensive strategy has resulted in the alleviation of Cd toxicity with increased growth of plants. These findings may be helpful in developing the tolerance of Cd heavy metal in chilli plants growing in Cd-contaminated soils and water sources. Same investigation may be extended to other species of the same family like brinjal & tomato etc.

5. CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Proceedings of the Pakistan Academy of Sciences: B
Life and Environmental Sciences 60(3): 489-496 (2023)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(60-3)859

Pakistan Academy of Sciences

Research Article

Geoinformatics and Extrapolation-based Applications for Estimation of Shortwave Radiation Potential as a Sustainable Energy Source: Emphasis on Smart Cities

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Abstract: Smart cities are objectively developed for a sustainable and better life quality for their inhabitants. The present study is focused on the determination of downward shortwave radiation potential-based sites to develop smart cities based on the suitability and useable aspect of these radiations as a sustainable energy source. The downward shortwave radiation is estimated through MTCLIM-XL extrapolation with further spatial-based potential through spatial analysis of Geographic Information System (GIS) as a Geoinformatics application an applicable tool of Geoinformatics majorly helps in integration and processing of related geo-data and related critical factors for final visualization towards smart and applicable decision making. Hence, these properties make Geoinformatics a viable approach in the applications of sustainable energy estimation for the development of smart and sustainable cities. Prospectively, Geoinformatics with the integration of related critical parameters can be a reliable approach for application in the determination of suitable locations for harvesting the radiation potential as a sustainable energy source.

Keywords: Smart cities, Downward shortwave radiation, Sustainable energy, extrapolation, Geographic Information System, Geoinformatics

1. INTRODUCTION

The term "city" has its manifestation since ancient times (3500 to 3000 BC) and relates to the urban set-up and infrastructure based on certain legal terms. Since its basis, this term is changing progressively as the urban infrastructure has evolved due to urban population growth [1]. The United Nations (Department of Economic and Social Affairs) has estimated that by 2050, 68 % of the population will be residing in cities as, according to 2018 data, it has been calculated as 55 % of the global population [2]. Consequently, due to this large and exponential urban population, the consumption of energy resources is leading to challenges of environmental consequences. In addition, there will be a rise in major contributors to the degradation of the urban environment, particularly greenhouse gases (GHG) emissions [3]. In the last three decades, the concept of a 'Smart City' has emerged with a basis of enhancing the life and environment of the growing urban population. In this perspective, to consider ISO 17742 [4], the addition of green and renewable energy resources is an applicable and progressive approach towards the development of a 'Smart City'.

For a viable use of these energy resources, the determination of resource potential is significant to exploit it sustainably within urban infrastructure. For these integrative approach-based studies, geoinformatics is an applicable tool as it integrates

Received: February 2023; Revised: May 2023; Accepted: June 2023
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and processes spatiotemporal data required for the planning and designing of urban infrastructure. For integration as a renewable energy source for 'Smart Cities', the downward shortwave radiation (from herein: DSR) is one of the active and sustainable considering factors, and also essential for the energy systems [5].

These radiations, as an incident flux comprise 85 % of solar radiation, which is a major energy source that drives many critical processes related to energy and agricultural systems [6]. However, there is a challenge in its viable applications due to its intermittent nature, as well as, spatiotemporal and geographical variability. Hence, for its possible total harvesting and determination, the best decision tool is Geoinformatics.

Geoinformatics, including Geographic Information System (GIS), has the applicable ability to smartly manage, and retrieve spatial-based results and thus help in potential-based planning of the feasible locations (hotspots) and areas for harnessing the energy potential. This study proposes and reviews the integrative applications-based role of GIS, a geoinformatics tool, in the determination of DSR potential as an active solar energy source.

2. MATERIALS AND METHODS

2.1. Sustainable Exploitation of the DSR

For better and sustainable exploitation of the radiation energy for different purposes, its determination approach can be based on different phases including; Strategic layout-based estimation, geographical factors-based suitability, technical-based hotspots determination, and utilizability-based potential estimation, respectively. Concisely, for better and sustainable exploitation of the DSR as an active energy resource, the following top-down approach can be applicable.

2.1.1. Strategic layout-based estimation

The Strategic layout-based estimation is the start-up and comprehensive phase in which need and availability-based analysis can be followed to determine the availability and exploitation of resources in a focused area. For instance, the parameter to be considered as an energy resource is the DSR. Hence, the higher the DSR budget against relative need-based usage in the area, the higher will be the sustainable-based potential.

2.1.2. Geographic factors-based potential

The geographic factors-based potential is the analysis of potential after determining the locations where radiation energy can be exploited after consideration of physiographic factors. It entails the exclusion of areas with topographical constraints in the perspective of the best possible exploitation of radiation as an energy resource. These critical physiographic factors includ elevation, slope, and aspect, East and West horizon (which truncate direct irradiance). The solar zenith angle (SZA) can be considered as an additional factor as it has an inverse relation with the downward radiation of the sun (due to radiation decrease) with a rise of SZA.

Furthermore, other geographic constraints also need to be included for consideration of energy potential at the urban level. These include complex urban infrastructure, irregular terrains with high elevations, eco-diverse places including water bodies, etc. as well as, the proximity level towards the grid (base station).

2.1.3. Utilizability-based potential

The Utilizability based potential is the technical-based potential of available DSR in a target area which is based on the conversion of this radiation energy for multi-purposes i.e., electricity generation, thermal insulation of houses, etc. For the radiation to electric power conversion, the downward radiation can be harvested more efficiently during the 'peak sun hours (PSH). PSH refers to the determination and comparison of the amount of sunlight in different locations in any area or region. For instance, 1 PSH is an average of 1,000 watts (W) of energy per square meter i.e., W/m^2.

2.1.4. Utilizability-based potential

The determination of utilizable-based radiation potential gives insights and a realistic approach toward the economic potential. The factors which are critical to be considered include; land types and use, the overall set-up-based cost, and other required infrastructure, as well as, the cost for the

maintenance of set-up which may vary according to the applications of DSR energy for certain purposes. In addition, it can also include the related social, economic, and environmental factors of the urban area to be developed.

2.2. Data Acquisition

The incoming shortwave radiation is available only during the daytime, hence, dominates the overall radiation budget. However, these radiations have variations which are based on the amount of its direct and diffuse components having major hindering factors including atmospheric conditions and related topographic agents. On this, Angelis *et al.* [7] followed separate approaches for the determination of both direct and diffuse radiation. These approaches include the acquisition of field data from the meteorological base stations in the target area, as well as, filling the data gaps by different interpolation techniques. Subsequently, the satellite imagery-based data is obtained from MeteoSat (meteorological Satellite). Lastly, these approaches are integrated by using the imagery data for locations having no meteorological stations [7].

In the latest model approach for DSR simulations, particularly in a clear-sky condition, the atmosphere, and terrain-based algorithms retrieve good results for areas with pre-determined metrological data. Additionally, in the last decades, approaches that consider critical topographic factors have also been studied. In these models, some have been integrated as a built-in application in GIS software. However, many such models are based on a common radiative transfer approach which makes their applications unreliable [8].

In the context of solar radiation as an energy resource, Korfiati *et al*. [9] have applied the irradiance data for the determination of photovoltaic cost and its global energy potential. The data was acquired from the NASA database of surface meteorology and solar energy. Similarly, with the GIS-based analysis approach, Sun *et al*. [10] have determined the potential of photovoltaic cells with a focus on Fujian Province, China. For instance, the surface DSR and other related parameters have been derived from geostationary satellite observations [11]. Additionally, Moderate-resolution Imaging Spectroradiometer (MODIS) has also developed datasets of atmospheric parameters which is helpful for the estimation of surface shortwave radiation [12].

The developing trend of climatic and local topographic factors integration is critically significant for the generation of updated spatiotemporal data. Focusing on the estimation of DSR in particular, the impetus for integrating physiographic agents of a focused area has developed as a reliable approach on a local and regional scale. In this context, the MTCLIM (mountain climatic) model has been developed in 'Visual Basic for Applications' to determine the daily-based DSR and other microclimatic variables. The MTCLIM-logic works on extrapolation of daily available local meteorological data inputs from one or two base sites (stations) to a remotely located target site.

2.3. Data Processing

The DSR potential is initiated through DSR determination of the target area by integrating its results from MTCLIM-XL, topographic analysis, and production of the spatiotemporal-based maps in ArcGIS. The significance and reliability of MTCLIM are due to the relative convenience of its basic parameterization, as well as, the easy and fast processing of the modules embedded in the MS-excel-based workbook (version 4.3XL) [13]. The MTCLIM-XL takes into account the basic topographic factors including elevation, slope, aspect, and East/West horizon, as well as, daily weather observations; maximum and minimum temperatures, and precipitation.

In the context of the proposed study [from 5], topographic factors were mainly produced by the available high-resolution imagery of 'Shuttle Radar Topography Mission (SRTM)'. For the initialization file of the MTCLIM-XL, required physiographic information (latitude, elevation, slope, and aspect, East and West horizon of target sites, and elevation of reference site (base station) were generated in GIS environment (Arc GIS 10.1) using 'Spatial analyst' tools. For this process, open-access data of the digital elevation model (DEM) available at high-resolution (30 m) was obtained from the Shuttle Radar Topography Mission (SRTM) [14]. From these generated data layers, the cell-based elevation, slope, and aspect were extracted to the study area-based target points. Finally, spatial layers of the points-based (sites) with physiographic

information (slope, aspect, and spatial elevation gradient) of the study area were generated.

Once the DSR is spatiotemporally estimated, the strategic layout-based potential is determined through the production of spatial potential-based hotspot maps of the locations within the target area i.e., the city. Furthermore, the process of geographic factors-based potential follows a similar approach to exclusion-based criteria of the topographical constraints mentioned in section 2.2.

For estimating the utilizability-based potential of the DSR for specific applications, for instance, 250 W/m^2 (Watt per square meter) or 2 kW h/m^2 per day on sites or areas in a city are considered as the radiation-based hotspots with a minimum or exceed that range of surface radiation as a threshold level of any particular energy system [5].

For the source of energy and related applications, the determined radiation (W/m^2) values can be converted into a unit of kilowatt-hour per meter square area (kW h/m^2/day) by using the conversion formula, as given in Eq. (1):

$$E\ (kWh) = P\ (W)E(kWh) = P(W)\ \mathrm{x}\ \frac{t(hr)}{1000}\ as\ E = kW.\frac{h}{m2}\ Or, E = \frac{\frac{kWh}{m2}}{day} \quad (1)$$

'E' is the DSR energy potential (in Kw: kilowatt) on locations of focused area in square meter (m^2) and time 'h' (hours/day) available as a maximum daily mean 8 hours (considered as maximum peak or active sun-hours on daily mean based). For urban areas with complex topography, a spatial-based factor of the Hill-shade analysis may be considered for the local topographic effects on the DSR budget. Within the Arc GIS environment, the 3D Analyst tool can be applied to generate a shaded relief (from surface raster data) on any specified location [5].

Subsequently, the last level of the proposed approach, economic potential entails the production of a hotspot map which is assessed on the basis of the DSR spatial distribution that can be exploited in specific locations or areas of the target city. These spatial findings lead to a reliable analysis of the exploitable energy, hence; an economical layout can be produced for sustainable applications in the perspective development of a 'Smart city'. Thus, the overall approach is an applicable source of prospective framework and guidance for urban planners and decision-makers for prioritizing this renewable energy source towards sustainable development of urban infrastructure i.e., a smart city.

3. RESULTS AND DISCUSSION

The resultant spatial product of the presented approach of potential determination produces DSR spatial distribution-based maps which provide a clear result for 'need and availability-based analysis. To assess the potential of DSR spatial distribution, a spatial hotspot-based map of the target sites was generated. The next phase of the present approach entails the production of the geographic factors-based potential of DSR spatial distribution which is based on the exclusion criteria of physiographic factors i.e., shaded relief (with local horizon). Subsequently, technical-based potential maps are produced which are based on the utilizable potential of available DSR in a focused area. The utilizability-based potential map was produced based on the conversion of this radiation energy for multi-purpose applications. For instance, in the case of Quetta city, Pakistan, spatial hot-spots analysis depicts some locations including Hazar Ganji, Tor Shor, Hazar Ganji-Chiltan national park, and Chiltan reserved forest with a DSR potential above the threshold level of 2 kW h/m^2/day (Figure 1 and 2). Additionally, the potential of DSR (watt/m^2) every month (for instance, September), and the spatial variations-based hotspots were also derived from daily mean-based data (Figure 2) [5].

Finally, for economical-based potential the correlated analysis-based studies of the focused city i.e., urban planning and management which includes many critical factors including land types and use, and the proposed set-up-based cost which may vary according to the DSR as an energy source for particular purposes. However, in this study context, it is out of approach due to the variations in the cost-benefit analysis of renewable energy exploitation-based projects.

The spatial assessment-based final visualization in maps of the overall and utilizable potential of the DSR estimation produces reliable directions

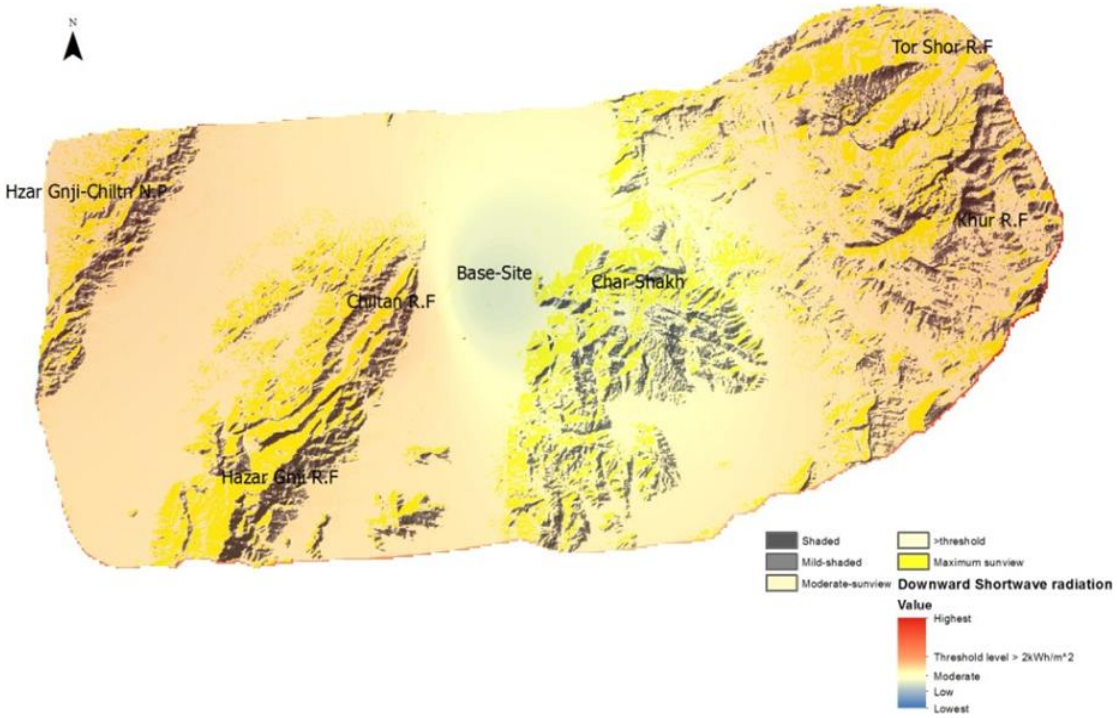

Fig. 1. Potential of DSR on basis of spatial distribution (Quetta city, Pakistan, Year: 2015) Source: Sardar *et al*. (2017) [5]

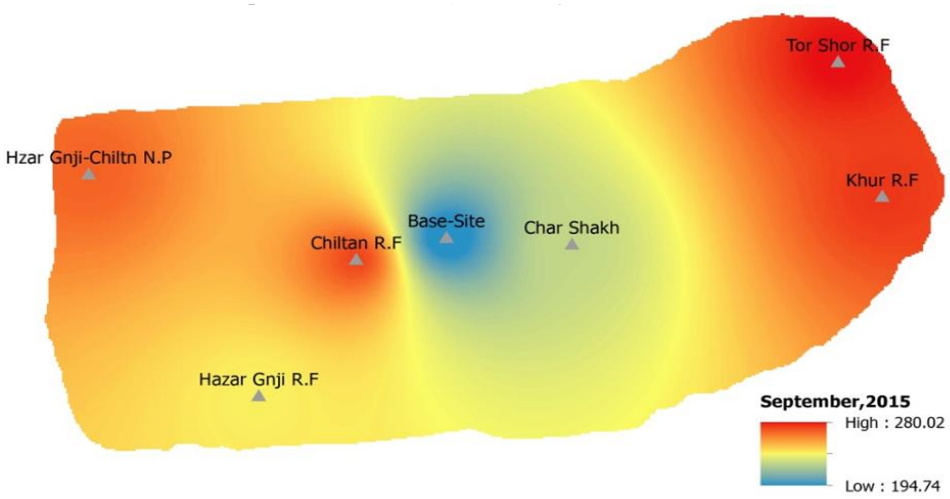

Fig. 2. Spatial distribution-based potential of DSR on target sites (monthly mean basis) (Quetta City, Pakistan, September 2015) Source: Sardar *et al*. 2017 [5]

for urban planners, developers, and policymakers toward the prospective initiation and development of smart and environmentally sustainable cities. For a prospective approach, the numerical-based results of daily mean DSR from MTCLIM-XL in this approach within a GIS environment can be developed as an integrated approach within advanced simulations for smart and updated access to multi-purpose exploitation of this active energy source.

3.1. Data Validation and Analysis on a Spatial Basis

The Daily mean incoming (downward) shortwave radiation (W/m^2); SIS from MeteoSat second generation data (obtained from EUMETSAT's Satellite Application Facility on Climate Monitoring (CMSAF) [15] was used for validation-based assessment of the MTCLIM-XL calculations of the daily mean DSR (W/m^2). This data (CMSAF) was

in NetCDF-file format (under MSG-Operational products covering full disk with a daily mean basis of temporal resolution and spatial resolution). From the obtained radiation data in NetCDF files, the EverVIEW-Data viewer [16] was used for retrieval and visualization of data values. Resultantly, a spatiotemporal data set of daily-based DSR was generated for the study data (Year 2015) (Fig. 3) [5].

The validation of the resultant data (daily mean: monthly average of the study year 2015) was carried out on a comparative analysis basis with the obtained data (from CMSAF). For this purpose, root mean square analysis (RMSE) has been proven to be a simple and reliable approach for the evaluation of differences between known and determined data values [17]. The RMSE-based comparative analysis resultantly shows low values for the base site and Char Shakh as compared to other target sites (Table 1). Due to the variable trend in estimated values of radiation for some sites, in contrast to its normal rise with the local change in weather during a year, variations in RMSE values for radiation is common and expectable. In addition, the satellite data can fluctuate from 10 to 50 W/m^{2,} particularly at irregular terrain surfaces with maximum anomalies of up to 600 W/m^2 [18]. For the study data (Year 2015), a relatively high correlation was found between the values of the base site, and Char Shakh site with correlative analysis to the standard data (CM SAF) as shown in Table 1. For Tor Shor reserved forest site, relatively negative values of correlation coefficient were analyzed for three months of data: January -0.23, April -0.35, and May -0.26. However, an agreeable

Fig. 3. EverVIEW Data Viewer (EU METSAT CMSAF) on study area and region-based spatial overlay based tabulated data: highlighted cells of the radiation data (September, 2015) Source: Sardar *et al*. 2017 [5]

Table 1. Root mean square errors (RMSE) between result-data (DSR: W/m^2) and CMSAF (Shortwave radiation products) for Study sites (Year 2015) (Source: Sardar *et al*. 2017) [5]

Site	January	February	March	April	May	June	July	August	Sep.	Nov.	Dec.
Base-station	3	2.89	3.19	4.08	3.99	4.46	3.93	3.94	4.13	3.44	3.04
Char Shakh	2.77	2.74	3.5	3.34	3.46	3.29	3.19	2.85	3.24	2.9	3.06
Tor Shor reserved forest	6.13	5.01	5.15	3.75	3.47	3.6	3.3	2.41	2.21	3.52	4.64
Khur reserved forest	7.4	5.56	5.24	3.62	3.64	4.04	3.52	2.81	2.33	3.91	5.32
Chiltan reserved forest	6.1	4.82	4.75	3.47	3.2	4.04	3.61	2.5	2.32	3.37	4.52
Hazar Ganji reserved a forest	6.95	4.99	4.63	3.42	3.95	4.79	4.21	3.59	3.15	3.52	4.21
Hazar Ganji-Chiltan national park	7.66	5.55	4.85	3.38	3.72	4.47	3.9	3.09	2.46	3.86	5.42

correlation was found for the data of other months [5].

4. CONCLUSION

The proposed integrated-based application of geoinformatics with extrapolation is a reliable and applicable approach for spatial-based determination of solar radiation, particularly DSR, as a sustainable energy source. For better exploitation of this renewable energy resource, the GIS-based approach is relatively significant in spatial visualization of energy for the identification and assessment of potential locations (hotspots) in city areas to assist urban planners and developers. Prospectively, the initiation of these renewable energy resources-based developments will lead to a sustainable and green energy fraction within urban infrastructure. Furthermore, in an economic context, it will be a smart alternative and additive energy supply to meet the growing demand for power consumption in urban areas. In accordance with the 'Goal 7 and 11' of the Sustainable Development Goals [19], such an approach will be an applicable step and standard for smart city development.

The spatiotemporal-based assessment of DSR also require the exclusion criteria of physiographic factors to be considered due to their variations, hence; retrieval of high-resolution data is critical. Furthermore, in the context of energy applications, the reliability of the DSR potential estimation significantly relies on updated spatiotemporal data of major meteorological parameters from sophisticated databases. We anticipate that integration of local physiographic analysis with an interpolation approach, the present study will be applicable for researchers and decision-makers toward real-time assessment of DSR for the potential exploration of potential in areas with sparse or no ground data. Conclusively, this approach will be insightful for the analysis of exploitable energy sources in the perspective development of a smart city. Hence, the determination of the real-time based potential of the DSR in any such area will lead to an alternative, green, and sustainable energy solution.

5. ACKNOWLEDGEMENTS

The corresponding author is thankful to the KUST University, Kohat 26000, Pakistan for providing a research-oriented environment.

6. CONFLICT OF INTEREST

The authors declared that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Proceedings of the Pakistan Academy of Sciences: B
Life and Environmental Sciences 60(3): 497-503 (2023)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(60-3)866

Pakistan Academy of Sciences

Research Article

Biorisk Management and Antibiotic Susceptibility Pattern of Biofilm Producing *Pseudomonas aeruginosa* Isolated from Broiler Chicken: A Public Health Concern

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Abstract: Control of biosecurity and biosecurity within poultry consists of a set of practical measures meant to prevent and control the spread of disease between people and animals. Infections, caused mainly by zoonotic agents, occur frequently due to the lack of safety monitoring regulations, as well as the inappropriate use of antimicrobial products, leading to the emergence of antimicrobial-resistant microorganisms. *Pseudomonas aeruginosa*, often known as the MDR pathogen has evolved resistance to multiple antibiotics. Because of its propensity to build biofilms in meat and other food products, *P. aeruginosa* is even more resilient to the phenomenon of drug resistance which is a major public health issue. Standard microbiological and biochemical tests were used to isolate and identify *P. aeruginosa* from a total of 100 meat samples (20 from each district from broiler chicken meat) gathered from various butcher shops and supermarkets. The Kirby Bauer method was used to identify antibiotic resistance, while the microtiter plate test was used to monitor biofilm formation. It was found that *P. aeruginosa* was identified from 22 % of the broiler chicken meat samples and showed resistance to Cloxacillin, teicoplanin, ciprofloxacin, imipenem, and meropenem, followed by linezolid, streptomycin, amikacin, compound sulphonamide, aztreonam and cefepime which showed intermediate resistance. Multiple Antibiotic Resistance Index (MARI) was calculated as 0.45 for a total of 11 antibiotics. Also, all 22 MDR isolates of *P. aeruginosa* tested positive for the presence of the biofilm. In conclusion, it was determined that chicken meat was contaminated with *Pseudomonas aeruginosa,* and these strains that produce biofilms are more resistant to antibiotics. Thus, there is a serious threat to public health from biofilm-forming isolates found in broiler chickens.

Keywords: *Pseudomonas aeruginosa*, Biofilm, Multi-Drug Resistance, Laboratory Infections, Biorisk Management, Antibiotics

1. INTRODUCTION

Aside from being one of the most innovative animal businesses and quickest manufacturers of meat worldwide, poultry has made important contributions to the country's agricultural economy. Protein from poultry products is necessary for human health [1]. Proper management and cleanliness on the farm have a significant impact on the quality of the chickens produced [2]. It is generally accepted that a chicken production system's main priority should be ensuring the highest possible levels of biosecurity and farm hygiene. Better flock health, lower medical costs, fewer losses, and greater profits are all possible thanks to effective biorisk management [3]. The FAO believes that the best strategy to prevent the spread of infectious illnesses is through the strict implementation of biosecurity measures [4]. High-risk areas for the transmission of illness could be mapped using biorisk management tools. This has crucial implications for the prevention and control of epidemics and facilitates their monitoring [5].

Received: March 2023; Revised: May 2023; Accepted: June 2023
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For the maintenance and improved health status and standards of livestock, reducing the spread of infectious diseases is imperative. Strict biorisk management policies will help to reach that goal. Over-prescribing antibiotics, which leads to antibiotic resistance in infectious organisms, is a prevalent practice that arises from the need to lower the prevalence of infectious agents [6]. Misusing biocidal agents in livestock farming also promotes the spread of bacteria that are resistant to antibiotics [7, 8]. Antibiotic overuse can be prevented through the implementation of stringent biosafety and biosecurity measures, which will also preserve the efficacy of traditional antimicrobials used to treat acute and chronic diseases [9]. The biorisk management measures for animal farms are specifically prepared to prevent the introduction and spread of diseases and considered as key-factors for increased farm productivity [10].

Pseudomonas aeruginosa is an opportunistic pathogen, with the ability to produce biofilm, that causes severe problems in chicken farms [11], while *Pseudomonas spp.* have historically been the most common pathogens found on chicken carcasses in industrial poultry processing facilities [12]. *Pseudomonas aeruginosa*, which causes serious respiratory infections [13] & cystic lung fibrosis [14] is widely believed to originate from chicken-based goods sold in grocery shops, especially among the immunocompromised. In the agriculture sector of Pakistan, and particularly in poultry farms and retail shops, there is a serious scarcity of understanding and application of biorisk management measures and protocols. This study is designed to establish a baseline regarding the presence of infectious agents, like *P. aeruginosa*, as a contaminant in poultry products. The research was also meant to aid in the development of an antibiogram of isolated strains to highlight the impact of over-use of antibiotics in farm settings and to identify the severity of the risk to which the producers and consumers of poultry products are exposed.

2. MATERIALS AND METHODS

2.1. Study Area and Sample Collection

The research was carried out in Karachi, Pakistan. Five meat markets from five districts in Karachi, Pakistan, namely Karachi East, Karachi West, Karachi Central, Karachi South, and District Malir, were randomly featured. In total, 100 samples of broiler chicken meat, 20 from each area, were randomly purchased from the meat shops in meat markets, and subsequently pooled. Briefly, five chicken meat pieces were taken randomly from each market, and samples of chicken meat were collected with sterile swabs, separately packaged, and sent within 2 to 4h to the research laboratory of the Department of Biosciences, Faculty of Life Sciences, SZABIST. In this laboratory, all microbiological investigations were undertaken.

2.2. *Pseudomonas aeruginosa* Isolation and Identification

Using a homogenizer, samples of broiler chicken meat were placed in 1ml Eppendorf tube containing one ml phosphate-buffered saline (PBS) and a 5-mm steel bead (Qiagen #69989) for 5 minutes at 30 Hz (Retsch; MM400). The meat homogenates were then plated on Cetrimide Agar and then allowed to incubate overnight at 37 ℃. Biochemical tests confirming the presence of *Pseudomonas aeruginosa* colonies were found positive for indole and negative for Simmons citrate. Twenty-two out of a hundred samples from all the districts were found to be positive for *Pseudomonas aeruginosa*, and isolates with visible colony morphologies were chosen for further examination.

2.3. Biochemical Identification of *Pseudomonas aeruginosa* isolates:

On cetrimide agar plates, the putatively *Pseudomonas aeruginosa* colonies were streaked for biochemical confirmation. For additional phenotypic screening using the Quick Test Strip (QTS)-24 kit, conventional biochemical assays such as Gram staining, indole, catalase, citrate, oxidase, and motility were done on chosen pure *Pseudomonas aeruginosa* colonies.

2.4. Bacterial Growth and Media Conditions

For long-term preservation, bacterial isolates were kept in 20 % glycerol at -80 ^{o}C. From frozen stocks, isolates were inoculated onto Tryptic soy agar (TSA; Becton Dickinson) and incubated at 37 ^{o}C for 18 h. A single colony was inoculated into 5 mL

of LB broth and cultivated overnight at 37 °C with 200 rpm of shaking. All subsequent tests utilized overnight cultures.

2.5. Phenotypic Antibiotic Resistance Pattern

To determine antibiotic resistance among *Pseudomonas aeruginosa* strains, the Kirby Bauer disc diffusion method was used in accordance with CLSI recommendations. To determine which antibiotics would be effective against each potential positive isolate, a panel of 11 antibiotics was used. For the test, we took one colony of the *Pseudomonas aeruginosa* isolates from plates of cultures grown overnight established on Cetrimide Agar (Oxoid, Basingstoke, United Kingdom) and transferred it to plates of nutrient agar, where it was incubated at 37 °C for 24 h. *Pseudomonas aeruginosa* colonies were then emulsified into sterile saline until the turbidity reached 0.5 McFarland standard which is correspondent to 10^8 CFU/ml. Mueller Hinton (MH) agar plates (Oxoid, Basingstoke, United Kingdom) were prepared by spreading suspensions with sterile cotton swabs, and antibiotic discs were placed aseptically on the surface of the MH agar with sterile forceps; the plates were then incubated at 37 ℃ for 24 h. The widths of the inhibitory zones were measured and recorded; the values were then interpreted following CLSI guidelines.

2.6. Multiple Antimicrobial Resistance Indices (MARI)

The multidrug resistance level was enumerated using the multiple antibiotic resistance indices (MARI) as per the formula defined by the previous author [15].

Where a = total number of antibiotics to which an isolate shows resistance and b = total number of antibiotics to which the isolate was exposed.

2.7. Detection of Biofilm Production

Microtiter plate test was used to determine the biofilm-forming phenotype (MPA). There was a 100-fold dilution of the fresh cultures into tryptic soy broth. After that, the 96-well (flat bottom) plate was loaded with 250 µL aliquots of isolates and left to incubate at 37 °C overnight. Agitating and shaking the wells during washing helped get rid of any bacteria that had not properly detached. Because biofilms are so easily fixed by heat, the plates were heat dried to achieve this. Afterward, a crystal violet (0.1 %) stain in 250 µL was applied and left to set for 20 minutes. Once again, washed and dried the wells before treating them with 250 µL of 50 % acetone. The un-inoculated wells still filled with sterilized tryptic soy broth served as negative controls. The biofilms' OD values were evaluated at 594 nm using an ELISA reader from (BioRad, USA). In addition, a threshold value (ODc) was calculated as follows:

According to Saxena *et al*. a lack of opacity was observed in non-biofilm producers (OD<ODc), a lack of opacity was observed in weak producers (ODc<OD<2×ODc), a lack of opacity was observed in moderate producers (2×ODc<OD<4×ODc), and a lack of opacity was observed in strong producers of biofilm (4×ODc<OD).

3. RESULTS AND DISCUSSION

3.1. Bacterial Identification, Biochemical, and Growth Characterization

A total of 22 morphologically distinct colonies of non-lactose-fermenting bacteria were identified as *Pseudomonas aeruginosa* as shown in Table 1. The remaining 78 strains were found to be of *Serratia fonticola, Pseudomonas gessardii, Pseudomonas mucidolens, Lysinibacillus fusiformis, Pseudomonas stutzeri, Bacillus aryabhattai, Pseudomonas viridiflava,* and *Bacillus megaterium.*

3.2. Prevalence of *Pseudomonas aeruginosa* and the Multidrug Resistance Profile in Broiler Chicken Meat

The overall prevalence of *Pseudomonas aeruginosa* positive samples after isolation was 22 %. MDR profile revealed that the strains were highly resistant to 5 out of 11 antibiotics as shown in Figure 1. Table 2 presents the antimicrobial resistance profile of the isolates to the antibiotics and multiple antibiotic resistance Index (MARI) estimated as 0.45 for all of the MDR isolates (ranging from 0.2 to 0.5). Cloxacillin, Teicoplanin, Ciprofloxacin, Imipenem, and Meropenem showed 100 % resistance, followed by Linezolid, Streptomycin, Amikamicin, Aztreonam, Compound Sulphonamide, and

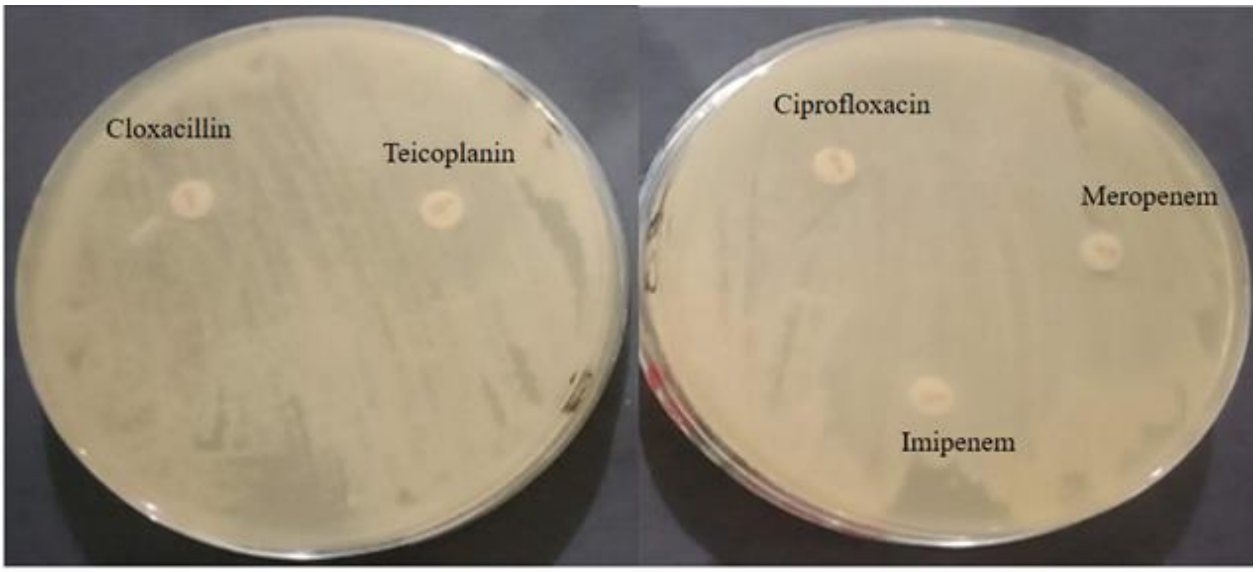

Fig. 1. Antimicrobial activity of *Pseudomonas aeruginosa* against antibiotics (Cloxacillin, Teicoplanin, Ciprofloxacin, Imipenem, and Meropenem) showing complete resistance.

Table 1. Sampling data of meat samples collected from different districts of Karachi

Districts of Karachi	Total Sample Collected	*Pseudomonas aeruginosa*	Positive Percentage
East	20	5	25 %
West	20	2	10 %
South	20	4	20 %
Korangi	20	6	30 %
Central	20	4	20 %

Table 2. Prevalence of AMR pathogens in livestock-sourcefood products

Antibiotics	Zone of Inhibition (mm)
Cefepime (Fep)	18
Compound Sulphonamide	15
Cloxacillin	6
Imipenem	4
Teicoplanin	6
Ciprofloxacin	6
Aztreonam	16
Meropenem	7
Linezolid	11
Amikamicin	14
Streptomycin	11

Multiple Antibiotic Resistance Index (MARI) *for the above antibiotics is found to be* ***0.45.*** *Zone of Inhibition (ZOI): 0-10 mm: Resistant; 10.1-16 mm: Intermediate; 17+ mm: Sensitive.*

Cefipime which showed intermediate resistance.

4. DISCUSSION

In this study, we compared the antibiotic resistance profile of *P. aeruginosa* that was isolated from poultry sources from 5 different districts of Karachi. There were a total of 100 samples were collected, and there were 22 % of the isolates showed the presence of *P. aeruginosa*. After antibiotic resistance profiling we found that strains exhibited high levels of resistance to five of the eleven antibiotics tested. MDR isolates were calculated to have a (MARI) of 0.45 overall (ranging from 0.2 to 0.5) All strains showed resistance to Cloxacillin, Teicoplanin, Imipenem, Meropenem and Ciprofloxacin but showed intermediate resistance towards Linezolid, Streptomycin, Amikamicin, Compound Sulphonamide, Aztreonam and Cefipime.

Antibiotic uses excessively in poultry to accelerate the development of broiler chicken is causing the spread of MDR *P. aeurginosa*, a harmful form of bacterial resistance, as evidenced by significantly greater numbers of MDR isolates. Isolates exhibit resistance to types of antibiotics, which contributes to their progression toward MDR status. The study's authors conclude that the widespread prevalence of multidrug-resistant *P. aeruginosa* strains in chickens poses serious risks to human health. Preventative actions that can be taken to reduce the transmission of MDR *P. aeruginosa* from chicken to humans include the proper management of raw products and the boiling of meat to sterilize it. These precautionary actions can be implemented to lessen the likelihood that multidrug-resistant *P. aeruginosa* will be transferred from chickens to people. The presence of biofilm promotes the growth of bacterial communities that are resistant to biocides and antibiotics [16]. Antibiotic resistance was caused by multiple factors, including biofilm formation, metabolic processes within in the biofilm, efflux pumps, and even outer membrane structures that prevented antimicrobials from penetrating the biofilm. Changes in bacterial phenotypic, gene expression, antibiotic resistance, and metabolic activity that result in the generation of virulence-associated proteins are all hallmarks of biofilm-forming microorganisms [17]. Significant economic losses as a result of deterioration, illness epidemics, and even deaths are caused by biofilm-producing bacteria's impact on the cattle and food industries [18]. *Pseudomonas*, along with *Clostridium, Campylobacter, Bacillus, Salmonella, Staphylococcus, Acinetobacter, Listeria, Acinetobacter, Klebsiella, Enterococcus, E. coli*, & *Aeromonas spp*. are a major health risk in chicken farms [19]. Maintenance and raising standards for livestock health require a reduction in the transmission of infectious diseases among livestock. High biosecurity standards are used to attain this goal, along with several preventive strategies that work to regress the prevalence of infectious pathogens and, as a result, decrease the requirement for the overprescription of antibiotics [20]. High biosecurity measures will reduce antibiotic use and preserve the efficacy of traditional antimicrobials for acute or chronic illnesses [18]. Similarly, the improper use of antimicrobials in farm animals contributes to the evolution of antimicrobial resistance [21]. Controlling the invasion of pathogens and their subsequent spread is the primary goal of biosecurity. They are also "important factors" in lowering infection rates, boosting agricultural output, and reducing antibiotic consumption. Experts have also proposed regular animal testing for different illnesses, accompanied by quarantine of diseased animals [22].

The primary risk factors for contamination of the environment in poultry farming with pathogenic bacteria that produce biofilms are contact with chicken feed, plants, pipelines, air, utensils, contact surfaces, and equipment. Furthermore, there are numerous points of entry for contamination of chicken products like meat and eggs along the food chain, from production to processing to distribution to retail to handling to preparation.

5. CONCLUSION

In conclusion, the findings of this study emphasize the importance of biorisk management and antibiotic susceptibility pattern of *Pseudomonas aeruginosa* biofilm-forming bacteria found in broiler chicken. The presence of antibiotic-resistant strains of this species highlights the need for further research to understand the mechanisms of resistance and for the development of procedures to slow the spread of bacteria resistant to many drugs. These strategies should include measures such as the implementation of appropriate hygiene and sanitation practices,

the careful use of antibiotics, and the adoption of antibiotic stewardship programs. To safeguard public health by lowering the risk of zoonotic illnesses, it is particularly critical to monitor the overall antimicrobial susceptibility of *P. aeruginosa* strains from animals, notably poultry.

6. CONFLICT OF INTEREST

The authors declared that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Proceedings of the Pakistan Academy of Sciences: B
Life and Environmental Sciences 60(3): 505-513 (2023)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(60-3)875

Pakistan Academy of Sciences

Research Article

Space-borne Air Quality Monitoring of Nitrogen dioxide (NO_2) over Karachi and Lahore using Remote Sensing Tools

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Abstract: In this study, we used Sentinel-5P TROPOMI satellite data to examine the NO_2 and gas concentrations in the cities of Lahore and Karachi, Pakistan, and to use environmental valuation methods that focus on air quality problems. Furthermore, the causes and main sources of NO_2 are discussed with its effect on the environment and the health of humans. This study examines the correlation between the tropospheric NO_2 collected from the recently launched Sentinel-5 Precursor, a low-earth-orbit atmospheric mission dedicated to observing air pollution and outfitted with the spectrometer TROPOMI (Tropospheric Monitoring Instrument). The average amount of NO_2 that was gathered between May 2018 and May 2022. The results showed higher levels of NO_2 concentrations were recorded in both, Karachi and Lahore. The concentrations exceed the WHO standard levels for NO_2 in ambient air. The NO_2 concentrations in Karachi ranged from 3.0e-6 mol/m² being the minimum average concentration to 4.0e-1 mol/m^2 being the maximum concentration. However, in Lahore, the minimum average value of NO_2 was ranging from 4.0e-5 mol/m^2 to 5.5e-1 mol/m^2 as the maximum average, which was higher than the minimum and maximum values of Karachi. The study also revealed that the NO_2 concentrations measured for both cities were higher than the WHO's yearly limit threshold, which is 53 ppb/year. Thus, it was crucial to take action to address this issue before it poses a severe risk to the local people. This study's identification of the key regions with the greatest NO_2 concentrations will aid in understanding the significance of satellite data for monitoring NO_2 concentration. Thus, the originality of the study lies in the fact that using the example of Karachi and Lahore, the dynamics of the deterioration of the environmental situation was revealed, and the main reasons for what was happening were also established. In this case, an available tool was used - remote sensing tools. The competent authorities can assist this study in managing and regulating the air quality in the most densely populated areas.

Keywords: Metropolis, Pollutants, Ecological air state, Public health, Sentinel 5p, GIS

1. INTRODUCTION

The problem of air pollution is getting worse as the world's population is growing so [1-3]. Urbanization, energy use, transportation, and motorization are some of the major contributors to air pollution. The environment's quality and people's health are also negatively impacted by population increase and exposure to air pollution [4]. Urbanization and transport have a negative impact on public health, air quality and, due to rapid population growth, contribute to global warming [5, 6]. Air pollutants

Received: March 2023; Revised: May 2023; Accepted: July 2023
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are usually caused by industrial facilities and other activities. [7]. Nitrogen dioxide (NO_2), one of the worst air pollutants, is primarily produced when fossil fuels are burned, particularly in the exhaust emissions from moving vehicles. Satellite remote sensing data has been used to track air pollution over time [7]. In cities with large populations where CO, CH_4, NO_2, particulate matter $PM_{2.5}$ and PM10, as well as ozone and other gases, contribute to the deterioration of the population's health situation due to the occurrence of cardiovascular diseases, respiratory diseases, and even fertility diseases. These diseases affect people of all ages, including children, which has been confirmed by various studies on the relationship between road and industrial pollution and these diseases [8]. As a result, it is essential to continuously and accurately monitor the air quality in order to reduce the impact of air pollutants and ensure that modern discharges are given in accordance with administrative requirements [1].

Different ground-based and satellite-based observing techniques are used as a result. Due to a number of considerations, remote sensing or satellite-based monitoring has an advantage over conventional estimations and ground-based techniques [1]. In order to secure data about the Earth, such as land and sea surface temperature, vegetation cover, air quality, and even to predict and assess catastrophic events like wildfires, remote sensing techniques and GIS are suitable [1]. Sentinel-5P was used for this study of space-borne air quality monitoring of nitrogen dioxide (NO_2) [9]. The Sentinel-5P satellite mission, also known as the Sentinel-5 Precursor, was launched on October 13, 2017 [9-11]. The Sentinel-5P mission, a single-payload satellite in low Earth orbit, is the first in a line of atmospheric observation systems within Copernicus, the European Union's programme for Earth observation with the primary goal of examining the composition of the Earth's atmosphere [9].

The European Space Agency operates various Earth observation satellites that are available under the Copernicus program. These satellites are used to map and monitor the Earth's chemical and physical changes [7]. Sentinel, one of the largest Earth monitoring programmes, uses a variety of satellites. The TROPOMI (Tropospheric Monitoring Instrument) spectrometer on the Sentinel-5p measures CO, CH_4, NO_2, O_3, HCHO, as well as SO_2 in various wavelength ranges (short-wavelength infrared, near, visible, and ultraviolet [12, 13]. Through the Copernicus Open Access Data Hub, all data, including offline and reprocessed data, is publicly available [7]. The former study [14] analyzed, using the ozone-monitoring instrument (OMI) dataset 2004-2008, the spatiotemporal variability of monthly averaged vertical tropospheric columns (VTCs) of NO_2 over Pakistan. A study compared the air pollutants of smog near the border and other sites in Lahore [15]. This research work was performed based on space-borne air quality monitoring of nitrogen dioxide (NO_2) over Karachi and Lahore using remote sensing tools. Karachi and Lahore of Pakistan are two metropolitan areas with their own unique culture. Karachi is often referred to as a mini-Pakistan as it is Pakistan's largest populous city due to the significant influx of migrants. Lahore is the capital of the Punjab province with a rich history. Both cities are rapidly developing and are the center of technological progress, which certainly affects the environmental performance of the environment.

Up-to-date, very limited research work has been done on NO_2 concentration in the mega cities of Pakistan. Such work on NO_2 has not been done in the mega cities including Karachi and Lahore, Pakistan by using remote sensing yet. The Sentinel 5p mission has been used for the collection of data. Furthermore, the results are analyzed and displayed using Arc Map in the research paper. Therefore, the current study's purpose was to investigate the NO_2 pollution spatiotemporal patterns and the data availability for the operational TROPOMI NO_2 product over Karachi and Lahore. Based on the findings, concentrations of NO_2 were determined. Seasonal variations were analyzed to assess the status of nitrogen dioxide (NO_2) using Sentinel 5p data.

2. MATERIALS AND METHODS

2.1. Study Area

The two research areas that were chosen for this study are both in Pakistan. Karachi and Lahore as shown in Figure 1, two cities in Pakistan, are among the sites. These cities were chosen because they

represent the majority of Pakistan's large, highly populated cities. Pakistan, one of the most populous nations on earth, is home to several megacities. Karachi is situated on a shoreline between the Arabian Sea (AS) and the continent of South Asia (SAC), which is known for its sea-land breezes and dominant northeast and southwest winds [16]. Due to this, Karachi, a typical coastal metropolis, is vulnerable to a variety of air pollution sources that are both terrestrial and oceanic in origin [16]. Lahore, Pakistan's Punjab Province's most populous city, has a total area of 1772 km^2 [17]. From May to September there is a hot, rainy summer and from November to February there is a cold, dry winter in Lahore [18]. Since it is a semi-arid region, pollution from industry, work, and transportation is a major cause of a number of environmental issues [19].

2.2. Materials and Mapping

Data from the TROPOMI device of the SENTINEL-5P satellite mission, which monitored nitrogen dioxide levels for the Pakistani area, were used. Through a number of websites, data gathered during the SENTINEL-5P mission is freely available and can be downloaded without charge. Table 1 shows the different properties of Sentinel 5p. Data collected from May 2018 to May 2022, on a daily basis, was downloaded and processed. The methodological network was indicated in Figure 2.

2.3. Data Acquisition

The desired study area, for which data was needed to be downloaded, can be drawn or designated with a point, rectangle, or polygon on the interface for showing and downloading data. The time window during which the data must be downloaded is an optional choice. Additional filters can be chosen, including those for product type and processing technique. First, a rectangle designates the region of Pakistan's territory. In the event that a specific portion of the boundary data is lost during processing, a little broader region than the default

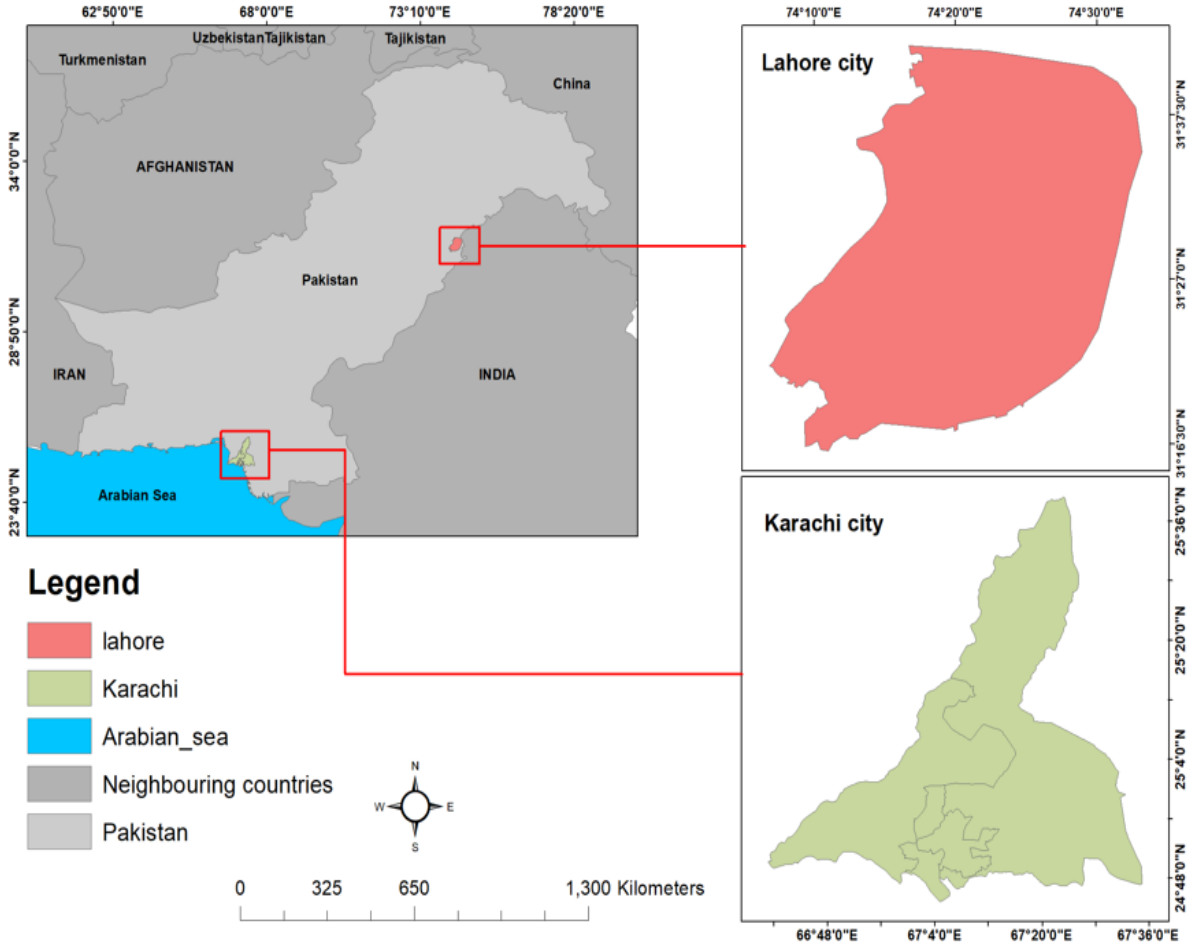

Fig. 1. Study areas, Karachi and Lahore

Table 1. Properties of Sentinel 5p

Spectral band		Spectral coverage, nm	Aperture width, km	Spectral res, nm	Time resolution	Spatial res, km2
Ultraviolet	1	270 - 320	2600	0.49	daily	7 x 28
	2					
Visible	3	320 - 495		0.54		7 x 3.5
	4					
Near-infrared	5	675 - 775		0.38		
	6					
Shortwave infrared	7	2305 - 2385		0.25		7 x 7
	8					

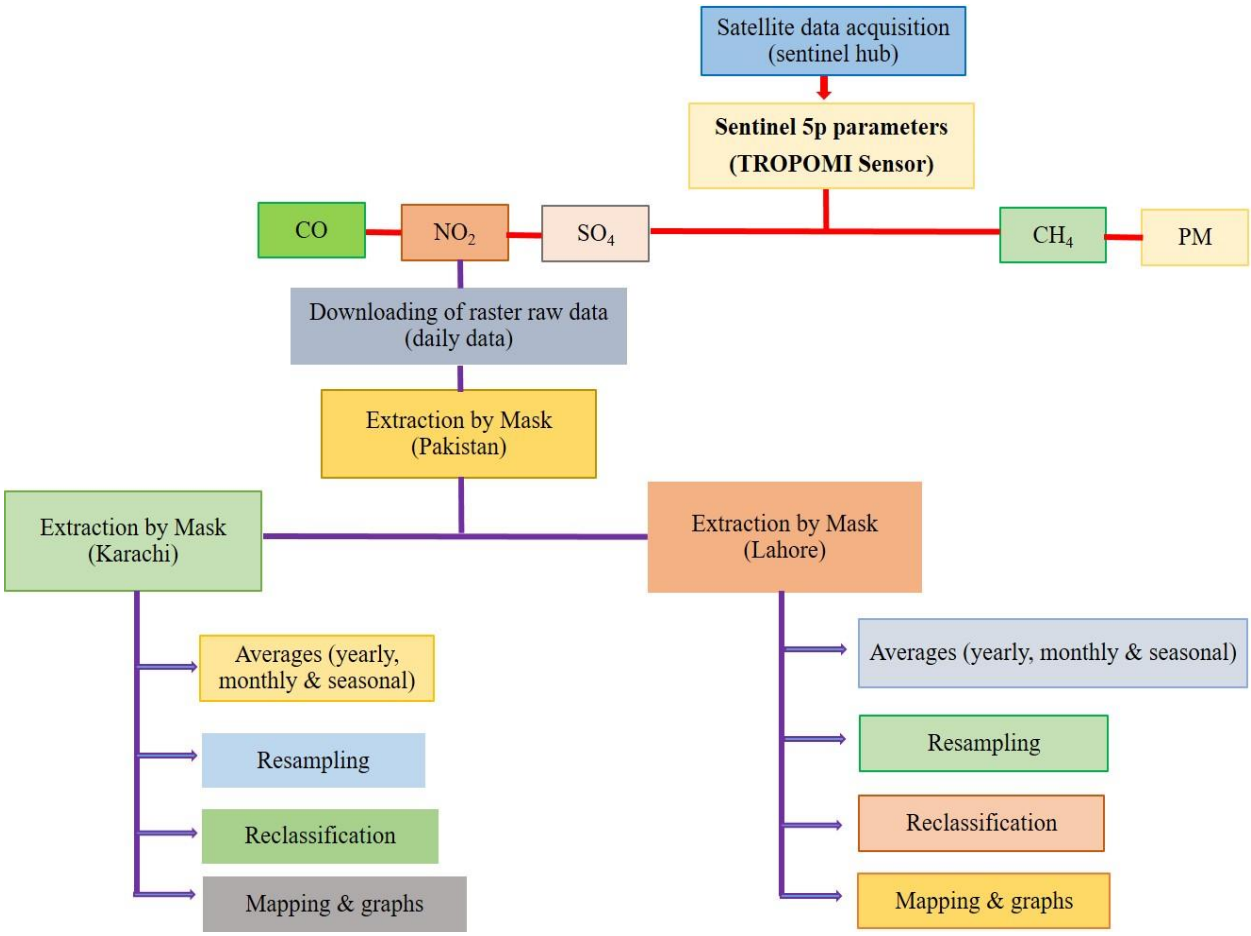

Fig. 2. Methodological network

one is drawn. Data on a daily basis was downloaded from May 2018 to May 2022, and the time span for each individual file was chosen in accordance with this. Every piece of information is correctly geo-referenced, shown using the correct coordinates for the WGS84 (World Geodetic System 1984) coordinate system, and converted into raster TIF format.

2.4. Data Processing

Pakistan's border is first removed from the layer of all administrative borders during data processing. In order to accomplish this, the Pakistan boundary was chosen and stored in certain individual layers, keeping in mind that alone certain objects should be kept. After that, just the data that was located on

the territory of Karachi and Lahore was obtained by cutting all other vector layers to each individual boundary layer of Karachi and Lahore, using the spatial operation crossing. The Clip raster by mask layer operation was used to remove the raster layer. Averages were calculated to cover the missing locations and smooth the data.

2.5. Presentation of Data

Mapping was done of the averaged data maps for monthly, seasonal, and yearly data. For mapping, maps were classified into twelve classes according to their highest and lowest values overall. This way, a legend can be prepared to designate different colours for different values of NO_2 concentrations in both cities as shown in Figure 3.

For making graphs, python coding was used to collect the minimum and maximum concentrations of the map automatically and produce an Excel sheet. These graphs helped in demonstrating the rising or falling trend of the CO concentrations over the year or month. The amount of atoms per surface is expressed in the data's unit of measurement, which is mol/m^2. Finally, the graphs were plotted by using the mean values of the four seasons from the year 2018 to 2022.

3. RESULTS

The results are described for seasonal nitrogen dioxide concentrations over the city of Karachi and Lahore, for the years, 2018 to 2022. Besides, the results of this research study are divided into three major parts such as results of Karachi, the results of Lahore, and the causes and impacts posed by nitrogen dioxide pollutants, and the discussion for each of them is done in accord with it and completely fulfills all the objectives narrated above.

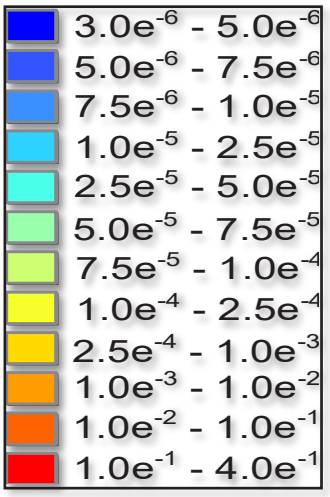

Fig. 3. Legend used in maps

3.1. Karachi Seasonal Average

The NO_2 concentrations for Karachi were assessed through the data collected from Sentinel 5p. The results displayed much higher concentrations than the standards set by WHO or NAAQS, which was 53 ppb. Using NO_2 satellite data products, the NO_2 air pollutant around Karachi city has been examined on a seasonal basis winter (DJF), spring (MAM), summer (JJA), and autumn (SON)) from 2019 to 2022. Seasonal changes have a great impact on the concentrations of the pollutants. Therefore, they must be examined seasonally to assess their scale of risks and impacts.

The map in Figure 4 shows the seasonal mean average NO_2 surface concentration varies from $6.42e^{-6}$ to 0.037 mol/m^2 overall. Concentrations are seen high in winter and summer due to the accumulation of pollutants in atmosphere. However, lower concentrations are seen in spring and autumn, which are the seasons of wind and rain. The graph in Figure 5 shows a uniform shift in values from winter and summer to spring and autumn respectively. This sudden concentration shift between the seasons is due to the different atmospheric conditions in all four seasons. The study reported that Karachi has shown the maximum and minimum mean monthly average values of NO_2 by 11.33×10^{15} molecules/cm^2 and 0.98×10^{15} molecules/cm^2, accordingly, furthermore, the annual increasing concentration of NO_2 was noted at 3.29 % [14].

3.2. Lahore Seasonal Average

The second study area, Lahore, was assessed for its NO_2 concentrations from May 2018 to May 2022. The data acquisition was done through Sentinel 5p and later assessed in yearly, seasonal, and Monthly manner. Lahore is a well-known city for its smog during winter. The major contributor to this smog is NO_2 which reacts with sunlight and produces smog. The results below show that the concentrations of NO_2 in Lahore are higher than the WHO International standards for NO_2. Our results are in line [20].

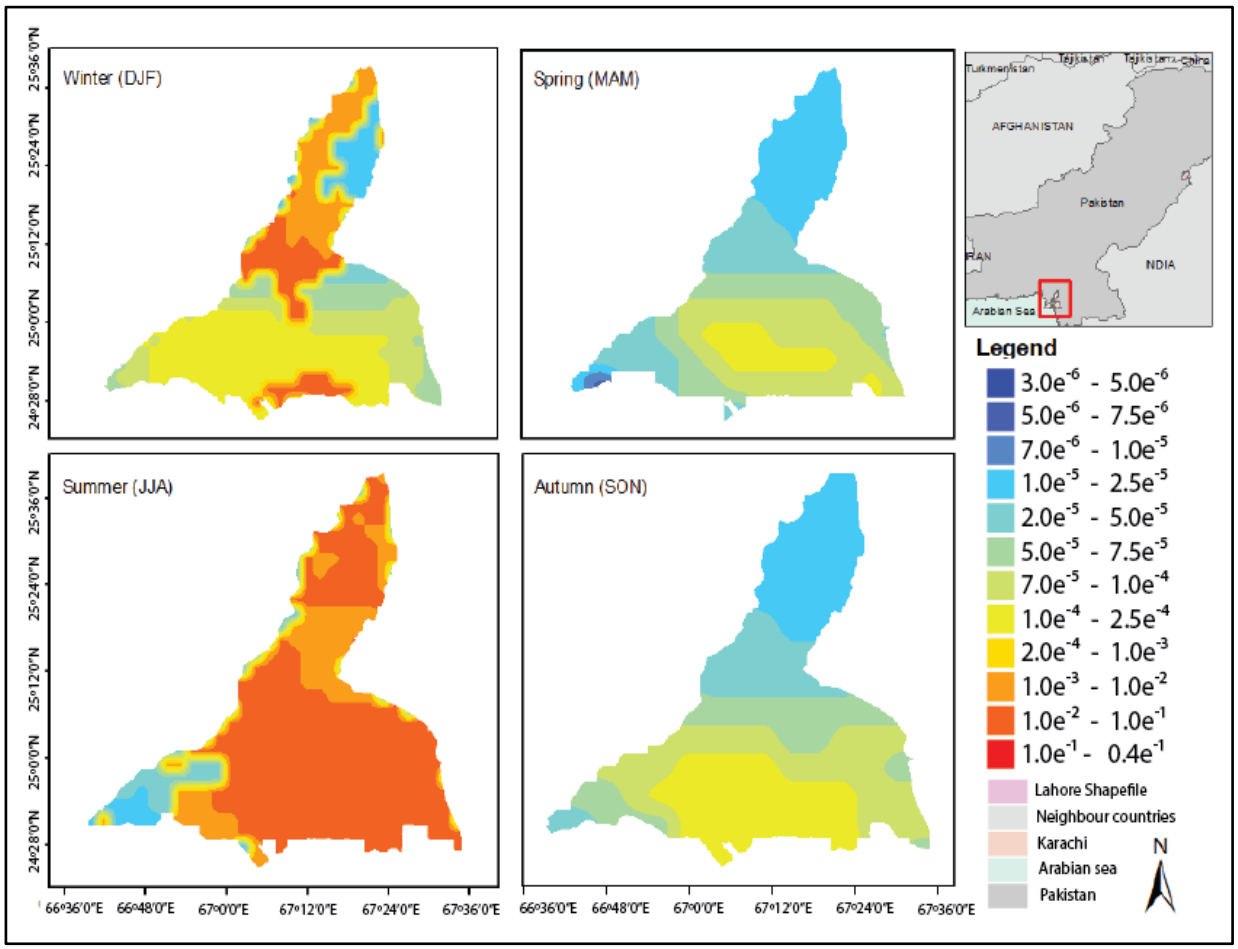

Fig. 4. The seasonal average concentrations of NO_2 in Karachi for last five years (2018 – 2022) in mol/m^2

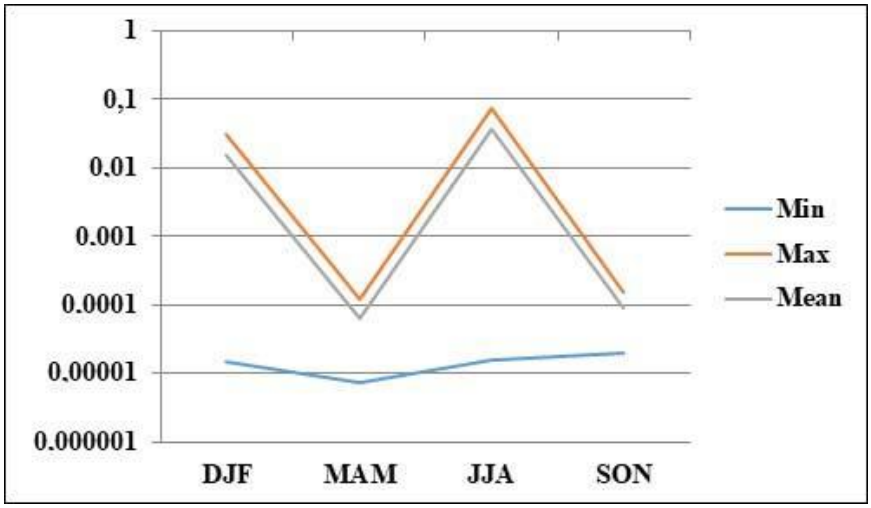

Fig. 5. The logarithmic graph of seasonal average concentrations of NO_2 in Karachi for the last five years (2018–2022)

The NO_2 air pollution in the area of Lahore city has been studied from 2019 to 2022 on a seasonal basis winter (DJF), spring (MAM), summer (JJA), and autumn (SON). Pollutant concentrations are greatly impacted by seasonal fluctuations. As a result, they must be evaluated every season to gauge the severity of the dangers and effects. As it was obvious, the concentration of NO_2 was higher

in winter, which cause the formation of smog in the Punjab province. The map in Figure 6, shows the seasonal mean average NO_2 surface concentration varies from 5.5 e^{-5} to 0.6 mol/m^2 overall. Due to the accumulation of pollutants in the atmosphere, concentrations are higher in the winter and summer. However, the windy and rainy seasons of spring and fall show lesser quantities. The burning of fossils, waste from crops and agriculture, and burning in industries and vehicles contribute to these high-level NO_2 concentrations which result in environmental and human health degradation. Some researchers [15] observed a higher concentration of NOx, whereas SO_2 concentration was found to be lower in the air as compared to national environmental quality standards (NEQS).

The data in Figure 7 demonstrates a consistent change in values from winters and summers to, respectively, springs and autumns. Due to the various atmospheric conditions in each of the four seasons, there is a dramatic concentration change between the seasons. Previously, the researchers [21] performed a case study, and, as a consequence, significant NO_2 concentrations could be observed for high-density housing estates (like high-rise buildings) and food businesses.

4. CONCLUSION

Pollution associated with road traffic and transport is a problem in both developed and developing countries, showing increased levels of NO_2 pollution in the air. At the same time, such areas are more extensive compared to industrial and urban areas, which requires additional modelling of the development of the situation. Since the COVID-19 epidemic began, NASA has published findings showing that the COVID-19 lockdown measures have resulted in a drop in NO_2 concentrations. According to numerous additional researches, the limited human activity during the lockdown led to a considerable decrease in surface NO_2 emissions in cities and megacities. Thus, the originality of the study lies in the fact that, using the example of Karachi and Lahore, the dynamics

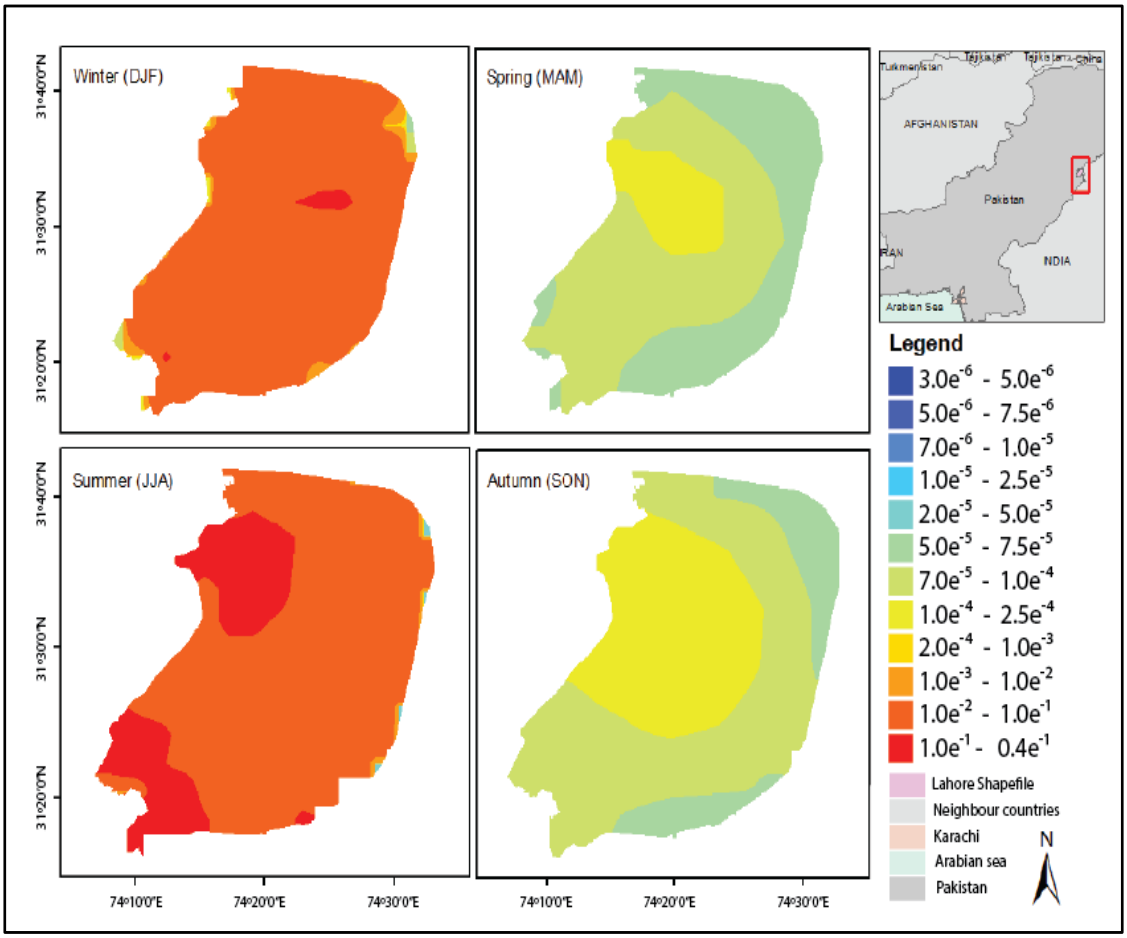

Fig. 6. The seasonal average concentrations of NO_2 in Lahore for last five years (2018 – 2022) in mol/m^2

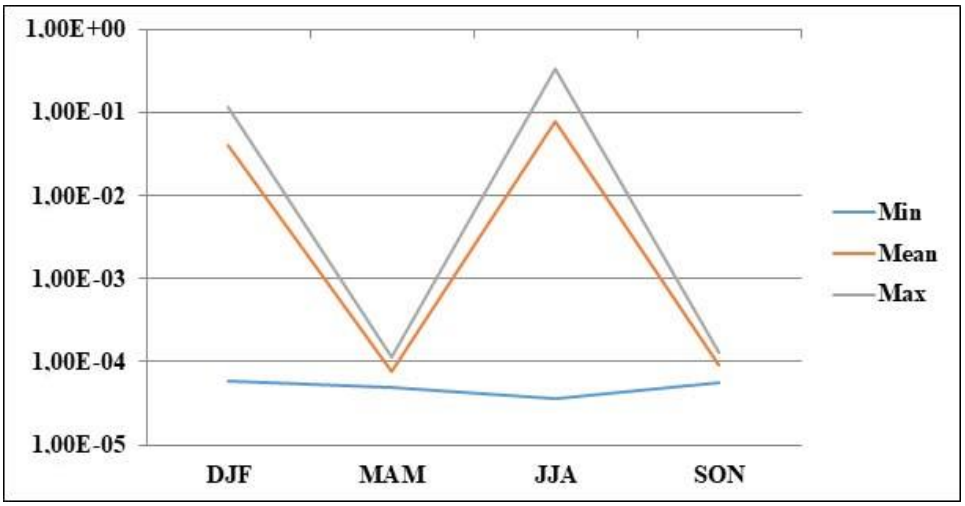

Fig. 7. The logarithmic graph of seasonal average concentrations of NO_2 in Lahore for the last five years (2018 – 2022)

of the deterioration of the environmental situation was revealed, and the main reasons for what was happening were also established. In this case, an available tool was used - remote sensing tools. Government and policy makers are encouraged to take into account the results of the current study when developing NO_2 emission reduction and air pollution management plans.

5. CONFLICT OF INTEREST

The authors have no relevant financial or non-financial interests to disclose.

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Proceedings of the Pakistan Academy of Sciences: B
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ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(60-3)882

Pakistan Academy of Sciences

Research Article

Amending Soil with Rhizobium carrying Biochar Ameliorates Drought Stress on *Phaseolus vulgaris*

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Abstract: As a consequence of climate change/global warming earth's agriculture output is under rigorous stress. There is a growing need to develop strategies to cope with these abiotic stresses. Biochar exhibiting many beneficial qualities appeared to alleviate these problems by improving soil fertility by adding carbon and preventing nutrient losses etc. Biochar can also enhance BNF and could be used as a carrier for rhizobium by providing a suitable microenvironment. The current study is aimed to find the ameliorative potential of different biochar types to be used as rhizobium carriers for *Phaseolus vulgaris* L. exposed to drought stress. Both types of biochar were analyzed for physico-chemical and morphological parameters. Presence of Silicon content remains the key finding for rice husk biochar which was absent in *Lantana* biochar. Increased C, K, and Ca weight percentages were found in *Lantana* biochar as compared to their proportions for rice husk biochar. On the contrary, the oxygen content was higher in rice husk biochar as compared to that in *Lantana. Phaseolus* seeds were used for the pot experiment where stress treatment was applied by FTSW (Fractionable Transpirable Soil Water) technique. One isolated strain along with two types of biochar carrier was applied to the plants in combination with water stress treatment. Plants were analyzed for growth and physiological parameters including plant height, leaf area, biomass, photosynthesis, transpiration rate, stomatal conductance, and water use efficiency, where rice husk biochar responded better than the one obtained from *Lantana.* Plants responded positively for all the growth as well as physiological parameters when treated in combination with the inoculum for both stress levels i.e., 100 % and 60 % field capacity F.C. The present study advocates rice husk biochar for its ability to enhance tolerance in *Phaseolus* against drought stress through its role as an inoculum carrier contributing suitable habitat for the microorganism.

Keywords: Biochar, *Phaseolus vulgaris,* Rhizobium carrier, drought, arid climate

1. INTRODUCTION

Sustainability of global agriculture is inherently linked with climatic transformations. Shift in weather patterns, rainfall intensity, and frequency has diverse effects on ecological systems especially soil health [1]. These worldwide environmental changes are a matter of great concern due to their potential effects on our future ecosystems [2] and demand thoughtful learning along with the quantification of the anthropogenic processes involved [3]. Fahad *et al.* [4] have emphasized on employing various mitigation strategies to combat climate change. The prolonged drought can pose a wide range of abiotic stresses for plants [5–6]. Therefore, plants exhibit a vast range of morphological and physiological variations in response to such water-deficit situations [7]. The plant regulates its diverse mechanisms ranging from leaf area reduction to root/shoot ratio increase, stomatal conductance, or osmotic adjustment [8–10] to cope with the water stress condition.

Many rhizobial strains show tolerance towards certain stress effects thus forming effective (N_2-fixing) symbiotic association with their host legumes under heat, salt, and acid stress environments. However, the stress tolerance

Received: March 2023; Revised: May 2023; Accepted: June 2023
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levels vary with strains depending on their natural habitats. It becomes difficult for rhizobium to infect root hairs at temperatures greater than 40 °C, while nitrogenase activity also decreases even at 35 °C [11]. Similarly, nitrogen assimilation was also found to be limited by low temperatures [12]. To sustain the rhizobia in the soil different carrier materials have been used for the inoculation of legumes with reasonable beneficial results in protecting the bacteria and enhancing the survival of inoculants for longer durations. The use of organic carriers offers additional benefits over chemicals of physical carriers of nutrient provision to the soil.

Biochar could be used as an imperative intervention for increasing soil organic pool for the reason of organic matter deficiency being considered as a major factor limiting plant growth. In addition to this, biochars from different feedstocks exhibit divergent effects on soil fauna diversity, activity, and transport owing mainly to the indirect changes in the chemical properties of soil. Studies have documented positive influences of biochar addition on soil productivity enhancing nutrient uptake, water retention capacity and soil structure, etc. [4,13]. Biochar could also be employed in the reduction of soil acidity by exploiting its alkaline nature [14–15] and could also benefit alkaline soils by utilizing some modification techniques [16]. Biochar augments nutrient availability thus being effectively engaged in getting nutrient uptake benefits increasing the soil pH, P, K, and Ca whereas subsiding free Al [17–18]. Owing to its high porosity and surface-to-volume ratio it easily captures exchangeable cations.

Biochar has been found to be used for the improvement of soil fertility and simultaneously it can also create an opportunity to carry plant-growth-promoting rhizobacteria (PGPR). Its unique characteristics are responsible for making it conducive for being used as an inoculum carrier. Rondon *et al.* [19] have obtained increased BNF results by common beans with increasing rates of biochar additions.

The bean crop is generally grown in rain-fed conditions therefore drought stress could be a common problem. The problem is further exacerbated if the soil is shallow and low in essential nutrients and organic matter [20–21]. Nitrogen fixation by common beans has been found to be limited by environmental stresses like drought and adverse soil conditions. It becomes difficult for rhizobium to infect root hairs at temperatures greater than 40 °C, while nitrogenase activity also decreases even at 35 °C [22]. Moisture stress has been observed to have a greater impact on nodulation and nodule activity of legumes with varying adaptabilities at rhizobial strains level. In this context the different carrier materials have been used for the inoculation of legumes with reasonable beneficial results.

Rhizobium-legume compatibility is very much significant for getting successful inoculation benefits. If any legume crop is grown for the first time in certain area, there is a possibility that rhizobium strains which are compatible with that specific legume may not inhabit that soil. Sometimes many rhizobia already exist in soils, but often these rhizobia are fewer in number or not effective enough for attaining desired nitrogen fixation and increased yield. Such problems can be alleviated by inoculating with compatible strains of rhizobium and spectacular expected yield increase benefits can be achieved.

Keeping in view the multiple benefits of biochar we want to manipulate and optimize biochar properties as an inoculum carrier for certain rhizobial strains which have not been fully acknowledged in the past. The current research aims to evaluate the suitability of biochar as rhizobial carrier material. The present study has been planned to apply biochar (obtained from two different feedstocks) as a rhizobium carrier for *Phaseolus vulgaris* L. to achieve the following objectives. 1) Comparative assessment of different types of biochar (prepared from rice husk and *Lantana*) as an improved type of inoculum carrier specifically for *P. vulgaris* L. and 2) Comparison of the growth and physiological responses of *P. vulgaris* L. grown in drought stress under different biochar treatments.

2. MATERIALS AND METHODS

Biochar from two different feedstocks (i.e., Rice Husk and *Lantana camara* L.) was used as inoculum carrier for *P. vulgaris* L. to cope against drought stress.

2.1. Isolation of Rhizobial Strains

Few healthy plants of *P. vulgaris* L. were brought from Molen Gol valley near Chitral city, KP province, Pakistan to the laboratory for isolation of rhizobium strains. The collected specimens were compared with the herbarium collection at Herbarium of the Quaid-i-Azam University Islamabad (ISL) and the Herbarium of Pakistan Museum of Natural History Islamabad. A voucher specimen was also deposited in the Herbarium (ISL) against the Herbarium collection Number: ISL-A573 (This is available in the Angiosperms section of the Herbarium at the Department of plant sciences. Nodules of freshly uprooted *P. vulgaris* L. plant were used for the isolation. Healthy pink nodules were selected for the isolation of nodule-associated bacteria (NAB). Individual nodules were dissected from the roots. Subsequently, these were surface disinfected. These surface-disinfected nodules were then crushed. The obtained bacterial suspensions were streaked on YEM-agar medium and incubated at 28 °C. Each colony was then purified by streaking repetitively on YEM agar plates. All obtained pure isolates were sustained on YEM media and well-preserved at 4 °C [23].

2.2. Morphological Characterization and Gram Staining

After 2, 4 and 6 days of incubation at 28 °C, different colonies were analyzed for morphological (form, size, margins, elevation, texture and opacity) and biochemical characterization. Gram staining was performed for all the obtained isolates and finally observed under microscope (magnification: 100X) with immersion oil.

2.3. Biochemical Characterization (Confirmatory Tests for Rhizobium)

The isolated colonies were also characterized for their biochemical characteristics [24] mentioned below and specifically their growth on YEM (Yeast Extract Mannitol) Agar medium incorporated with Congo red dye [25]. Isolates that developed white colored colonies (with very low absorption of dye were considered as rhizobium. The isolated colonies were also grown on YEM medium containing Bromothymol Blue (0.00125 mg/kg) for the detection of acid growth reaction and growth responses [26].

For further confirmation of being gram-negative and lactose fermenters, all the isolates were streaked on MacConkey agar medium as well as Eosine Methylene Blue (EMB) agar medium. Development of pink colonies (due to acid production) on MacConkey agar medium was taken as positive result indication for lactose fermenters while on EMB dark purple colored colonies indicated the positive results.

For further confirmation for nitrogen utilization the isolates were grown on minimal salt agar medium (MSA) containing ammonium sulphate. Triple Sugar Iron (TSI) media was used to perform TSI test in order to check production of H_2S gas and carbohydrate fermentation by the isolates. Color alterations in slants (red/yellow) and the butts were observed and used as indication of specific carbohydrate fermentation, acid and gas production.

All isolated strains were screened for oxidase test using Kovac's reagent prepared according to [24]. For the detection of enzyme catalase, catalase test (using 1-2 drops of 3 % H_2O_2) was performed with isolated colonies (24-48 hrs. incubation). For the differentiation of isolates on the basis of urease activity, urea agar base was utilized. After incubation the inoculated slants were observed within 2-6 hrs. for valid rapid urease-positive results. While for delayed urease reaction observations were taken after 24-48 hrs. In case of positive urease reaction exhibited by the isolates intense pink color development was observed.

All the isolated strains were tested for their citrate utilization ability (as carbon source) using slants of simmon citrate agar medium. Fluid thioglycolate medium designated to check the aero-tolerance of the isolates was used. All the test isolates were stab-inoculated on motility indole urea medium in order to check for motility. Diffused growth/turbidity was considered as indicator for motile organisms while growth along stab line was taken for non-motile ones.

2.4. Preparation of Biochar

Biochar of rice (*Oryza sativa*) husk was prepared by the pyrolysis in the muffle furnace at 300 °C with the rise in temperature of 17 °C per minute. After removing the sample from furnace, it was (kept in a desiccator) weighed and stored in air tight plastic

containers. Small pieces (5-8 cm, dried at 105 °C for 1 hour) of *Lantana* (stem and branches) were pyrolyzed at 450 °C for 20 minutes. Finally, the prepared biochar was crumpled into small particles before use [27–28].

2.5. Biochar Analyses

Biochar was analyzed after preparation for following physico-chemical parameters and morphological analyses for surface morphology, phase and structural properties (Table 1).

2.6. Analyses of Soil used for Pot Experiment

Soil (used for the pot experiment) was characterized for its electrical conductivity, moisture content [30], pH, texture [35] and organic matter [36]. All the measurements were taken both before and after the pot experiment.

2.7. Screening of Rhizobial Strains

The screening for the efficient rhizobial strains was carried out in two parts. Initially the nodule forming strains were screened which were then used for their stability on biochar.

2.8. Screening of Nodule Forming Strains

Pure cultures were tested for nodule formation in *P. vulgaris* L. displaying a potential to enhance nodulation, growth and yield of legume plants when co-inoculated with Rhizobium. This study genetically characterizes bacteria isolated from bean root nodules in Cuba and investigates the effect of Rhizobiume *Pseudomonas* co-inoculation on common bean (*P. vulgaris* L.) [23]. Seeds of *P. vulgaris* L. were treated with 10 % ethanol solution and rinsed with sterile distilled water. The sterile seeds were placed on sterile paper towel for 4-5 days. After germination five seedlings were planted in pots filled with sterile sand for jar experiment. At time of sowing each seedling was dipped in broth solution containing isolated strains separately (three replications). The duration of jar experiment was six weeks. After six weeks plants were uprooted and checked for nodule formation. Strains which showed development of healthy nodules in bean plants were used for further experimentation.

2.9. Inoculum Stability on Biochar

Inoculum was prepared by inoculating strain (performing good in screening test) in 10 ml YEM broth [37] and incubated in shaking incubator (150 rpm) overnight at 28 °C [38]. Sterile biochar (Rice husk & *L. camara*) was added in this broth culture in 1:10 ratio (1 g biochar in 10 ml culture) and placed on an orbital shaker (150 rpm) at 28 °C [39]. Optical density of these combinations (inoculum + carriers) at 600 nm was observed at regular intervals to get substantial results. All the procedures were conducted under strict aseptic conditions.

2.10. Effect of Stabilized Inoculant on Seed Germination

The biochar inoculum combinations were tested for their efficacy in germination of *Phaseolus* seeds. Seeds were surface sterilized with mercuric chloride (0.1 %) for 2 min., then thoroughly rinsed and soaked in distilled water for six hours. Soaked

Table 1. Details of methods used for analytical parameters

Parameters	Methods
Moisture content (%)	Gravimetric method [29]
pH	pH meter [30]
Electrical Conductivity (μS/cm)	EC meter [30]
Ash content (g)	Weight loss after heating method [31]
Bulk density (g/cm^3)	ICARDA manual [32]
Nutrients (N, P, K, Ca, Mg)	ICP-OES [31] Nutrients in weight and atomic percentage were also determined by EDS
Scanning electron microscopy	MIRA3 TESCON scanning microscope
Energy dispersive x-ray spectroscopy surface functional groups	IRTracer-100 Fourier Transform Infrared Spectrophotometer (Shimadzu) [33]

seeds were then mixed with the combination of biochar and inoculum. After mixing, ten seeds from each combination were placed on sterile filter paper and incubated at 25 °C for 1-2 weeks. The detail of the treatments applied is given as follows:

C: Control (without Biochar and strain)
CS: (Control with strain)
B_1 S: (Biochar of rice husk + strain)
B_2 S: (Biochar of *L. camara* L. + strain)

All the treatments were applied in three replications.

2.11. Pot Experiment

Pot experiment was conducted to check the efficacy of the formulated biochar inoculums combination to increase the water use efficiency in *Phaseolus*. The experiment was conducted with the germinated seedlings [27].

2.11.1. Experimental Setup

For each pot (~5 kg capacity & dimension: 18 cm high × 22 cm diameter) one percent combination (biochar + inoculums) was mixed to the soil (4 kg air dried, ground & sieved) on weight basis and germinated seeds (Control) were planted in pots. After six weeks of plantation (after nodule formation) water stress was applied to the plants by maintaining their field capacities (F.C.) at 100 % and 60 % [40]. The same treatments were used in well watered condition by returning 100 % transpired water to the plants.

2.11.2. Application of drought stress

An evening before the start, stressed plants were soaked with water up to their saturation levels and were then left to drain out. The next day the plant pots were weighed. But before weighing, all plants were concealed within polythene bags which were tied to the bottom of the shoot in order to avoid the evaporative loss. Afterwards, each plant was weighed daily and required percentage of the daily weight loss was returned to it by adding distilled water in order to maintain its field capacity. This practice was continued till their stomatal conductance reached zero.

Following method of Masinde *et al*. [41], the initial pot weight referred to the weight of a pot at 100 percent water holding capacity (WHC) while final pot weight referred to the weight of that pot when the transpiration of the stressed plants was almost zero in comparison with the well-watered plants. Daily values of FTSW (Fractionable Transpirable Soil Water) for each pot in the water deficit treatment on each day for each pot was calculated using the following formula, Where

$$\text{Daily FTSW} = \frac{\text{daily pot weight} - \text{final pot weight}}{\text{initial pot weight} - \text{final pot weight}} \quad \text{(eq. 1)}$$

The stress was imposed to evaluate the resistance of *P. vulgaris* and biochar was applied (along with rhizobial strain). Detail of stress treatments is given in Table 2.

2.12. Measurement of Morphological and Physiological Parameters

The plants under pot trial were subjected to various morphological (plant height, plant biomass, leaf area) after 21st and 42nd day after stress (DAS) treatment [27, 41, 42].

All the plants of control and stress treatments were measured for the changes in their physiological parameters i.e., net photosynthesis (Pn), stomatal conductance (gs), transpiration rate (Tr) in morning

Table 2. Application of drought stress treatments

Treatments	Water Levels (%)	Biochar	Inoculation
T_1	100	0	0
T_2	60	0	0
T_3	100	Rice Husk	0
T_4	60	Rice Husk	0
T_5	100	*Lantana camara*	0
T_6	60	*Lantana camara*	0
T_7	100	Rice Husk	Inoculated
T_8	60	Rice Husk	Inoculated
T_9	100	*Lantana camara*	Inoculated
T_{10}	60	*Lantana camara*	Inoculated
T_{11}	100	0	Inoculated
T_{12}	60	0	Inoculated

with (IRGA) Infra-Red Gas Analyzer (CIRAS-2 Portable Photosynthesis System, software version 1.70). For measurement of these physiological parameters the uppermost fresh and fully expanded leaves were placed in the cuvettes of IRGA to obtain the values [43]. Water use efficiency (WUE) was calculated by dividing net photosynthesis by transpiration rate as done by Jianlin *et al*. [44], where

$$WUE = Pn/Tr \quad \text{(eq. 2)}$$

2.13. Statistical Analysis

All data were statistically analyzed by analysis of variance (ANOVA) in SPSS version 16.0 and graphed using OriginPro software. All the treatments were compared using least significant differences at the significance level of $P < 0.05$ to determine the effects on plant growth and physiological properties. While factorial experiments was used to establish the significance of the results, and individual means were tested by LSD.

3. RESULTS

The study was aimed to explore the ameliorative potential of biochar (derived from two different feedstocks) acting as rhizobium carrier to be used for *P. vulgaris* L. exposed to drought stress.

3.1. Physicochemical Analyses of Biochar

The results of physic-chemical characterization of biochar prepared from two different feedstocks are presented in Table 3.

3.2. Morphological Characterization of Biochar

3.2.1. Scanning Electron Microscopy (SEM)

The SEM images of rice husk at magnifications: 200 x, 500 x, 1.00 kx, 5.00 kx is shown in (Figure 1).

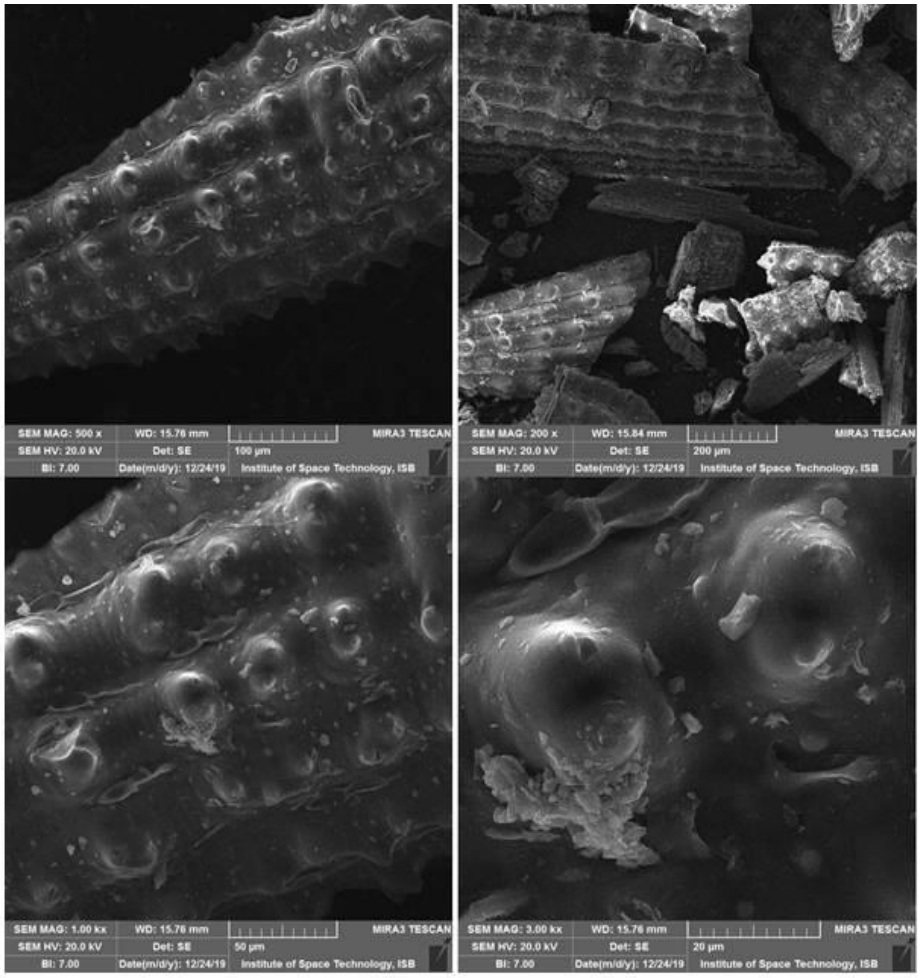

Fig. 1. Scanning electron microscope (SEM) images of rice husk biochar

Table 3. Physicochemical analyses of biochar prepared from two feedstocks

Parameters	**Biochar (Rice husk)**	**Biochar (*Lantana camara*)**
Moisture content %	24.08±0.01	0.88±0.01
pH	6.05±0.01	6.64±0.01
Electrical Conductivity (µS/cm)	898±0.01	1894±0.01
Ash content (g)	0.91±0.01	0.52±0.01
Bulk density (g/cm^3)	0.24±0.02	1.44±0.02
Nitrogen (%)	2.651±0.01	0.724±0.01
Phosphorus (%)	0.029±0.01	0.317±0.01
Potassium (%)	0.197±0.02	0.621±0.02
Calcium (%)	0.047±0.01	0.181±0.01
Magnesium (%)	0.018±0.01	0.986±0.01

While the SEM images of *Lantana* biochar at magnifications: 200 x, 500 x, 1.00 kx, 5.00 kx is shown as under (Figure 2).

3.2.2. Energy-Dispersive X-Ray Spectroscopy (EDS)

In addition to SEM, information regarding the

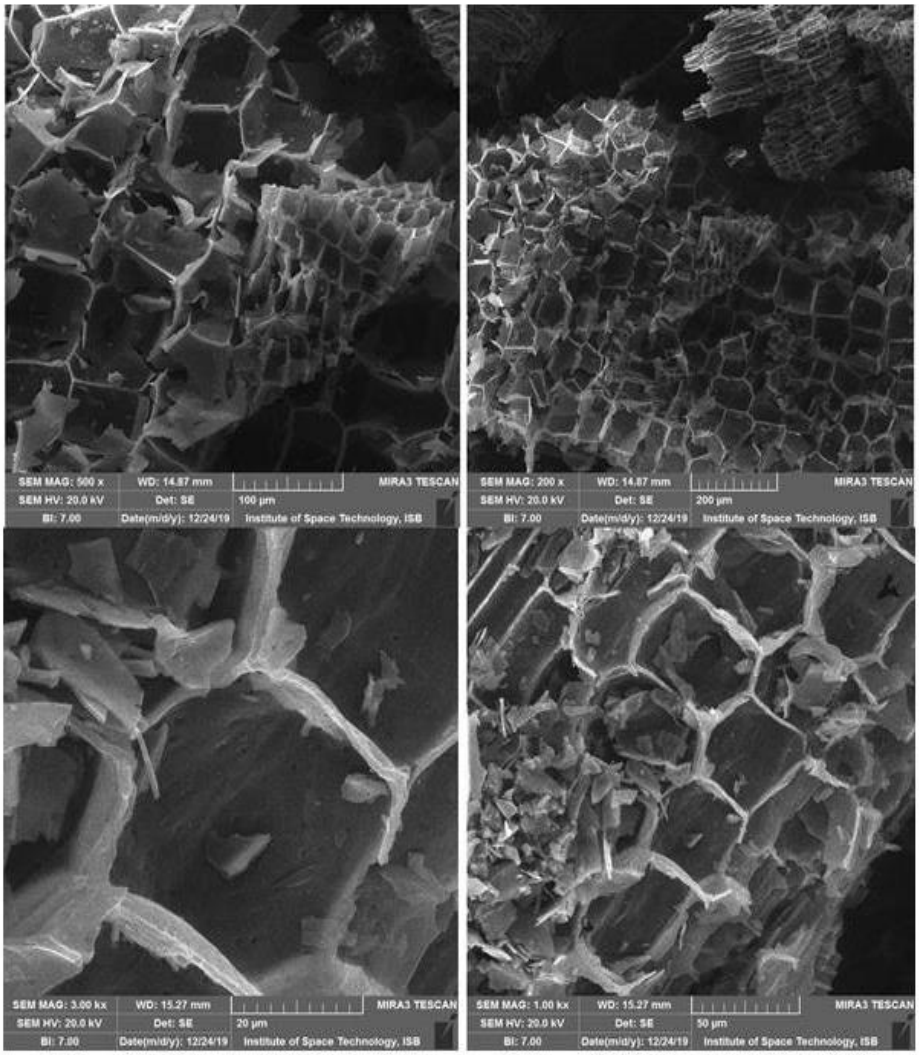

Fig. 2. Scanning electron microscope (SEM) images of *Lantana* biochar

spectral and chemical characteristics of the two biochars was also obtained through energy disruptive X-ray spectroscopy analysis. The noticeable result for rice husk is the presence of Si content with 7.45 % which was absent in *Lantana* biochar. *Lantana* biochar was richer in carbon, potassium and calcium with weight percentages of 77.8 %, 2.15 % and 0.67 % respectively as compared to the proportions of these contents) in rice husk biochar. Oxygen content (33.40 %) was found to be higher in rice husk biochar as compared to 19.20 % in *Lantana* (as shown in Figure 3a and 3b).

3.3. Surface Functionality by Fourier Transform Infrared Spectroscopy (FTIR)

All the FTIR measurements of the biochar samples were taken over the wavenumber range of 4500-400 cm^{-1} and 16 co-added scans, with spectra in transmittance units (%). The detailed description of chemical bonding, peak ranges/band ranges for both the biochars are presented in Table 4 showing the following surface functional groups: aromatic C=N bond, hydroxyl, carboxyl, N–H bond etc.

3.4. Soil Analyses

The pH of control soil remained unchanged while pH of soils of the treatments (i.e. rice husk biochar, *Lantana* biochar, rice husk biochar + inoculum, *Lantana* biochar + inoculum and inoculum) was changed (i., 8.9, 8.5, 7.8, 7.7 and 6.9 respectively). Noticeable changes in the electrical conductivity were also observed i.e., from 3.0 µS/cm to 13.0, 10.0, 6.8, 5.6 and 8.5µS/cm respectively in soils with treatments mentioned above. Moisture content and organic matter of pot soil also showed increasing percentages (Table 5).

3.5. Results of Pot Experiment

Results of morphological and physiological parameters are described as under:

3.5.1. Plant Height

The plant height was measured at twenty one and forty two days after stress and the contrast between the treatments under stressed (60 % F.C.) and non-stressed (100 % F.C.) conditions and the controls is depicted in Figure (4a). Highly significant ($P \leq 0.01$) results for these height measurements were recorded in plants treated with biochar as compared to the control. Length of plants was better in the non-stressed (100 % F.C.) plants as compared to the plants at 60 % F.C. The rice husk biochar treated plants (35 cm) were taller plants treated with *Lantana* biochar (23 cm) and the controls (29 cm). Similar was the case of 60 % F.C. more plant height

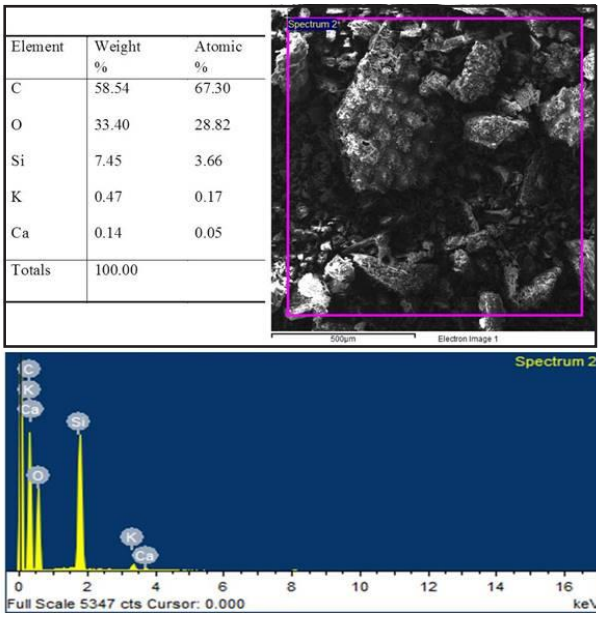

Fig. 3a. EDS Spectra of rice husk biochar showing elemental weight percentages.

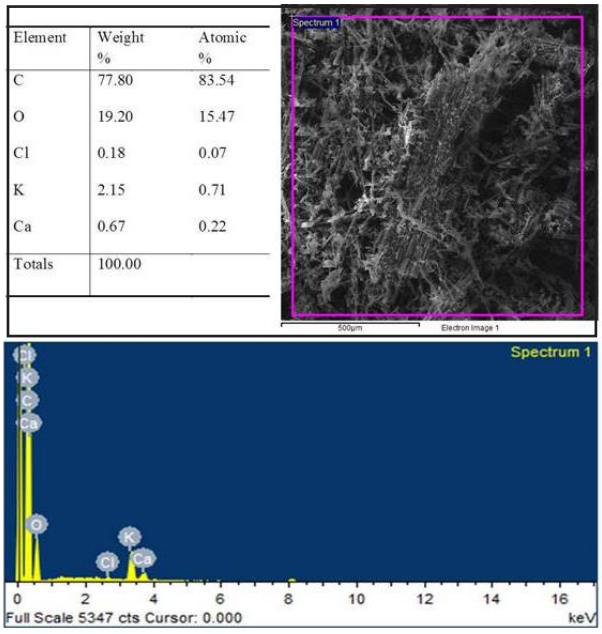

Fig. 3b. EDS Spectra of *Lantana* biochar showing elemental weight percentages.

Table 4. The functional groups existing in two biochar samples (i.e. rice husk and Lantana) determined by FTIR spectrogram.

Wave number (cm^{-1})	Rice husk biochar	*Lantana* biochar	Vibration characteristics
3902-3703	–	+	
3614-3570	–	+	
3643-3599	+	–	
3599-3565	+	–	
3376-3342	+	–	O-H stretching
3036-2830	–	+	C-H stretching
2929-2818	+	–	C-H stretching
2384-2335	–	+	
2383-2305	+	–	
2260-2238	+	–	C≡C stretching
2210-2151	–	+	C≡C stretching
1881-1859	+	–	
1847-1836	+	–	
1680-1635	–	+	N-H bond C=O stretching
1580-1435	+	–	Methyl C-H asymmetric Carboxylic acid
1554-1499	–	+	Carboxylic acid
1488-1444		+	Methyl C-H asymmetric
1435-1357	+	–	Nitro compound stretching
1260-1204	–	+	Aryl-O stretching
1212-1178	+	–	C-N stretch of tertiary amine
1133-1066	+	–	Primary amine of C-N stretch Secondary amine of C-N stretch
1104-1038	–	+	Primary amine of C-N stretch Secondary amine of C-N stretch
1000-883	–	+	Substituent of aromatic rings
877-844		+	Substituent of aromatic rings
732-654	+	–	Substituent of aromatic rings
622-588	–	+	Substituent of aromatic rings
542-520	+	–	
498-486	+	–	

Table 5. Results of soil analyses after pot experiment.

Treatments	**Moisture** (%)	**pH**	**EC** (µS/cm)	**Soil Texture**	**Organic matter** (%)
Control	1.2±0.1	7.1±0.01	4.0±0.01	Sandy Loam	0.5±0.03
Rice husk biochar	15±0.02	8.9±0.01	14.0±0.01	Sandy Loam	1.9±0.07
Lantana biochar	16±0.01	8.5±0.01	9.0±0.01	Sandy Loam	2.0±0.01
Rice husk biochar + inoculum	26±0.03	7.8±0.005	7.0±0.01	Sandy Loam	3.5±0.05
Lantana biochar + inoculum	30±0.07	7.7±0.01	5.0±0.01	Sandy Loam	3.1±0.01
Inoculum	15±0.06	6.8±0.01	8.5±0.01	Sandy Loam	4.0±0.01

was observed with rice husk biochar as compared to control and with the addition of *Lantana* biochar. Therefore, rice husk biochar treatment was seen to show nominal growth in plant height than others.

Biochar along with inoculum of isolated strain has been found to have a significant effect on the height. This comparison between control and combinations of biochar and inoculum treatments is presented in the Figure (4b). Elevated measurements in the height were observed with biochar and inoculum added plants as compared to the plants with no soil amendment and inoculum were smeared showing highly significant (at $P \leq 0.0$) effects for all the treatments.

While comparing treatments: 100 % F.C + without biochar + without inoculum, 100 % F.C. + rice husk biochar + inoculum and 100 % F.C + *Lantana* biochar + inoculum the plant showed considerable increase throughout the experiment. Nevertheless, the observed increase was higher in plants with BR + I (33 cm). In case of more stressed plants i.e. with 60 % F.C, BR + I treatment proved to be more beneficial for height increase in a similar fashion. Inoculation also has substantial influence on the height of the bean plants. The comparison between control plants and plants with inoculum (isolated strain) addition for the parameter of plant height is presented in Figure (4c).

Inoculum treatment has also enhanced plant height with high significance as compared to the plants with no added inoculum. With all these treatments i.e., 100 % F.C. + no added inoculum, 60 % F.C. + no added inoculum, 100 % F.C. + inoculum and 60 % F.C. + inoculum, considerable height escalation was observed in inoculum added plants (32.6 cm) as compared to the control (28.9 cm) plants.

3.5.2. Plant Biomass

Biomass measurements of the control and biochar treatments in terms of dry matter is presented in (Figure 5a) showing higher values of all non-stressed plants which were sustained with 100 % field capacity. While among these treatments (i.e., BR and BL) dry matter was found to be higher (3 g) with BR treatment as compared to the stressed (60 % F.C.) and non-stressed ones (100 % F.C).

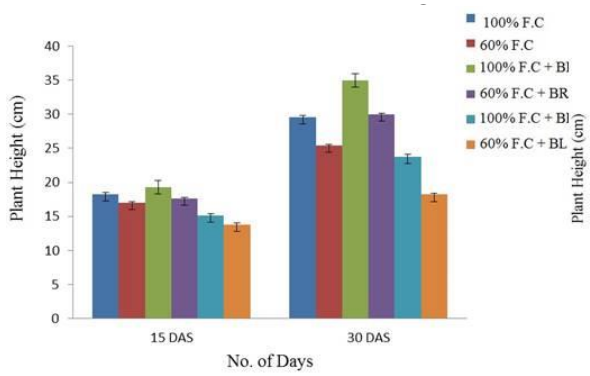

Figure 4a: Comparison of control and biochar treatments

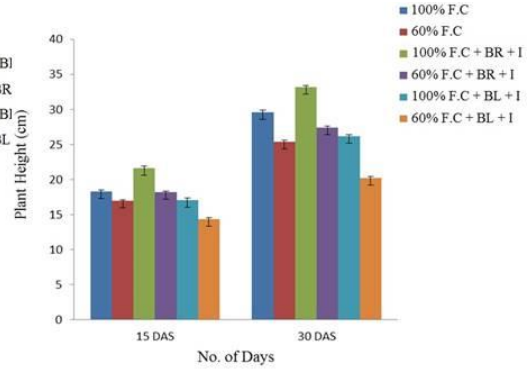

Figure 4b: Comparison of control and biochar with inoculum treatments

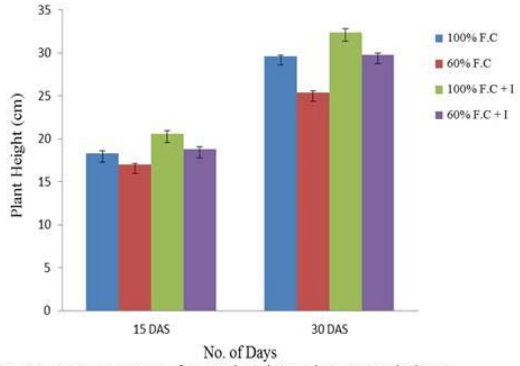

Figure 4c: Comparison of control and inoculum treated plants

Fig. 4 (a, b, c). Changes in plant height of control and treatments under stressed (60 % F.C) and non-stressed (100 % F.C) conditions

Comparison of control and the combinations of biochar and inoculum addition treatments is shown in (Figure 5b) where maximum value of dry matter (i.e., 2 g) was obtained at 100 % F.C. with BR + I. While in case of stressed plants with 60 % F.C. less value was observed. Figure (5c) is presenting the comparison of control and inoculum treatments. It is obvious from the figure that biomass increased in non-stressed conditions and comparatively higher values of dry matter (0.5 g) than control plants.

3.5.3. Leaf Area

Measurements for leaf area are presented in (Figure 6a) depicting highly significant ($P \leq 0.01$) results. As seen in figure all the treatments (BR and BL), which were retained at 100 % field capacity, showed increasing trends but with BR at 100 % F.C, 120 cm^2 leaf area was obtained which was the maximum value. Similar is the case of stressed treatments (i.e., 60 % F.C., 60 % F.C. + BR, 60 % F.C. + BL) where higher measurements were acquired with the addition of rice husk biochar.

It is noteworthy that greater values of leaf area were observed in all the plants kept at 100 % F.C. as compared to the stressed ones (60 % F.C.). Outcomes of combination of two types of biochar and inoculum were established while comparing all the treated plants with the controls (Figure 6b). When we look at the leaf area measurements of all the plants maintained at 100 % field capacity along with the treatments of biochar (BR and BL) and inoculum addition, higher values were obtained with BR + I. Therefore, at 100 % F.C rice husk biochar + inoculum was found to be more effective in increasing this growth parameter as compared to others. Furthermore, the same rice husk biochar at 60 % F.C. displayed similar increasing trend. Apart from biochar, inoculum addition alone was found to be effective for treated plants as depicted in Figure (6c).

3.5.4. Stomatal Conductance (gs)

Measurements for stomatal conductance (gs)

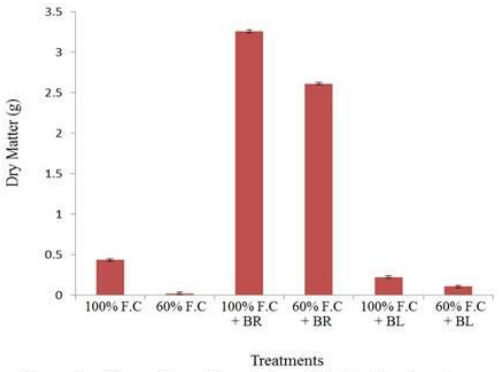

Figure 5a: Comparison of control and biochar treatments

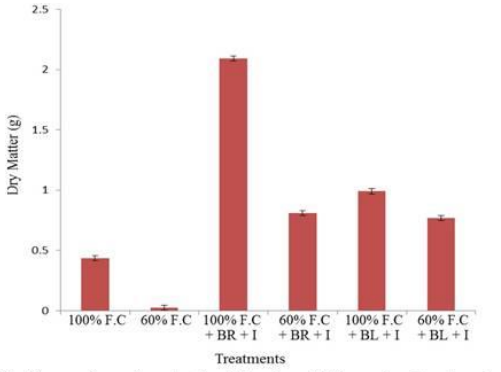

Figure 5b: Comparison of control and biochar with inoculum treatments

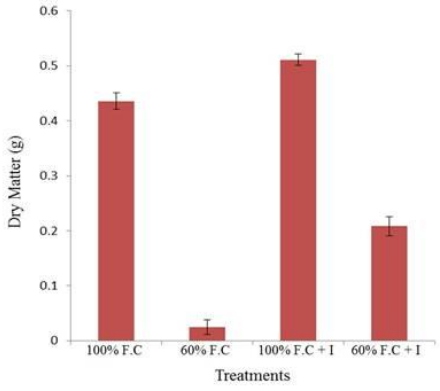

Figure 5c: Comparison of control and inoculum treated plants

Fig. 5 (a, b, c). Changes in dry matter of control and treatments under stressed (65 % F.C) and non-stressed (100 % F.C) conditions.

in all treated and control plants are shown in (Figure 7a). Highly significant ($P \leq 0.01$) differences were observed while comparing all the treatments. In case of non-stressed plants (i.e., 100 % F.C. + BR, 100 % F.C. + BL), stomatal conductance measurements persisted at constant value till week 6 due to daily maintenance of field capacity (100 %). It's noteworthy that the stomatal conductance was higher (365 mmol H_2O $m^{-2}s^{-1}$) with the addition of rice husk biochar in contrast to BL where it was 310 mmol H_2O $m^{-2}s^{-1}$. While, in case of stressed plants (i.e., with 60 % F.C, 60 % F.C. + BR, 60 % F.C. + BL) the values for stomatal conductance decreased very slowly in initial three weeks. After that when the stress was increased, a swift drop was observed in the values of stomatal conductance. Hence, biochar provided stability at such extreme stress level and thus stomatal conductance decreased upto 70 and 61 mmol H_2O $m^{-2}s^{-1}$ in BR and BL treatments, respectively contrasting to the treatments with no biochar additions where it touched closer to zero value till the sixth week.

Besides the treatments with biochar of two different feedstocks, the amalgamation of biochar with inoculum of the isolated strain also exhibited significant results ($P \leq 0.01$) for the responses in the stomatal conductance (Figure 7b). Just like biochar responses, similar effects were found when inoculum was added along with the two biochars. At 100 % filed capacity the stomatal conductance maintained almost at same readings throughout the experimental time period for all the treatments (0 % B + 0 % I, BR + I, BL + I). On the contrary, in case of stressed treatments (60 % F.C) for all the treatments (0 % B + 0 % I, BR + I, BL + I) the value of stomatal conductance declined till zero for 0 % B+ 0 % I as compared to the others where it didn't touch the figure of zero till the end of experiment. As a result, biochar in combination with inoculum contributed in increasing plants' resistance to drought stressed conditions.

Inoculum addition alone also had affirmative effects in improving the stomatal conductance (Figure 7c) with highly significant differences at

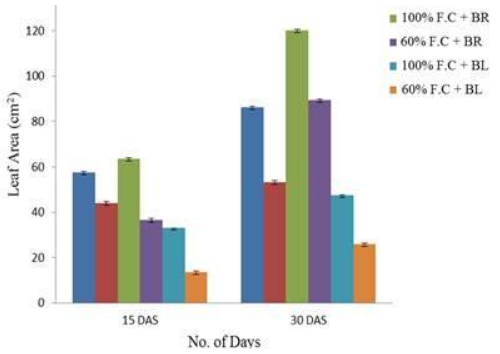

Figure 6a: Comparison of control and biochar treatments

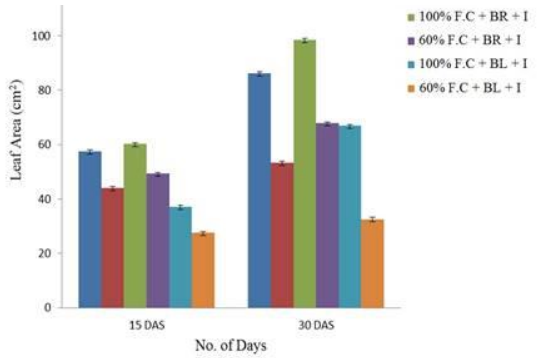

Figure 6b: Comparison of control and biochar with inoculum treatments

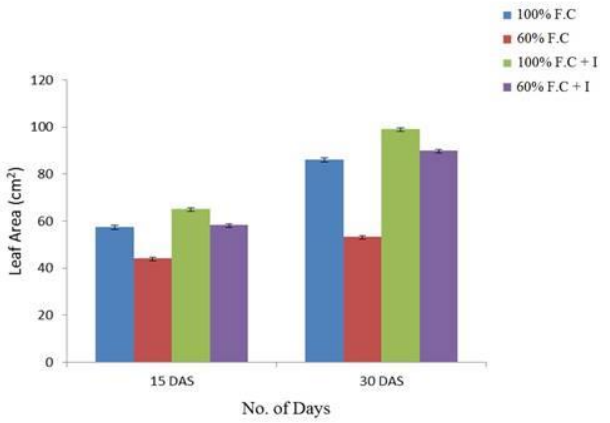

Figure 6c: Comparison of control and inoculum treated plants

Fig. 6 (a, b, c). Changes in leaf area measurements of control and treatments under stressed (65 % F.C) and non-stressed (100 % F.C) conditions.

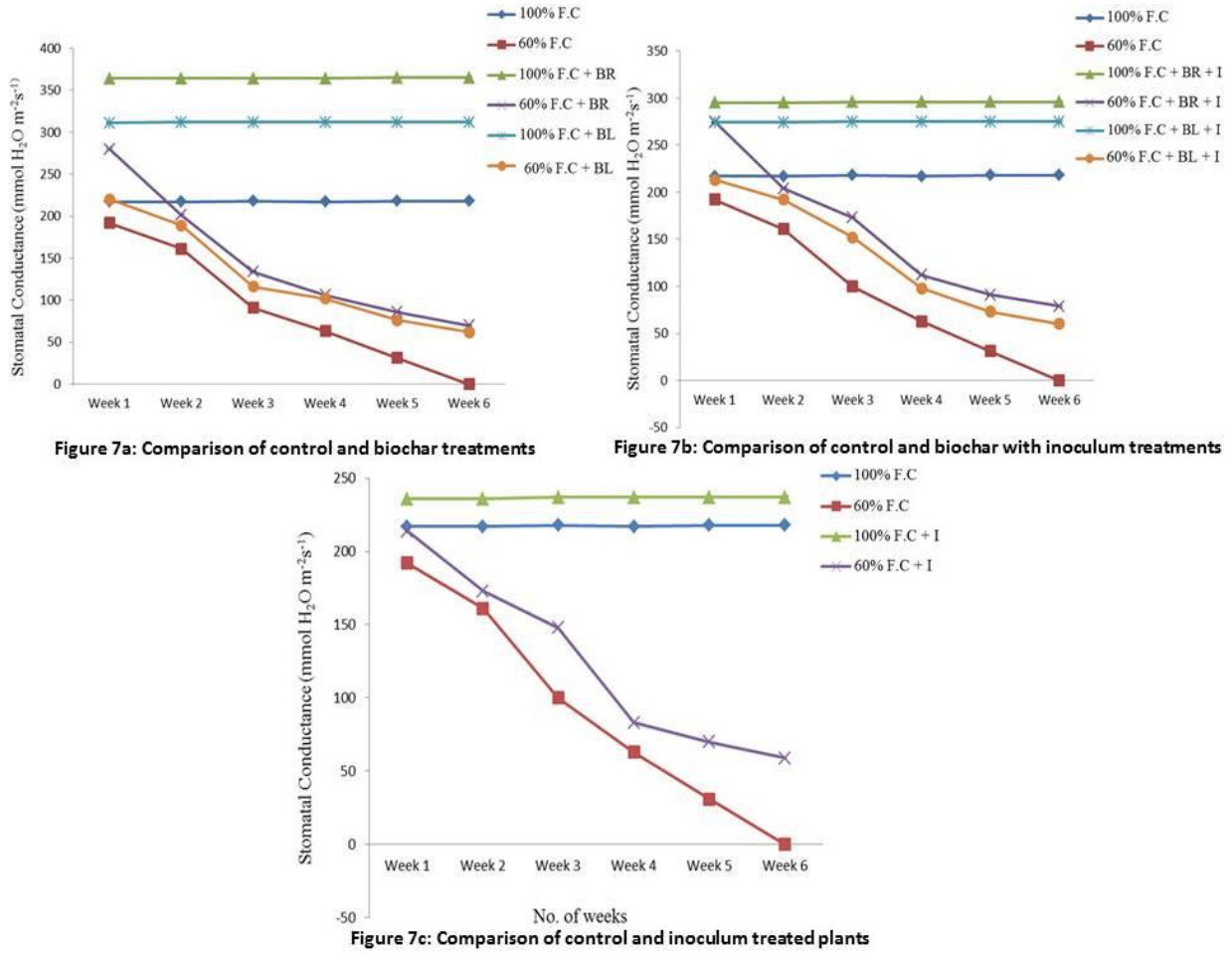

Figure 7a: Comparison of control and biochar treatments

Figure 7b: Comparison of control and biochar with inoculum treatments

Figure 7c: Comparison of control and inoculum treated plants

Fig. 7 (a, b, c). Changes in stomatal conductance of control and treatments under stressed (65 % F.C) and non-stressed (100 % F.C) conditions.

$P \leq 0.01$ among all the treatments. No noticeable changes in stomatal conductance were observed in plants maintained at 100 % field capacity and inoculum treatment. Additionally, higher value of stomatal conductance was obtained for inoculum added plants (235 mmolH_2O $m^{-2}s^{-1}$) than the control plants (215 mmol H_2O $m^{-2}s^{-1}$). In contrast to this, a gradual reduction in the stomatal conductance value of stressed plants (60 % F.C. + I & 60 % F.C) till the third week. After that a rapid drop up to zero was seen in plants at 60 % F.C. with no inoculum addition in contrast to the inoculum treated plants where the value was 60 mmol H_2O $m^{-2}s^{-1}$.

3.6. Net Photosynthesis

Responses in the form of net photosynthesis of control and biochar treated plants have also shown highly significant differences. At 100 % F.C. for the treatments T1, T3 & T5, no significant change was witnessed in the photosynthesis rates. Comparatively higher rates were witnessed in T3 (5 µmol CO_2 $m^{-2}s^{-1}$) than 2.5 µmol CO_2 $m^{-2}s^{-1}$ for T1 and 4.5 µmol CO_2 $m^{-2}s^{-1}$ for T5. On the other hand at 60 % field capacity an increasing trend was noticed with two types of biochar as compared to control (without biochar addition) where the rate decreased at week six. Photosynthesis rate was higher with rice husk biochar and further increased from 4.5 to 7 µmol CO_2 $m^{-2}s^{-1}$ from start till the end (Figure 8a). It is obvious from the results that rice husk biochar has significantly influenced the photosynthetic rate under stress conditions as an effective treatment.

Photosynthetic rates were also compared for the plants with biochar treatments along with inoculum additions (Figure 8b) and significant ($P \leq 0.01$) results were observed for all the comparisons. Higher photosynthetic rates were witnessed in plants maintained at 100 % field capacity with the

treatment combinations of biochar and inoculum as compared to the controls. In case of plants with 100 % F.C., rice husk biochar addition plus inoculum was found to be more effective than the others. In contrast to this, a gradual rise in the photosynthesis rate was witnessed in plants with 60 % F.C. with biochar inoculum treatments (T_8 & T_{10}) than control (T_2). Similarly, rice husk biochar along with inoculum has also shown better effects than *Lantana* biochar.

Photosynthesis rates of control plants and inoculated plants were also compared with significant results ($P \leq 0.01$) shown in (Figure 8c). Very little alteration was detected in the photosynthesis rates of plants which were kept at 100 % field capacity (T_1 and T_{11}) throughout the pot experiment. But inoculated plants showed higher rates of photosynthesis (4 µmol CO_2 $m^{-2}s^{-1}$) than the control plants (2.6 µmol $CO_2m^{-2}s^{-1}$). While for the plants at 60 % F.C. (T_2 and T_{12}) the photosynthesis rates were higher. In plants with inoculum treatment, it increased from 2.6 to 5 µmol CO_2 $m^{-2}s^{-1}$ and in control plants the value decreased from 2.5 to 0.7 µmol CO_2 $m^{-2}s^{1}$.

3.7. Transpiration Rate (Tr)

Plants of the pot experiment were compared for their transpiration rates of control and biochar treatments (Figure 9a) with highly significant ($P \leq 0.05$) results. In case of all non-stressed plants (i.e. T_1, T_3 & T_5) there appeared no significant change in transpiration rate throughout the experimental period, as the 100 % field capacity was sustained daily. On the contrary, in stressed plants (i.e. T_2, T_4 & T_6) a slow reduction was seen till three weeks. After that a rapid decline was observed. The values changed from 3.7, 5.1, 4.0 and 2.9 to 4, 3.6 and 3.2 in plants of T_2, T_4 & T_6, respectively. Finally, the value nearly touched zero value at the termination of experiment in plants without biochar addition. Significant impact was witnessed on the rate of transpiration of plants which were treated

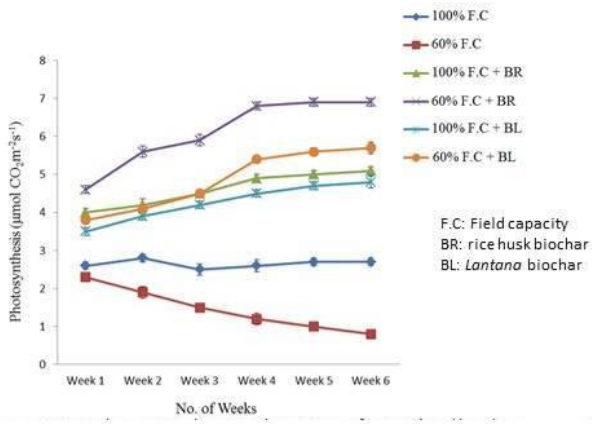

Figure 8a: Comparison of control and biochar treatments

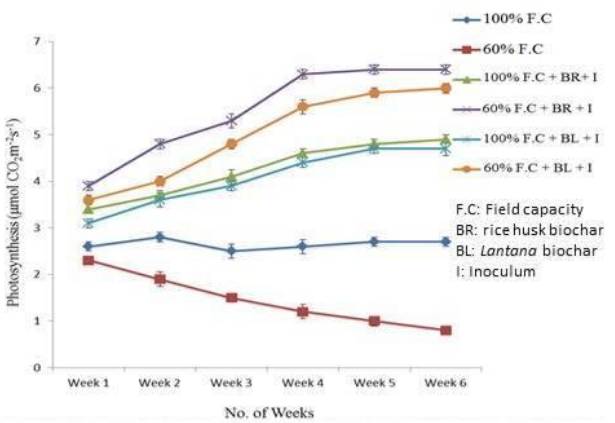

Figure 8b: Comparison of control and biochar with inoculum treatments

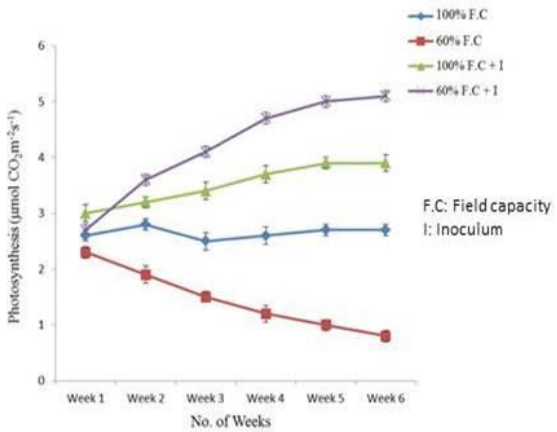

Figure 8c: Comparison of control and inoculum treated plants

Fig. 8 (a, b, c). Changes in photosynthetic rates of control and treatments under stressed (65 % F.C) and non-stressed (100 % F.C) conditions

with combination of biochar and inoculum. The comparison between control and this combination treatment is depicted in (Figure 9b) with obvious significant results ($P \leq 0.01$). In case of treatments at 100 % field capacity (T_1, T_7 & T_9) no substantial changes were observed during the course of experiment. Whereas in case of T_2, T_8 & T_{10} kept with 60 % F.C. the transpiration rate gradually declined till the third week with sudden decrease afterwards with proximate zero values at the end.

The inoculum-biochar combination treatments also showed significant results ($P \leq 0.01$) on the transpiration rates of the plants when compared with the controls (Figure 9c). Similar trends were experienced by the plants maintained at 100 % F.C (T_1 & T_{11}) with almost constant transpiration rates. However, stressed plants at 60 % F.C (T2 & T_{12}) showed a gradual decreasing transpiration rates and the values further reached proximate zero values at week 6.

3.8. Water Use Efficiency (WUE)

Water use efficiency was also calculated simply by dividing net photosynthesis (Pn) by transpiration rate (Tr) and the obtained values for the control plants and biochar treated plants were compared (Figure 10a) at P value of ≤ 0.01. As depicted in previous comparisons rice husk biochar has shown improved results for the water use efficiency of Bean plants as compared to the controls. In case of treatments T_1, T_3 & T_5, which were kept with 100 % field capacity minor changes, were observed in the obtained water use efficiency values weekly till the end of the experiment. But it improved in plants with biochar treatments as compared to less value for the controls. For 60 % F.C different trends were observed for the treatments T_2, T_4 & T_6 as increased values were calculated. Plants with rice husk biochar treatment have shown the maximum value for WUE i.e. 4.7 mmol mol^{-1} as compared to

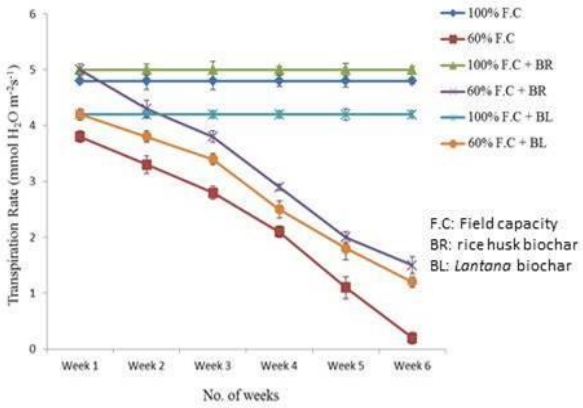

Figure 9a: Comparison of control and biochar treatments

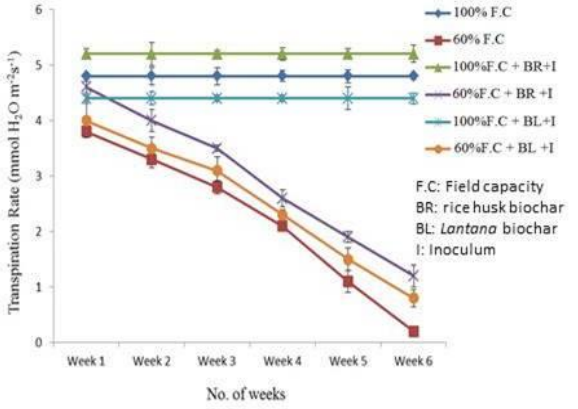

Figure 9b: Comparison of control and biochar with inoculum treatments

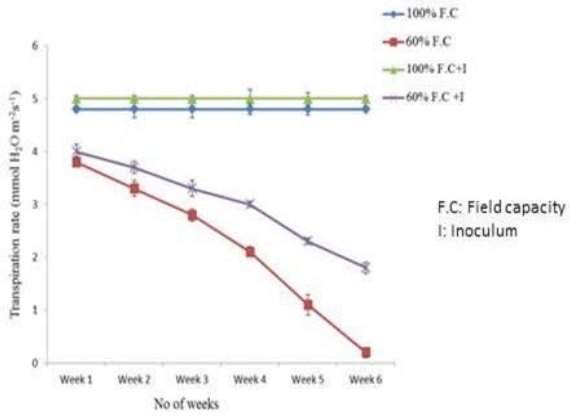

Figure 9c: Comparison of control and inoculum treated plants

Fig. 9 (a, b, c). Changes in transpiration rates of control and treatments under stressed (65 % F.C) and non-stressed (100 % F.C) conditions

control and BL (1.5 and 3.7 mmol mol^{-1}).

Figure 10b is presented for the comparison of WUE between control and the combined treatment of biochar and inoculum additions and it is showing highly significant results ($P \leq 0.01$) for all the treatments. Plants which were returned with 100 % transpired water i.e. T_1, T_7 & T_9 similar trends were observed with maximum calculated value of WUE for T_7. In contrast to this, the other group of plants which were returned with 60 % transpired water (i.e. T_2, T_8 & T_{10}), water use efficiency did increase during the course of experiment with similar WUE improvements in plants with rice husk biochar along with inoculum additions.

The third comparison between control and inoculum applied treatment for the parameter of water use efficiency (WUE) is shown in (Figure 10c) with highly significant results ($P \leq 0.01$). Water use efficiency varied slightly in plants maintained at 100 % field capacity for both controls and inoculum treatments. But in case of plants with 60 % F.C significant rise in WUE values were observed for T_2 & T_{12} throughout all the weeks of experiment. However comparatively higher efficiency was seen in inoculated plants rather than control plants.

4. DISCUSSION

The present study was designed with basic aim of evaluating the ameliorative potential of biochar as a rhizobial carrier for the bean plant with specific focus on increasing tolerance against drought stress by improving its water use efficiency. Another aspect of the study was to compare two types of biochars which were obtained from rice husk and a wild invasive shrub *L. camara.* The study also tried to assess the potential of this carrier material for the delivery of isolated strain and assisting the plant in achieving tolerance against water stress.

The addition of biochar provides habitat fulfilling one of the basic microbial requirements.

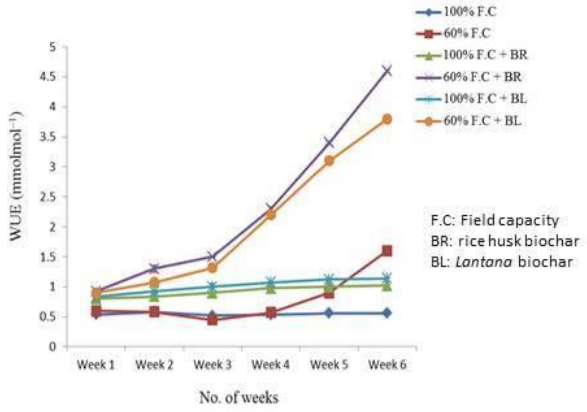

Figure 10a: Comparison of control and biochar treatments

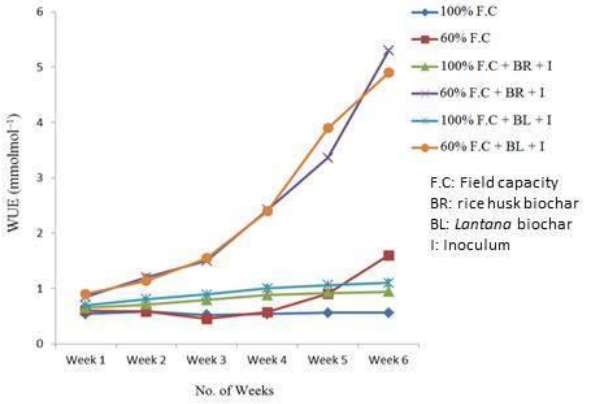

Figure 10b: Comparison of control and biochar with inoculum treatments

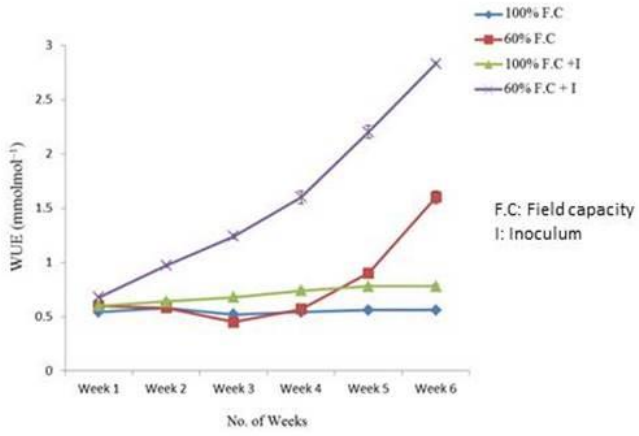

Figure 10c: Comparison of control and inoculum treated plants

Figure 10 (a, b, c). Changes in water use efficiency (WUE) of control and treatments under stressed (65 % F.C) and non-stressed (100 % F.C) conditions.

Jaafar *et al*. [45] confirms our hypothesized potential habitat contribution of different types of biochar, for soil microorganisms, owing to their high porosity and surface area as indicated by scanning electron microscopy micrographs. The high porosity of biochar allows it to maintain water, nutrients and also microbes for a considerable long duration [46].

Scanning electron microscopy along with EDS provides valuable information related to the spectral as well as chemical characteristics of biochar [47]. The analysis showed highly heterogeneous microstructure of biochar upto the magnifications of 5.00 kx. Presence of silicon (Si=7.45 %) mineral agglomerates in rice husk biochar presents typical structure having biomass origin. Rice husk biochar exhibited high degree of macro-porosity with contents of carbon (58 %), oxygen (33 %), potassium (0.47 %) and calcium (0.14 %). On the other hand, the results of SEM-EDS analysis for *Lantana* biochar indicated that these particles consisted of comparatively higher percentage of carbon content (77 %), potassium (2015 %), calcium (0.67 %), chlorine (0.18 %) and lesser oxygen (19 %).

Silicon is one the non-essential elements, that is considered to boost plant growth by improving certain physiological processes in them [48]. Datnoff *et al.* [49] documented inefficient tolerance of both biotic and abiotic stresses when plants were grown in Si-deficient environment. However, if provided with Si, plants showed high tolerance by retaining leaf water potential, stomatal conductance and photosynthetic activity. So it comes in the category of functional nutrients [50] and is regarded as essential (agronomically) for achieving sustainable crop productivity. Silicon plays a similar role for rice and other crops of grass family. Large amount of silicon (in the form of silica gel ($SiO_2.nH_2O$) is accumulated by *Oryza sativa.* This accumulated Si performs the functions of cell wall support, reduction water losses through evapotranspiration, imparting resistance against pathogens and heavy metal and drought tolerance etc. Rice crop specifically takes up greater quantities of Si (even more than essential elements). Rice husk is rich in lignin in combination with opaline silica (apporx. 20 %) and its confirmed by our EDS analysis results for rice husk biochar. Varela Milla *et al*. [33] applied rice husk biochar achieving improved nutrient conditions in Ultisol and increased plant biomass production as compared to wood biochar.

Mostly FTIR transmission analyses provide information about the organic functional groups representing the organic components present in bichar. The peak in the band range 3376-3342 cm^{-1} is expected due to hydrogen bonded hydroxyl groups stretching, designating phenols and alcohols. It may be due to any retained water in the BR sample which is found absent in BL. Another vibration i.e. of C–H stretch between 3036-2830 cm^{-1} indicated presence of alkene groups in BL. A peak observed at 2929-2818 cm^{-1} demonstrates the presence of the aliphatic C–H stretching vibrations.

Stretching band of 2260-2238 cm^{-1} represents C≡C group in BR and 2210-2151 cm^{-1} indicate the same group in BL. The N–H bond with peak range of 1680-1635 cm^{-1} was present in the case of BL. The band at 1580-1435 cm^{-1} is attributed to carboxylate functional group and C–H asymmetric band in BR. Presence of such oxygenated groups have been found to be efficient nutrient adsorbers for the plants capable of releasing these nutrients controlled by various pH driven mechanisms. Presence of variable surface groups enables biochar particles to harbor both positive and negatively charged agri-inputs [51]. The biological effectiveness could further be upgraded through conversion into nan-forms.

The FTIR spectrum of BL was also characterized with the presence of inorganic functional groups as evident by the peaks around 1680-1635 cm^{-1} which could be due to the presence of C=O stretching of aromatic rings. Purakayastha *et al*. [52] suggested that highly stable biochar might have great potential for long-standing carbon sequestration in the soil specifically attributed to the stronger surface groups of aromatic C–C stretching.

Rhizobium strains are reported to have great potential for plant growth promotion and nutrient procurement. Nevertheless, the persistence and proliferation is greatly affected by drought. Protection against this environmental stress could be achieved by different carrier materials such as biochar and their shelf life could further be prolonged. A similar study by Ardakani *et al.* [53]

compared different materials as potential carrier for the survival and viability of bacterial inoculants over an experimental period of six weeks. They proposed wood biochar as efficient inoculum carrier. Saranya *et al*. [54] also compared biochar from two different feedstock and declared coconut shell biochar as better microbial inoculant carrier in sustaining highest number of viable cells.

Besides known qualities of biochar for assisting plants in increasing tolerance against water stress conditions, it is expected to act as efficient carrier for certain rhizobial strains. The present study is based on this hypothesis of ameliorative potential of biochar (obtained from two different feedstocks) as rhizobial carrier besides its role in granting drought tolerance to a leguminous plant (*P. vulgaris*). During the last part of the study, biochar from two different feedstocks were compared in attaining the assigned objectives for all the morphological and physiological parameters. Sandy loam textured pot soil was collected from dry sub-tropical conditions characterizing distinctive water scares and stressed environment for the experimental plants. It was low in organic matter content with neutral pH having electrical conductivity of 4.0 µS/cm. Rice husk biochar alone as well as in combination with the inoculant gave encouraging responses in improving all the studied parameters of the plant.

Our findings regarding plant height evaluation, rice husk biochar was found to be influencing the plant height positively more than *Lantana* biochar. Studies by Dong *et al*. [55] and Win *et al*. [56] have also found significant increases in shoot length as a result of biochar amendment in soil. The results of another study by Pratiwi and Shinogi [57] revealed enhanced shoot height by the application of commercial rice husk biochar that greatly support our results. These findings have further elucidated that such progressive outcomes are partially accredited to the nutrients contribution of the biochar with high electrical conductivity values that represent the quantity of water soluble nutrients. The changes in our EC values are comparable to these studies. Even at 60 % field capacity plant height was more with the addition of BR as compared to control and that of with the addition of *Lantana* Biochar coinciding with the findings of Shashi *et al*. [58].

The rate of increase in leaf area of plants usually decrease due to reduction in the quantity of water provided as reported by McGiffen *et al*. [59] who observed 50 % drop in the leaf area increase as they decreased the amount of water. In the present study a significant rise in the leaf area was seen till the three weeks and afterwards the leaf expansion of all the droughty plants (at 60 % field capacity) began to decline in comparison to the expansion of all the control i.e. well watered plants. In a similar study conducted by Głodowska *et al.* [60] biochar-coated seeds showed significant higher fresh weight, leaf area and plant height compared to the other treatments.

A greater increase in biomass production was observed with the application of fertilizer and rice husk biochar [33]. A similar output was achieved in plant biomass with the application of biochar along with fertilizer [61]. Głodowska *et al*. [60] also found significant higher fresh and dry weight in maize crop when seeds were coated with biochar along with inoculum treatment. Bio-char additions by Rondon *et al*. [19] were found to significantly increase (39 %) total biomass production with the biochar addition to the N-fixing bean. This achievement of getting better biomass production by N-fixing beans is mainly due to greater leaf biomass as a result of biochar and inoculum treatments [19]. A comparable finding of improvements in dry weight with the inoculum addition treatment for the controls as well as stressed plants, was done by Jones *et al*. [62] in which he concluded that dry matter reduction in the stressed plants was observed due to stumpy transpiration and lesser leaf area.

Win *et al*. [56] have supported the impression that biochar do promote the survival of inoculated bacteria performing the role of a stable shelter for them. Furthermore, if crops are provided with inoculum in combination with the biochar, significant yield enhancements could be achieved utilizing its property of acting as a carrier. A review by Sashidhar *et al*. [51] suggested varying inoculum responses of different types of biochar prepared from different feedstocks with varying process settings. Biochar has a supplementary advantage that enables to be utilized as inoculum carrier ensuring survival of the microbes for maximum duration [63].

Purakayastha *et al*. [52] have advocated rice biochar for its ability to restore bio-fertility of soils by enhancing their microbial activities. According to them the rice biochar has being found to be fairly labile in the soil contributing to the microbial biomass proliferation supporting our better results with rice husk biochar. Being a major rice grower continent, Asia gets copious amount of rice residues with the estimates of about 560 million tons of straw and 112 million tons in the form of husk. These huge quantities of residues could be efficiently utilized or in other words recycled in the form of biochar and in return be benefitted by its enormous qualities [33] with additional benefit of getting rid of winter smog problem caused by rice residue burning. This could be the reason that makes husk biochar the most cost effectively considered biochar for a long time.

Limited studies have been conducted about the synergistic utilization of biochar and rhizobacteria. Hafez *et al*. [64] conducted a field study on alleviating water deficit in rice crop using biochar and rhizoacteria in combination with soil treatments i.e. control, biochar, PGPR as well as combination of PGPR with biochar especially in the field. According to their results this integrative use augmented the physicochemical properties of the soil. Significant increase in the stomatal conductance occurred with decreasing proline content in *Oryza sativa* being treated simultaneously with PGPR and biochar confirming the efficacy of our study approach for the bean plant. Studies have shown that the photosynthetic machinery is impaired under drought stress and application of PGPR have been found to overcome this impairment along with the enhancement in the chlorophyll content and shoot length [65].

Supporting our findings with reduced conductance in water stress treatment, Galmés *et al.* [66] compared different plant species for reduction in stomatal conductance under water deficient environment. On the contrary, a plant showing higher rates of stomatal conductance even under drought conditions depicts its greater tolerance level [67]. Studies have linked such closure response of stomata to the moisture content of the soil than the leaf water status.

According to Jones *et al*. [62], there is a linear relation between the decline in transpiration rate and dry matter reduction. Signals are transmitted by the roots towards the shoots with a measure of reduced water consumption through transpiration reduction enabling plants to grow in drying soil. This transpiration decline can be achieved either by reduction in leaf expansion or by leaf shedding in case of severe drought stress [68]. Liu and Stuzel [69] attributed such transpiration declines as the major cause of reduction in stomatal conductance. Transpiration rates of control plants reached zero at the end as compared to a bit higher with biochar and inoculum treatments. This shows that biochar and inoculum has enabled plants to respond better in water deficient conditions.

Our findings of improvements in photosynthestic rates of bean plant in response to the biochar application under drought stress conditions are confirmed by the studies of Solaiman *et al*. [70]. In accordance with Akhtar *et al*. [71], biochar amendment specifically prepared from rice husk had positive influence on the photosynthesis, transpiration rate, stomatal conductance and water use efficiency in relation to respective *Lantana* biochar and non-biochar control treatments. Hattori *et al*. [72] found by bluegrass under drought stress with the application of Si and concluded that it happened due to improved water uptake, root growth and leaf erectness contributed by Silicone.

Similar to our results for getting enhancement in water use efficiency with biochar applications, Romdhane *et al*. [73] concluded about getting considerable reduction in drought sensitivity and improved water use efficiency in maize plant. Water use efficiency (WUE) depicts the performance of a crop facing any environmental constraint. Several studies have demonstrated similar results of contribution of declined transpiration rate (Tr) and increased photosynthetic rate (Pn) towards water use efficiency [74–75]. In our experiment, we achieved WUE enhancement through reduction in transpiration rate and escalation in photosynthesis rate.

5. CONCLUSION

Water, in certain quantity, is the basic requirement of all the life forms existing on planet earth for optimal growth and survival. Water use efficiency (WUE) acts as the most crucial factor that can limit plant growth. Current scenarios of

rising temperatures (contributed mostly due to anthropogenic reasons) with the consequences of climate change are contributing severe weather patterns with flash floods, droughts and threatened watersheds are going towards extreme water scarcity. Soil amendments like biochar have proven their vital role as one of the paramount methods in overcoming stress due to drought conditions. Along with its contributions as soil amendment, biochar was evaluated for its ameliorative potential as microbial carrier for rhizobial strain in *Phaseolus* plants exposed to water stressed condition. The present study advocates rice biochar for its ability to enhance tolerance in *Phaseolus* against drought stress through its role as inoculum carrier contributing suitable habitat for the microorganism. The huge quantities of rice residues produced in Pakistan could be utilized efficiently in the form of biochar and could solve the problem of biomass burning and the resultant smog. In the arid regions of Pakistan, where drought is prevalent, application of rice-husk biochar can significantly enhance the plants' tolerance against this climate stress by improving their water use efficiencies. In conclusion the synergistic use of rice husk biochar and rhizobacteria could be a promising strategy in improving growth and productivity of *P. vulgaris* L. under drought stress as compared to the biochar prepared from *Lantana camara*.

The beneficial role of biochar prepared from crop residues of the same plant (i.e., *P. vulgaris*) invites further research to explore its true potential for growth as well as grain yield enhancements of this plant using isolated strain inoculum in water deficit areas focusing on plant's physiological responses. Comparative evaluation of biochar over other known carrier materials like peat and lignite for the isolated strains could be done. The inoculation effects on Nitrogen retention and BNF by *P. vulgaris* with different application rates of biochar as well as for long term applications in the field could further be explored. Study could be focused on micronutrient uptake of the bean plant as affected by biochar inoculum combination.

6. ACKNOWLEDGEMENTS

Authors are thankful to all who have contributed in research, data analysis, write-up and publication of this manuscript.

7. CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Proceedings of the Pakistan Academy of Sciences: B
Life and Environmental Sciences 60(3): 539-548 (2023)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(60-3)927

Pakistan Academy of Sciences

Research Article

Role of Plant Growth Regulator and Organic Fertilizer in Growth Stimulation and Quality Enhancement of Muskmelon (*Cucumis melo* L.)

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Abstract: The application of Plant Growth Regulators (PGRs) and certain types of fertilizers can enhance plant growth, production as well as sugar content of muskmelon. Aiming to determine the role of PGRs in stimulating growth and to ascertain the ability of both organic fertilizers and biofertilizers to raise the level of sweetness in muskmelons, this study put a split-plot design into work – the main plots were assigned to fertilizers (P0 = no organic fertilizer, P1 = liquid organic fertilizer, P2 = organic biofertilizer) while sub-plots to PGRs (Z0 = no PGRs (water), Z1 = coconut water, Z2 = GA3). The result concluded that biofertilizer (P2) increased the fruit weight by 11.76 % at an average of 2.66 kg. It also boosted the sugar content by 29.53 %, much higher than organic fertilizer at 15.46 %. As for PGRs, GA3 (Z2) was proven to enhance the sweetness in muskmelon by 23.62 %, higher than coconut water at 16.63 %. The net pattern on the rind of the P2 treatment was smooth, while the ones of P1 were rough. The very fragrant aroma was obtainable by applying biofertilizer (P2) and coconut water as PGRs (Z1).

Keywords: Biofertilizer, Gibberellin, Integrated farming, Rind net pattern, Sweetness level

1. INTRODUCTION

Muskmelon (*Cucumis melo* L.) is widely consumed due to its sweet juicy flavor. It contains polyphenols, organic acids, lignan, and other polar compounds beneficial to health [1–4]. The appearance of the netted rind texture and the sweet flavor of the fruit are the most captivating features for end-users, and its nutritional aspects have made it one of the most demanded cucurbitaceous products.

The factors responsible for muskmelon's taste are genetic as well as non-genetic such as microclimatic environment, pest, disease, and inappropriate fertilization [5] – may be beyond the farmers' awareness, making them inattentive to maintain or even enhance the sweetness. Such purpose calls for plant growth and harvest quality management by administering organic fertilizers and plant growth regulators (PGRs).

An agreeable alternative both economically and environmentally is organic fertilization which supports nutrient cycle and supplies carbon to soil [6, 7] better than mineral fertilization. Muskmelon can extract > 80 % of total applied nutrients Nitrogen (N) and Potassium (K) are the most vital compounds. N particularly affects rooting system, fruit ripening, and K absorption in the muskmelon plant [8], therefore influential towards yield,

Received: January 2023; Revised: May 2023; Accepted: June 2023
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quality, juice quantity, total dissolved solids (TDS), and total acidity [9] as well as flesh thickness and fruit quantity [10, 11]. Organic media of bokashi, cocopeat, and rice husk charcoal (60:20:20) has been proven effective in increasing the sweetness intensity in muskmelon of Action 434 F1 variety [12].

A hormone naturally produced in small amounts, gibberellin is able to boost germination, shoot formation, stem elongation, leaf growth, flower budding, fruit enlargement, and root growth as well as its differentiation. According to [13], applying 30 mg kg^{-1} of gibberellin enhances muskmelon growth and quality. Rahman *et al.* [14] has proven that both soaking the plant root in 50 mg kg^{-1} of GA3 and applying 25 mg kg^{-1} of it in the flowering stage equally increase the quantity of flowers and yields significantly in each tomato plant.

Coconut water is a source of natural PGRs. Containing zeatin – a plant growth hormone in the cytokinin family – it reserves the capacity to encourage cell division and certain tissue differentiation in shoot formation and root growth. Yet, the presence of other phytohormones – particularly auxin – is key for cytokinin's role in cell division [15]. Ansar and Paiman [16] state that coconut water of 40 % concentration as natural PGRs gives the best result in heightening the growth and yield of shallot.

Based on the functions of organic fertilizers and growth hormones, it is necessary to combine the two to be applied to melon cultivation to increase the quality of melons. Several previous studies [17–21] recommended mixed research between PGRs and organic fertilizers (solid and liquid). With these considerations, this research was conducted to see how well organic fertilizers containing gibberellin and coconut water as natural PGRs work in the quality enhancement of muskmelon.

2. MATERIAL AND METHODS

The research was conducted between April and July 2019 in the farmland of Klurahan Village of Kartoharjo District, Magetan Regency, East Java, Indonesia, coordinate S 7°32'47.184" E 111°28'47.1252". It is situated in 60 m to (60-75)m above sea level, the area's rainfall is rated at 1 616.9 mm and environmental temperature at 20 ℃ to 30 ℃ with approximately 82.0 % humidity. The soil characteristics for this study are detailed in Table 1 below.

The organic fertilizer was of fruit sweetening, applied once per day at 10 g L^{-1} water for 7 d during the generative period. The biofertilizer at 3 ml L^{-1} water was sprinkled once per 7 d. The seeds for the treatments were soaked in their respective PGRs for 8 h. Once 3 to 4 leaves were formed in each seedling, they were transferred to the beds and planted at a distance of (60 × 50) cm. The basic fertilizers administered were 15 t ha^{-1} manure, 250 kg ha^{-1} urea, 250 kg ha^{-1} SP-36, and 250 kg ha^{-1} KCl, followed up weekly. Referring to Prasetyo *et al.* [21], Table 1 shows the research locations is categorized as medium soil fertility (N, P, and K nutrients), while organic matter and CEC are classified as high.

Table 1. Soil characteristics analyzed for the study

Characteristics	Value
pH (H_2O)	5.88
C-organic (g 100 g^{-1})	4.16
Organic substances (g 100 g^{-1})	4.40
C/N ratio	9.01
N total (g 100 g^{-1})	0.46
P_2O_5 total HCL 25 % (mg 100 g^{-1})	35.05
P_2O_5 Bray (mg 100 g^{-1})	9.93
K_2O total HCl 25 % (mg 100 g^{-1})	36.81
CEC (me 100 g^{-1})	32.79

Three multiplications of the split-plot design were performed. The main plots were of fertilizers planned to be P0 (without organic fertilizer), P1 (with liquid organic fertilizer), and P2 (with biofertilizer), while the sub-plots were of PGRs devising Z0 (fresh water without PGRs), Z1 (coconut water), and Z2 (GA3). Liquid organic fertilizers (Brix up) that are applied are fertilizers containing sucrose, sulfate salts, K, Ca, and Cl. The biofertilizer used is Bio to Grow Gold (BGG) fertilizer, an organic fertilizer containing macro and micronutrients, microbes, and growth regulators. The microbes contained in BGG include *Actinomycetes*, *Azotobacter* sp, *Azospirillium* sp, *Rhizobium* sp, *Pseudomonas*, *Lactobacillus* sp, *Bacillus* sp, *Cytophaga* sp, *Streptomycetes* sp, *Saccharomyces*, Cellulotic, BPF, Mycorrhiza, Trichoderma. The elements contained in BGG include organic materials, including 2 %,

7.5 % organic, 2.35 % N, 3.5 % P_2O_5, 2.24 % K_2O, 1.1 % CaO, 0.1 % MgO, S 1 %, Fe 0.58 %, Mn 0.3 %, B 2 250.80 mg kg^{-1}, Mo 0.01 %, Cu 6.8 mg kg^{-1}, Zn 0.2 %, and Cl 0.001 %.

The observed variables were plant height, leaf size, chlorophyll content (determined with a spectrophotometer at harvest time), fruit weight, and sugar content (recorded using a hand refractometer at harvest time). Also tested organoleptically – involving 20 panelists – were taste, aroma, flesh texture, and net patterns on the rind. The data obtained were run through analysis of variance and statistically recorded using Statistical Product and Service Solutions (SPSS) version 25.0. Any significant difference that occurred should call for Duncan's test at the value of $\alpha = 5$ % [22, 23].

3. RESULTS AND DISCUSSION

In general, it is inferred that mixed treatments of PGRs and organic fertilizer administration are not significantly effective towards all studied variables.

3.1. Plant Height

The stem development represents muskmelon growth. While PGRs were insignificantly effective, fertilizers were considerably useful in boosting the plant height at the beginning and the end of the observation period (Table 2). While soaking muskmelon seeds in coconut water as PGR did not significantly affect the plant heights, their maximum growth of 131.10 cm was the highest – allowing them to reach up to 150.41 cm at 50 d old. Coconut water helps to boost metabolism and provides the required energy for seedlings to grow. The study of Zainudin and Adini [24] discovered that soaking in coconut water for 8 h promoted papaya seed germination and seedling time as well as improved the plant's height, leaf quantity, fresh mass, and dry mass. Muttaleb *et al.* [25] also confirmed that seed soaking enhanced both physiological and morphological features of vegetables, including germination and harvest. Yet, the absence of a positive response in the attempt to maximize their development potential is perceived to be the result of a clash between the seeds' endogenous auxin and the PGR's exogenous one, preventing the latter from contributing.

Organic fertilizer administration has significantly influenced the plant height at 50 d old. Muskmelon plants applied with organic fertilizer produced taller plants of 158.80 cm. It is convincing that substances in organic fertilizer have played a key role in improving the biological, chemical, and physical activities in the soil to suit it better for plants.

3.2. Leaf Size and Chlorophyll Content

Significant differences are recorded for both leaf sizes and chlorophyll contents after PGR soaking and fertilizer administering as detailed in Table 3. While GA3 immersion has expanded the leaves up to 705.75 mm^2, biological organic fertilizer administration has extended their spreads up to 695.82 mm^2. There are evidences that nutrient management involving gibberellin as PGR and biological organic fertilizer is feasible for better plant development. Specifically on PGR, gibberellin is proven to be more effective than coconut water as it advances the cellular expansion process by

Table 2. Effects of PGRs and fertilizers on plant height

Treatment	Plant height (cm)				
	10 d old	20 d old	30 d old	40 d old	50 d old
Plant Growth Regulator					
Without PGR (Z0)	19.81 a	27.53 a	65.16 a	123.71 a	150.30 a
Coconut water (Z1)	19.31 a	27.59 a	65.69 a	111.97 a	150.41 a
GA 3 (Z2)	20.95 a	29.60 b	66.86 a	124.82 a	146.84 a
Fertilizer					
Without organic fertilizer (P0)	18.19 a	28.31 a	65.98 a	121.98 a	139.20 a
Organic fertilizer (P1)	21.73 b	28.35 a	66.29 a	112.78 a	158.80 c
Biofertilizer (P2)	20.15 b	28.06 a	65.44 a	125.73 a	149.56 b

Note: Values shared by the same letters are not significantly different among the treatments at Duncan's test with $\alpha = 0.05$ %.

Table 3. Effect of PGRs and fertilizers on leaf size and chlorophyll content.

Treatment	Leaf width (mm^2)	Chlorophyll a ($mg\ kg^{-1}$)	Chlorophyll b ($mg\ kg^{-1}$)	Total Chlorophyll ($mg\ kg^{-1}$)
Plant Growth Regulator				
Without PGR (Z0)	479.01 a	584.14 a	849.43 a	1 432.41 a
Coconut water (Z1)	579.72b	626.18 b	933.95 b	1 558.87 b
GA 3 (Z2)	705.75 c	663.75 c	967.48 c	1 629.92 c
Fertilizer				
Without organic fertilizer (P0)	477.60 a	601.68 a	879.99 a	1 480.49 a
Organic fertilizer (P1)	603.73 b	62.16 b	912.92 b	1 533.85 b
Biofertilizer (P2)	695.82 c	650.24 c	957.94 c	1 606.88 c

Note: Values shared by the same letters are not significantly different among the treatments at Duncan's test with α = 0.05 %.

stimulating xyloglucan endo-transglycosylase – an enzyme component to the cell walls – to detach hemicellulose, allowing microfibrillated cellulose reposition and widen cell walls. This outcome agrees with Rahman *et al*. [14] stating that immersing the root of a tomato seedling in 50 mg kg^{-1} of GA3 increased flower, fruit, and harvest production.

Regarding chlorophyll, GA3 immersion has boosted the formings of chlorophyll a up to 663.75 mg kg^{-1} and chlorophyll b up to 967.48 mg kg^{-1} with a total of 1 629.92 mg kg^{-1} while biofertilizer administration has enhanced the amounts of chlorophyll a up to 650.24 mg kg^{-1} and chlorophyll b up to 957.94 mg kg^{-1} with a total of 1 606.88 mg kg^{-1}. Able to produce higher chlorophyll contents quite substantially, GA3 immersion is, therefore, more effective than biofertilizer administration for the purpose. It is the result of improved absorption of stimulated nutrients – particularly nitrogen and chlorophyll synthesis – essential in assimilating amino acids and nucleic acid to promote chloroplast in forming better chlorophyll [26].

Chlorophyll construction relies on sun rays and nitrogen as well as magnesium [13] therefore broader leaves should receive more sunlight and be able to produce more chlorophyll. Since GA3 and biological organic fertilizer optimize leaf size, muskmelon plants with such treatments contain more amounts of chlorophyll than ones with different treatments.

3.3. Fruit weight and sugar content

While PGR treatment made an insignificant difference in fruit weight, it was noteworthy regarding sugar content. Fertilizer administration, on the other hand, was significantly influential in determining fruit weight and sugar content (Table 4).

Bioto Grow Gold (BGG) – the biofertilizer employed in this study – has complemented the weight rate of muskmelon up to 0.15 kg compared to liquid organic fertilizer. Among the microbes contained in the product are phosphate-solubilizing

Table 4. Effects of PGRs and fertilizers on fruit weights and sugar contents

Treatment	Fruit Weight (kg)	Sugar Content (%)
Without PGR (Z0)	2.47 a	8.00 a
Coconut water (Z1)	2.56 a	9.33 b
GA 3 (Z2)	2.52 a	9.89 b
Fertilizer		
Without organic fertilizer (P0)	2.38 a	7.89 a
Organic fertilizer (P1)	2.51 ab	9.11 b
Biofertilizer (P2)	2.66 b	10.22 c

Note: The same letter following each number in a group signifies an insignificant difference in accordance with Duncan's test at α = 5 %.

bacteria (PSB) and *Lactobacillus*. PSB is beneficial in enhancing soil and plants by providing available phosphate, while *Lactobacillus* facilitates the organic decomposition of cellulose into monomer glucose pertinent to carbon and energy sources. In addition to weight increase, the aforementioned microbial activities are proven to intensify the fruit's sweetness by up to 10.22 %. These results are in agreement with the study of Aritonang, and Surtinah [27] which discovered the highest sugar content in the Sakata Glamour variety of muskmelon at 12.25 % after BGG application at 3 mL L^{-1}. References [28, 29] confirmed that higher K, Mg, and Mn absorption performed by microbes in fertilizer amplifies sugar content in fruit.

3.4. Organoleptic test

An organoleptic test involving 20 panelists was performed to reveal the taste, aroma, flesh texture, and rind pattern of muskmelons harvested from the treatments [30]. The results are graphically presented in Figure 1, Figure 2, Figure 3, and Figure 4.

The panelists have selected the fruit of plants with biological organic fertilizer administration (P2) to be the sweetest; Z0P2, Z1P2, and Z2P2 treatments each scored a > 50 %. This result goes along with the result of the sugar content test. It is therefore concluded that macro and micro substances in BGG – e.g., Organic = 7.5 %, Organic ingredients = 2 %, N = 2.35 %, $P_2 0_{5:}$ = 3.5 %, K_2O = 2.24 %, CaO = 1.1 %, MgO = 0.1 %, S = 1 %, Fe = 0.58 %, Mn = 0.3 %, B = 2 250.80 mg kg^{-1}, Mo = 0.01 %, Cu = 6.8 mg kg^{-1}, Zn = 0.2 %, and Cl = 0.001 % – are supportive towards sugar increase in muskmelon [31, 32].

Most treatments delivered enough aroma in muskmelon to the panelists to score > 50 % except for the fruit from plants treated with coconut water and liquid organic fertilizer (Z1P1) at 36.67 %. Most panelists declared the fruit from plants treated

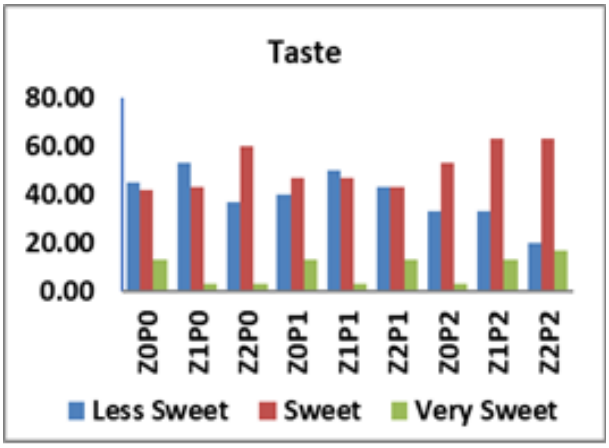

Fig. 1. Organoleptic test on taste

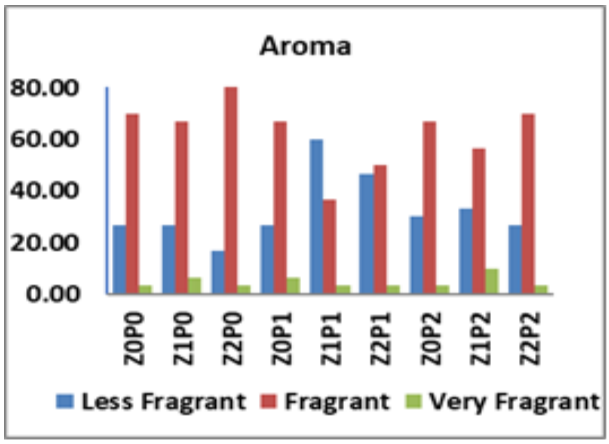

Fig. 2. Organoleptic test on aroma

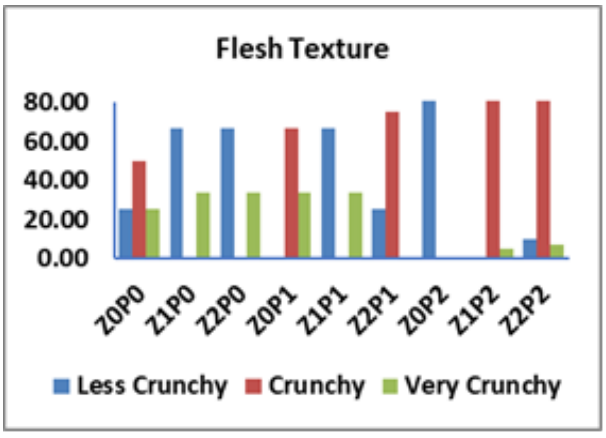

Fig. 3. Organoleptic test on flesh texture

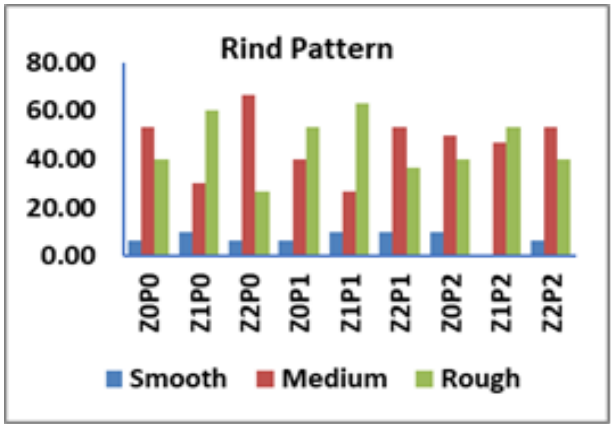

Fig. 4. Organoleptic test on rind pattern

with coconut water and biological organic fertilizer (Z1P2) to be very fragrant. Oh *et al.* [31] stated that the volatile aroma in muskmelon should change following the fruit development to ripeness, which appears in the muskmelon of the Pertiwi variety used in this study.

Assorted answers regarding flesh texture were recorded by the panelists. All muskmelons from plants with PGR and biofertilizer treatments are generally considered crunchy. Meanwhile, the ones from plants without PGR but with biofertilizer are declared less crunchy by all panelists.

Net pattern is formed on the rind of all muskmelons. Most panelists preferred the medium or rough patterns, while only a small number of them (≥ 10 %) chose the smooth ones. Daryono, and Maryanto [33] affirmed that net patterns differ after the quantity of water obtained by a plant. The effect of rind surface cracking is due to faster cell growth in the fruit center than in rind, a sufficient amount of water should accelerate the process, forming more distinctive net patterns on the rind.

Future research should be carried out in locations with low soil fertility which is expected to show mixed research performance between organic fertilizers (liquid and solid) and PGRs [17–21]. Likewise, the aim of farmers' self-reliance should be studied using local PGRs and liquid and solid organic fertilizers produced locally by farmers [34–41]. Integrating agricultural waste residue, leftover, kitchen waste, manure, and livestock urine in an anaerobic digester should be studied [42–45]. With this action, there are several advantages. Namely, farmers get high-quality biogas - renewable energy [46–48], a liquid and solid organic fertilizer [49–51]. It is a wise policy to implement toilet-linked anaerobic digesters (TLADs), *i.e.*, connect household-scale or communal-scale biogas digesters to household latrines/toilets [52–54] to improve environmental health and the quality of renewable energy and its residues in organic fertilizers (solids and liquids) [55–57].

4. CONCLUSION

PGRs immersion is conclusively supportive towards muskmelon plant growth as it increases the leaf size, allowing more chlorophyll to form and work in fruit bearing process. Further, organic fertilizer administration – be it liquid or biological – is beneficial in producing bigger, sweeter muskmelon. Since all favorable features of muskmelon – sweet taste, fragrant aroma, crunchy texture, and rough rind pattern – are overall achieved by administering biofertilizer, its use is therefore recommended to optimize the yield.

5. CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Proceedings of the Pakistan Academy of Sciences: B
Life and Environmental Sciences 60(3): 549-555 (2023)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(60-3)834

Pakistan Academy of Sciences

Research Article

Surfactant-Promoted Prussian Blue Analogues Fabricated Electrodes for Electrocatalytic Water Oxidation

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Abstract: Prussian blue analogues (PBAs) have unique structural and chemical behaviour and therefore have applications in various fields of catalysis as energy conversion materials for storage devices and molecular sensing. Herein we focused on the in-situ synthesis of three PBAs comprising cobalt hexacyanoferrate (CoHCF), nickel hexacyanoferrate (NiHCF), and cobalt-nickel hexacyanoferrate (CoNiHCF) through cation i.e. cetyltrimethylammonium bromide (CTAB) assisted drop cast method. The electrocatalysts were characterized through a multitude of spectroscopic techniques and were tested for water oxidation study. It was found that among the three electrocatalysts, CoNiHCF showed comparatively better catalytic performance with an overpotential value of 570 mV (at 1 mA cm^{-2})

Keywords: Prussian Blue Analogues, Coordination Polymers, Cationic Surfactants, Cetyltrimethylammonium bromide, Water Oxidation Studies

1. INTRODUCTION

We are heavily reliant on fossil fuels to meet the annual global energy demand [1]. However, the excessive burning of fossil fuels results in the emission of CO_2 into the atmosphere, which can be avoided by substituting non-toxic and renewable fuels for fossil fuels [2-3]. To convert solar energy into a usable form, the photovoltaic system has also emerged as a viable solution, but it still has significant drawbacks. As a result, finding a different source of energy is crucial [4-6]. Given all the options, hydrogen having the highest energy density and producing non-toxic byproducts is regarded as one of the most suitable energy sources [7]. Electrochemical water splitting is an intriguing method for producing hydrogen [6, 8]. However, oxygen evolution reaction (OER) has sluggish kinetics that needs more overpotential, and therefore, effective electrocatalyst that can reduce the activation energy, consequently, the overpotential of the reaction is required. The most common electrocatalysts utilized in the process are metal oxides based on Ir, Ru, and Pt [9-11]. However, their high cost and scarcity have significantly limited the usage of these metal oxides [11]. In the process of splitting water, several transition metals are also used as metal oxide electrocatalysts. First-row transition metal oxides, however, have several drawbacks, including the fact that metal oxide performance varies on different experimental conditions, such as morphology, temperature, etc [11]. Consequently, it is crucial to control them in order to obtain correct findings. Otherwise, it makes their applicability very challenging [11]. Additionally, the first-row transition metal oxides function best in an alkaline environment (Ph $\geq$ 13) but are less effective in acidic or neutral conditions [12]. Different non-oxide materials such as metal phosphides, sulphides, selenides, nitrides, based molecular-based organic metal-organic frameworks (MOFs), and polyoxometalates, etc. are known water oxidation catalysts (WOCs) with advantageous qualities, such as simplicity in synthesis, stability over a wide pH range, and durability during catalytic processes [13]. Among different coordination polymers, PBAs have been employed as heterogeneous electrocatalysts for OER [14]. The group of Galán-Mascarós have extensively studied the role of PBAs as WOCs

Received: January 2023; Revised: April 2023; Accepted: June 2023
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[15-18]. The group proved that PBAs are important electrocatalysts in water oxidation because of open metal active sites, the high porosity of the framework, and most importantly the easy oxidization of metal ions to their higher oxidation states [18]. The group of Karadas reported the preparation of amorphous PBAs through a novel synthetic route that exhibited high electrocatalytic water oxidation activity [4]. Recently we have reported the preparation of amorphous bimetallic PBAs following the pyridinium based surfactant assisted route to prove that surfactants play a significant impact on the film development (binder-free approach), better stability and charge transfer kinetics of OER [19]. We further extended the fabrication approach to electrodeposition method as well [20].

Herein, we examined the effect of a cationic surfactant on the electrocatalytic water oxidation performance of synthesized bimetallic and trimetallic PBA films. In the presence of cationic surfactant, PBA films have been deposited on the glassy carbon electrode (GCE). The fabricated electrode ultimately demonstrated a significant improvement in the electrochemical performance of synthesized catalysts toward OER.

2. MATERIALS AND METHODS

2.1. Chemicals

The chemicals that include sodium hexacyanoferrate (II) ($Na_4Fe(CN)_6$; HCF), sodium hydroxide (NaOH), and sodium nitrate ($NaNO_3$) were purchased from Sigma-Aldrich (St. Louis, MO, USA). For cobalt and nickel sources, the nitrate salts were used. Cetyl-trimethyl ammonium bromide (CTAB) as cationic surfactant and DMF as solvent was used. All the solutions were prepared in deionized water at room temperature.

2.2. Instrumentation

The Gamry Interface 1010E potentiostat/galvanostat is equipped with a standard three-electrode setup comprising platinum wire as a counter electrode, Ag/AgCl (3 M KCl) as a reference electrode, and modified glassy carbon (2 mm in diameter) as working electrode

The transmission spectrum of each powder sample was recorded in the frequency range of 4000 – 400 cm^{-1} and performed on a Thermo Nicolet-6700 FT-IR spectrophotometer. Powdered X-ray diffraction (PXRD) analysis of the synthesized materials was recorded on a PANalytical X'pert instrument equipped with CuKα X-ray source (λ = 1.5418 Å) in the range of 20 to 80◦. Scanning electron microscopy (SEM) was carried out using FESEM (NOVA-600) coupled with Bruker EDX system at an accelerating voltage of 3 kV.

2.3. *In-situ* Synthesis of Catalyst on the Electrode Surface

Before modification of the GCE, it was initially washed with alumina powder slurry on a polishing pad. The electrode was dipped in acetone and sonicated for about 30 min to remove solid particles from the electrode surface if any. After sonication with acetone, it was sonicated with distilled water. Finally, it was dried in an oven.

To develop the corresponding PBAs films (i.e. of CoHCF, NiHCF, or CoNiHCF) films, a drop of surfactant in DMF was added onto the GCE surface using a micropipette. Once the electrode dried out, a 10 µL of 10 mM aqueous HCF and 15 mM aqueous corresponding metal salt solutions were added simultaneously (schematically presented in Figure 1).

3. RESULTS AND DISCUSSION

3.1. Synthesis and Characterization

The synthesis of PBAs through a surfactant-assisted mechanism is the key factor in generating an efficient electrocatalyst for OER in this work. The surfactants form hemimicelles onto the surface

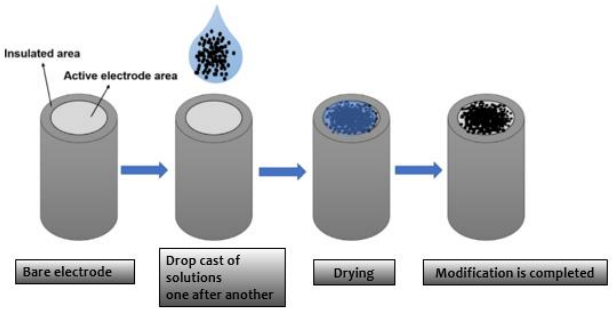

Fig. 1. Schematic presentation of electrode modification.

of the GCE such that the headgroups are exposed to the aqueous solution. This overall supports the binding of the anionic site of PBAs with surfactant. The synthesis of electrocatalysts was confirmed through FT-IR spectroscopy and the overlaid spectra of all catalysts are shown in Figure 2. The figure shows that synthesized electrocatalysts have all the characteristic peaks associated with PB-type systems. Cyanide stretching frequency is the distinctive feature of cyanide-based coordination compounds. All three compounds show sharp bands in the frequency range 2079-2093 cm^{-1}. The cyanide stretching frequency increases with the increase in the positive character of the metal. That is why it is slightly high for NiHCF and then for CoNiHCF. At about 1610 cm^{-1} there is a sharp band that corresponds to OH bending due to water molecules trapped inside the structure. While at 3200-3500 cm^{-1} a broadband represents OH stretch. At about 3000 cm^{-1} there is a hump that is because of the C−H stretch due to the presence of CTAB. 1500-1000 cm^{-1} is a fingerprint region, representing alkyl group bending. The band at 500 cm^{-1} corresponds to M−C stretching.

The crystalline content and phase of the synthesized compounds, PXRD was performed, and the spectra are shown in Figure 3. The spectra reveal that all the samples are iso-structural with the PB crystal system, having a face-centered cubic lattice and Fm3m space group symmetry. All the characteristic 2θ peaks are in good agreement with the reference databases for NiHCF and CoHCF [21]. The slight shift of the peaks' positions can be attributed to the presence of CTAB. The comparative spectra further reveal that the crystallinity of trimetallic CoNiHCF is reduced compared to bimetallic PBAs.

To further study the morphology of the PBAs scanning electron microscopy was performed. It appears in the form of two-dimensional images exhibiting the structural properties of the PBAs. For CoNiHCF Figure 4 shows the regular plate-like structures with uniform growth as shown in the close image with a resolution of 2 µm. The EDX spectrum for CoNiHCF confirms the presence of given metal atoms and hints at the atomic ratio of metals (Figure 5). The proposed molecular formula based on the stoichiometric ratio of the metals is $Co_{1.4}Ni_{0.3}CTAB_{0.3}[Fe(CN)_6]\cdot nH_2O$.

3.2. Electrocatalytic performance

LSV plots of all the PBAs in the buffer of neutral pH i.e., 7 with a potential range of 0-1.5 V Vs Ag/AgCl at a scan rate of 50 mV sec^{-1} were taken after IR compensation. From the graph, as shown in Figure 6 (a), we can say that the activity of NiHCF with CTAB is least toward water oxidation reaction. CoHCF with CTAB performs better towards water oxidation but the best results are obtained for CoNiHCF with CTAB giving the overpotential of 570 mV at 1 mA cm^{-2} current density. This can be attributed to the amorphous nature of CoNiHCF with CTAB compared to the others, hence providing more surface area for the reaction to occur. A comparison of the overpotential and Tafel slope for all three materials is given in Table 1.

To check the reaction kinetics Tafel plots were drawn between log j vs. overpotential. The slope of

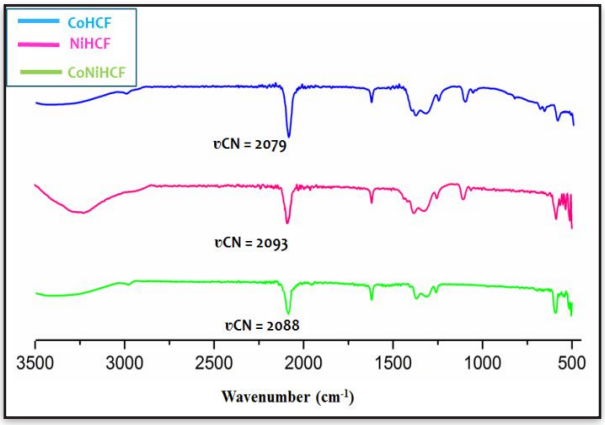

Fig. 2. Overlaid FT-IR spectra of synthesized electrocatalysts in the presence of CTAB.

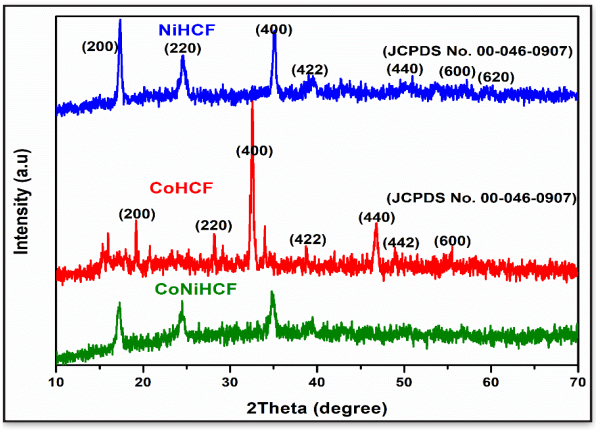

Fig. 3. PXRD patterns for all three synthesized PBAs.

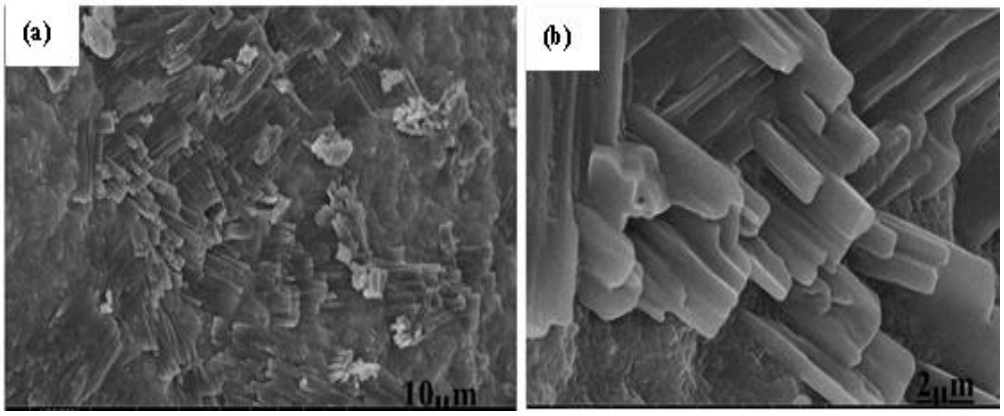

Fig. 4. SEM images of CoNiHCF at (a)10 μm and (b) 2 μm.

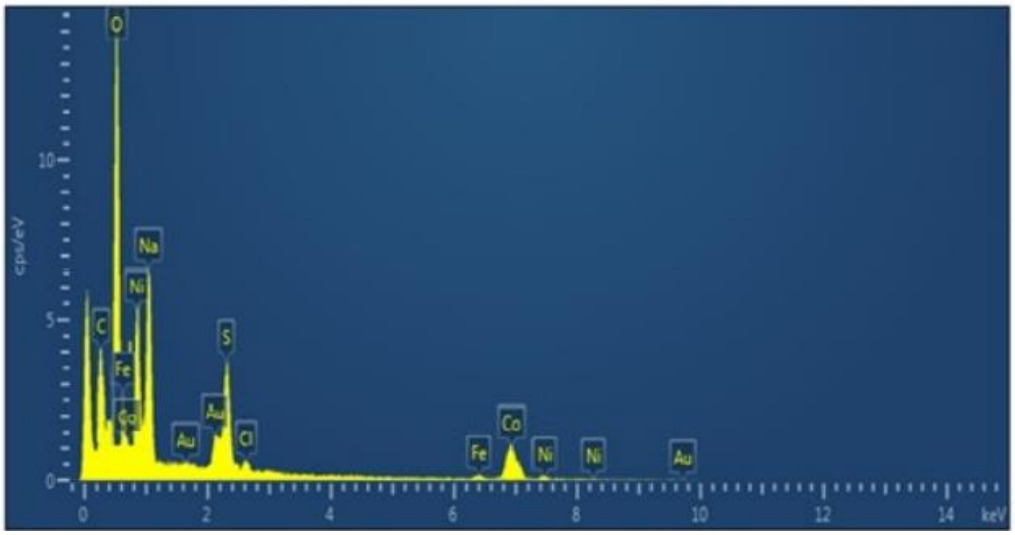

Fig. 5. EDX spectrum of CoNiHCF

the graph reflects reaction kinetics. The lower the Tafel slope, the faster will be the reaction kinetics. Figure 6 (b) shows the Tafel slope of all three compounds, where CoNiHCF has the lowest value of Tafel slope i.e., 133 mV/dec which indicates the fast reaction kinetics of trimetallic CoNiHCF for the water oxidation reaction considering more active sites on its surface than the other two bimetallic PBAs.

Cyclic voltammetry (CV) is used to measure the electrochemical surface area (ECSA) of the fabricated electrode in the non-faradic region by varying scan rates and the graph is shown in Figure 6 (c). It is clear from the graph that with the increasing scan rates the value of the current increases. From CV measurements, double-layer capacitance (C_{dl}) is calculated. The slope of the graph between scan rates ($mV.s^{-1}$) *vs.* the corresponding current (mA) provided double-layer capacitance as 45μF for CoNiHCF, (Figure 6 (d)). From the double-layer capacitance, ECSA reflecting the catalytic performance is determined. More active sites are reflected with higher ECSA possible electrochemical processes. ECSA is found to be 2.25 cm^2 for CoNiHCF. The ECSA further provides the roughness factor (RF) to be 32.1, associated with the electrocatalyst and is another important parameter to determine the catalytic efficiency. A

Table 1. Comparison of overpotential and Tafel slope for all three catalysts.

Electrocatalysts	Overpotential (mV) @ 1 mA cm^{-2}	Tafel slope (mV/dec)
CoHCF	590	139
NiHCF	750	154
CoNiHCF	570	133

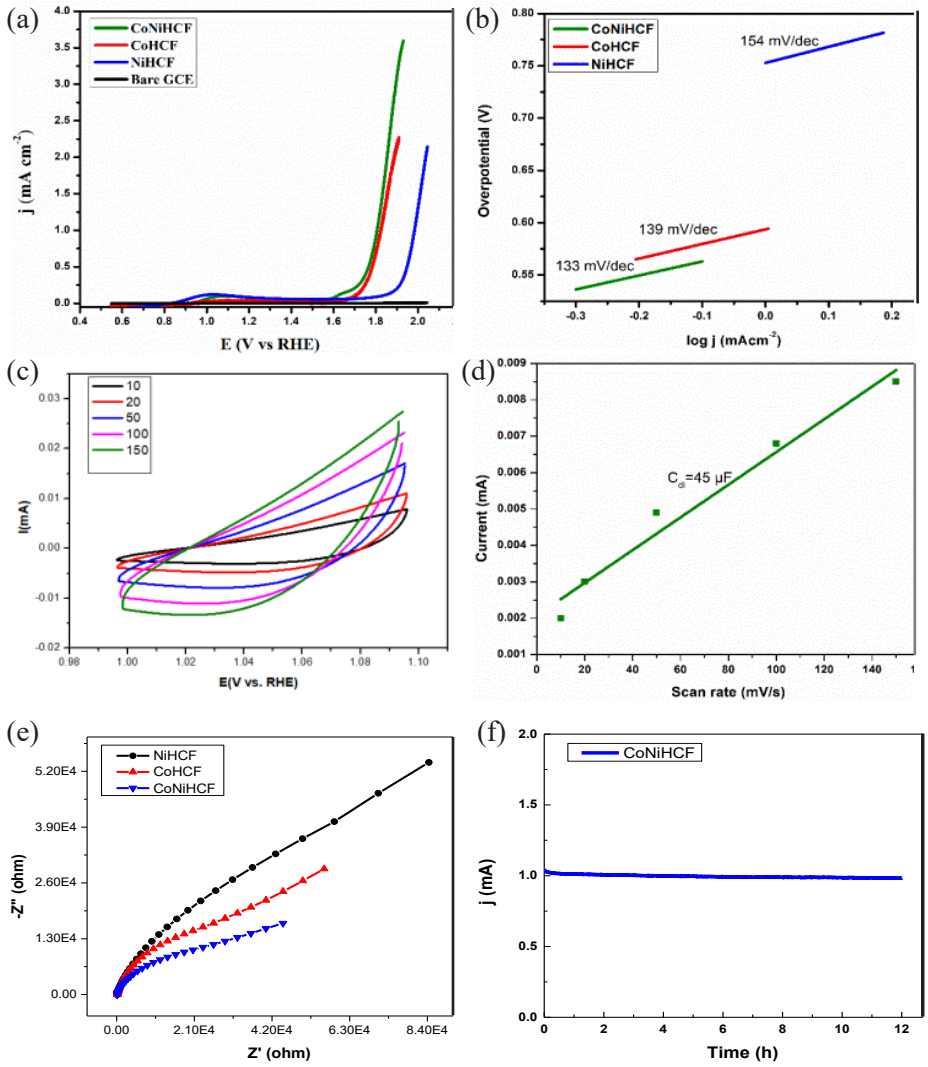

Fig. 6 (a) LSV curves for all three synthesized PBAs compared with bare GCE, (b) Tafel plots of the three PBAs, (c) CV cycles in the non-faradaic region for CoNiHCF at scan rates varying from 10-150 mV sec^{-1}, (d) measurement of double-layer capacitance from changing current and scan rate plot for CoNiHCF, (e) Nyquist plots from 0.1Hz to 100KHz frequency range for all three PBAs, and (f) chronoamperometric stability plot at potential correlated with 1 mA cm^{-2} current density for CoNiHCF.

high value of the RF means a more active surface and efficiency for the water oxidation mechanism. Electrochemical Impedance Spectroscopy is used to investigate the changes that take place in the interfacial properties of the electrode upon their encounter with the analyte species [22]. The plots in Figure 6(e) illustrated a smaller semicircle in the high-frequency region corresponding to the lower value of the charge transfer resistance (R_{ct}). CoNiHCF has a very efficient charge transfer process with a very small resistance because of which it has high water oxidation catalytic efficiency.

During the water oxidation process, the stability of the working electrode is tested by using chronoamperometry 1.8 V vs. RHE (constant potential) for the trimetallic CoNiHCF. The electrocatalyst produced a corresponding constant current density for 12 hours as shown in Figure 6 (f).

The comparison of electrochemical performance is given in Table 1. The data shows that the CoNiHCF has a lower overpotential and Tafel slope value which reflects more active sites

available for water oxidation. This can be attributed to the synergistic effect of the presence of Ni and Co, where both are available as active sites for the water oxidation process with faster kinetics and lower R_{ct}.

4. CONCLUSION

In this work, two bi-metallic and one tri-metallic PBAs were synthesized onto the surface of a glassy carbon electrode by applying surfactant-promoted in-situ approach to investigate their performance as heterogeneous electrocatalysts for OER. The presence of CTAB as a cationic surfactant ultimately enhances the electrochemical performances of the PBAs. The electrochemical water oxidation studies of all the fabricated electrodes were performed at neutral pH (Na_3PO_4 buffer, $NaNO_3$ as electrolyte). The electrode fabricated with CoNiHCF required the lowest overpotential of 570 mV compared to CoHCF (required 590 mV) and NiHCF (required 750 mV) to achieve the current density of 1 mA cm^{-2}. Tri-metallic CoNiHCF being a better catalyst than the other synthesized ones, showed an ECSA of about 2.25 cm^2 with a roughness factor of 32.1. The surfactant-assisted approach reported herein provides a binder-free strategy for fabricating PBA-based electrocatalysts over the electrodes and the approach can be expanded to other related materials and substrates as well.

5. ACKNOWLEDGEMENT

A.H. and S.A. are thankful to Pakistan Academy of Sciences for Financial support in the form of project funds (2020-22).

6. CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Proceedings of the Pakistan Academy of Sciences: B
Life and Environmental Sciences 60(3): 557-564 (2023)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(60-3)Proceedings

Pakistan Academy of Sciences

Workshop Proceedings

Proceedings of the ANSO-PAS-QAU Workshop 2023 on "Ensuring Biosafety: Empowering Trainers in Risk Management and Biosecurity"

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Abstract: Three days workshop entitled **"**Ensuring Biosafety: Empowering Trainers in Risk Management and Biosecurity,**" (**August 12-14, 2023) was organized at Bara Gali Campus, University of Peshawar. The workshop consisted of practical lessons on biosafety in the lab and during fieldwork, risk assessment techniques, biosecurity practices, experiment design, and execution. In addition, risk management, policy-making, and the rising concerns of antibiotic resistance were also discussed by keynote speakers and trainers. The course included theoretical lectures and hands-on exercises, allowing attendees to put their newfound knowledge to use in realistic situations. A field excursion also highlighted several plant types and aspects related to handling possibly toxic plants. In summary, the event stresses the importance of having a thorough familiarity with biosafety and risk management while practicing laboratory procedures.

1. OVERVIEW

Education and training of laboratory staff are essential to gain sufficient awareness to handle biologically hazardous materials as per internationally documented strategies [1]. Such hazardous material can be biological agents (pathogens, toxic plants or animals and their products), chemicals/reagents, etc., which can pose a threat to the environment and human health. Laboratory workers especially post-graduate students need training sessions on biological safety and related issues. Keeping in view this critical need, a three-day Workshop was organized by the Pakistan Academy of Sciences (PAS), Quaid-i-Azam University (QAU), and the University of Peshawar; and sponsored by the Alliance of International Science Organization (ANSO) at the Bara Gali Campus of the University of Peshawar on August 12-14, 2023. Over 45 participants (mainly post-graduate students) from five different universities (namely Quaid-i-Azam University Islamabad, University of Peshawar, Hazara University, Riphah International University, and Khyber Medical University) attended the event. The workshop consisted of 11 lectures and 13 practical sessions.

2. INAUGURAL SESSION – DAY 1

The inaugural session was graced as Chief Guest by Prof. Dr. Mukhtar Ahmad (Chairman, Higher Education Commission) along with the Guest of Honor Prof. Dr. Jehan Bakht (Vice Chancellor of Agriculture University Peshawar). Prof. Dr. Sumera Afzal (Director Center of Biotechnology and Microbiology, University of Peshawar) formally inaugurated the event. Subsequently, Prof. Dr. Zabta Khan Shinwari presented a keynote talk on "Ethics, Education, and Training in Emerging Frontiers". Dr. Shinwari emphasized the significance of not only providing technical expertise, but also cultivating ethical principles, visionary thinking, leadership capabilities, collaborative aptitude,

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and proficiency in science communication. He espoused the importance of developing proficient science communication abilities, which serve as a vital means of connecting scientific progress with the general population. He provided illumination about the intriguing prospects presented by synthetic biology. Nevertheless, he also highlighted certain ethical dilemmas, including the concept of generating offspring without biological progenitors.

The Chief Guest, Dr. Mukhtar Ahmad emphasized shaping a self-reliant Pakistan through knowledge sharing and impactful research. In his address, he appreciated the efforts of QAU, PAS, ANSO, Pakistan Biological Safety Association, and the University of Peshawar for jointly organizing this useful event. He emphasized that every scholarly endeavor and academic effort ought to be motivated by a vision to create a concrete influence on our society. The genuine influence of our research resides in the constructive societal transformations it engenders. The honorable chief guest informed the audience that Pakistan currently has a student population of over 6 million individuals (enrolled in universities) and is stressed to capitalize on its potential to generate products, technologies, and breakthroughs. He stated "*We must channel this potential for the betterment of our nation and its citizens. This aims to undertake a critical examination of the underlying factors contributing to the challenges encountered by our nation. What are the reasons for our underperformance in specific domains? Why is utilizing our intellectual resources not resulting in acquiring advantageous outcomes? The solution can be found inside our shared obligation, selflessness, hard work, commitment, and devotion towards our beloved country".* In summary, it is imperative to adopt the idea of sharing, encompassing the dissemination of knowledge, allocation of resources, and assumption of responsibility. Collectively, we have the potential to mold a forthcoming era in which Pakistan can emerge as a symbol of self-reliance, ingenuity, and advancement. Figure 1 represents the guests, master trainers Keynote speakers, and participants.

2.1. Session I

Subsequently, the session commenced after a tea break, encompassing a total of five stations, each led by a respective trainer. The workshop centered around various topics within the realm of biosafety and biosecurity. The initial station (Station-I) focused on "Four Primary Controls of Biosafety," where Dr. Ikram Ullah elucidated the first control known as personal protective equipment (PPE), encompassing all protective gear designed to safeguard us from potential risks. Following this, the second primary control, referred to as engineering control, was discussed, shedding light on the meticulous manufacturing processes in laboratories based on specific risk assessments. Subsequently, attention turned to the third primary control, Standard Operating Procedures (SOPs), wherein Dr. Ikram Ullah emphasized the criticality of having proper emergency instructions and protocols documented comprehensively during unforeseen situations within the laboratory. The final aspect covered was leadership, delving into the implementation of SOPs, the necessity of rigorous training, precise guidelines, and continuous surveillance to effectively regulate biosafety measures.

Moving forward, we arrived at station II which focused on "Biological spill management", led by Dr. Faouzia Tanveer. She imparted insights into the management of spills within laboratory settings. She elaborated on the two types of possible spills, namely chemical and biological, outlining a series of steps for their effective containment. Emphasizing the paramount significance of spill management, she highlighted the necessity of a well-equipped spill kit containing absorbent materials, personal protective equipment (PPE) such as gloves, masks, booties, and a biohazard bag and forceps. Furthermore, she stressed that in the event of a spill occurrence, promptly notifying others and raising awareness about the specific contamination is of utmost importance for effective response and containment measures.

Subsequently, we proceeded to station III centered on "risk group classification" which was expertly presented by Dr. Ibrar Khan. He comprehensively explained what constitutes risk groups and elaborated on the classification criteria used by both the NIH and WHO. Dr. Khan expounded on the four risk groups resulting from risk assessments, categorizing pathogens in ascending order of harm (from the least to the most harmful), as well as current

preventive and therapeutic measures. Following that, Dr. Ali Talha Khalil conducted a training session on "Containment Levels: Engineering and Biosafety" (Station-IV). He expounded on how the determination of biosafety levels in labs follows risk assessment. Within a Biosafety Level 1 lab, pathogens are handled that do not pose harm to humans, while Biosafety Level 4 labs manage organisms causing significant health impacts. Dr. Khalil also emphasized that the levels correspond with distinct infrastructural designs of the labs. The final station (Station-V) was led by Dr. Irum Iqrar, focusing on editorial policies and paper submission ethics. She provided insights into key aspects of article submission. Dr. Iqrar underscored that aligning the aims and objectives of articles with the goals of the chosen journal is of paramount importance during both article submission and journal selection. Additionally, she highlighted the significance of elements such as the cover letter, author contributions, and declaration of interest in the submission process.

2.2. Session II

In the next session, Dr. Faouzia Tanveer gave a presentation on "Bio-risk Management." She expounded upon the three stages of bio-risk management: assessment, mitigation, and performance. Dr. Tanveer also elucidated the distinction between hazard and risk, underscoring the importance of biological risk assessment due to the diversity and intricacy inherent in biological agents. She shared a risk assessment strategy, which commences with describing the activity, followed by risk identification, characterization of the identified risk, and determining the acceptability of the risk. She stated, *"Generally speaking, the risk can never be zero, try your level best to bring it to an acceptable level".*

Following that session, Dr. Javed Muhammad conducted a practical session on "Donning and Doffing of PPEs". During this session, he guided the participants through the process of properly donning personal protective equipment (PPE) after

Fig. 1. Participants of the ANSO-PAS-QAU Workshop on "Ensuring Biosafety: Empowering Trainers in Risk Management and Biosecurity" with Prof. Mukhtar Ahmad Akhtar (Chairman HEC), Prof. Dr. Jehan Bakht (Vice Chancellor AUP), Prof. Dr. Zabta Khan Shinwari (Distinguished National Professor, Prof Emeritus at QAU), Prof. Dr. Sumera Afzal (Head, Center for Biotechnology and Microbiology, University of Peshawar), Dr. Muhammad Ali (PI ANSO Project), and workshop trainers Dr. Ali Talha Khalil (Assistant Professor & Consultant Molecular Biologist at LRH-MTI, Peshawar), Dr. Ikram Ullah (Assistant Professor, Hazara University, Mansehra), Dr. Irum Iqrar (Journals Associate Editor, ORIC, The University of Lahore), Dr. Faouzia Tanveer (Senior Lecturer, Shifa Tameer-e-Millat University Islamabad), and Dr. Ibrar Khan (Associate Professor, University of Peshawar).

conducting a risk assessment and, subsequently, the recommended procedure for doffing once the task was completed. Furthermore, he provided insights into the appropriate use of lab coats, surgical suits, and Tyvek suits, including when and how to utilize them.

3. DAY 2 - SESSION I

Dr. Muhammad Ali welcomed the participants and gave an overview of Day 1 presentations and activities. Prof. Dr. Mushtaq Ahmad (Fellow, PAS) and Dr. Irum Iqrar served as session moderators. Dr. Javed Muhammad and Dr. Afreenish Amir, Technical Officer, AMR & Project Coordinator at the National Institute of Health, Islamabad, and the Chapter Head of the Pakistan Biological Safety Association in the ICT region, were the speakers in the first session of Day 2.

Dr. Javed Muhammad (General Secretory, Pakistan Biological Safety Association) discussed biosafety cabinets, their types, and their functioning. Laminar flow and biosafety cabinet categories were reviewed in the lecture. He stressed the importance of choosing the right biosafety cabinet for the specific risk groups. He also explained the pros and cons of chemical fume hoods and horizontal and vertical laminar flow benches. The discussion then proceeded to the several types of biosafety cabinets and the protection of products, environment, and users. He further suggested cost-effective biosafety cabinets like wooden ones and ventilated workstations could be designed and used during emergencies like pandemics and epidemics.

Dr. Afreenish Amir discussed "Risk Assessment" in the form of simplified illustrations. The talk was meant to give the audience an understanding of the core concepts and methods of risk assessment, focusing on their application to workplace safety. The speaker broke down risk into its parts, highlighting the relationship between the likelihood of negative outcomes, the severity of the risk, and effective methods for mitigating that risk. Aerosolization processes, sharp material, and possible negligence as factors that could increase the likelihood of a hazard. The lecture covered the basics of risk assessment and mitigation, including the dynamic nature of initial, tolerable, and residual risks. Incorporating risk assessment ideas, using a risk assessment matrix, and implementing a systematic method can be used as tools to ensure the health and safety of laboratory workers.

Fig. 2. Participants of Day 2 of ANSO-PAS-QAU Workshop on "Ensuring Biosafety: Empowering Trainers in Risk Management and Biosecurity" with the Prof. Dr. Mushtaq Ahmad (Chairman, Department of Plant Sciences, QAU & Fellow, PAS), Dr. Muhammad Ali (PI ANSO project, Assistant Professor, QAU, Islamabad, Member, PAS), Dr. Shujaul Mulk Khan (Associate Professor, Quaid-i-Azam University, Islamabad & Member, PAS), and workshop trainers Dr. Javed Muhammad (General Secretary of PBSA and Assistant Professor Department of Microbiology University of Haripur), Dr. Ikram Ullah (Assistant Professor, Hazara University, Mansehra), Dr. Fouzia Tanveer (Senior Lecturer, Shifa Tameer-e-Millat University, Islamabad), Dr. Ibrar Khan (Associate Professor, University of Peshawar), Dr. Ali Talha Khalil (Assistant Professor & Consultant Molecular Biologist at LRH-MTI, Peshawar), and Dr. Irum Iqrar (Journals Associate Editor, ORIC, The University of Lahore).

3.1. Training Session

The second day of the workshop consisted of a diverse range of instructive and interactive training sessions facilitated by renowned specialists in the respective departments. The participants were allowed to participate in vital aspects of biosecurity and biosafety, learning essential information about best practices and protocols.

Dr. Ikram Ullah ran a thorough session (Station-I) on the fundamentals of biosecurity primary controls. He stressed the significance of establishing physical safeguards to protect restricted areas and biological materials from unauthorized access. There was an emphasis on personnel screening and verification processes to reduce the risk of unauthorized entrance and enhance security generally. The dangers of losing or accidentally spreading biological material were discussed, and the significance of thorough inspections and detailed records was stressed. To reduce the likelihood of data breaches, the training highlighted the need to safeguard private information and data related to biological research. After that, we talked about the basics of transfer agreements and why they're so important for facilitating the safe and legitimate transfer of biological materials across organizations.

Dr. Javed Muhammad focused on "biosafety cabinets" (Station-II). Participants were briefed on the significance of sticking to the purge time, a period of four minutes during which the biosafety cabinet starts. This method guarantees the construction of a clean and controlled setting. The concept of "spray in and out," emphasizing the need for a steady flow from clean to dirty areas within the biosafety cabinet, was introduced to the students. To further improve workflow efficiency and lessen the possibility of cross-contamination, the biosafety cabinet was divided into "clean", "working," and "dirty" parts. The importance of putting samples in the back of the biosafety cabinet and taking them out in the opposite order was emphasized for maximum efficiency. Participants were instructed on how to best position their hands within the cabinet for maximum ventilation and safety. The discussion centered on the value of alarms installed in biosafety cabinets to alert personnel to changes in pressure or potential problems with HEPA filters.

Then the 3rd training session "Medical and Incident Surveillance Programme" by Dr. Faouzia Tanveer was fascinating (Station-III). Participants learned why first aid packs and spill kits are necessary for medical emergencies and catastrophe safety. Clear emergency exit signage and evacuation protocols were stressed to protect persons escaping a disaster zone. She discussed the most important aspects of a comprehensive worker health program: medical history gathering, task analysis, pathogen management, training, symptom recognition for occupationally acquired infections, risk evaluation, knowledge, and SOP dissemination. The training was thought-provoking because it examined how aging, pregnancy, immunosuppressive medicines, diabetes, and infection risks affect workers.

Dr. Ali Talha Khalil facilitated a comprehensive examination of laboratory-acquired illnesses (Station-IV). Dr. Khalil began the conversation with a case study of a laboratory-acquired smallpox death. This occurrence highlighted the risks of biosecurity and biosafety shortcomings. The students learned a great deal about the ways that infections can be spread in controlled settings like laboratories. Dr. Khalil stressed the significance of immunizations, regular checkups, and a thorough medical history for all laboratory workers. An unfortunate incident that led to a student's death due to an ethanol spill was also discussed in class. This case demonstrated the significance of stress management and understanding the potential mental health consequences of events that occur in the laboratory.

The training (Station-V) by Dr. Ibrar Khan, provided attendees with valuable knowledge and practical skills necessary for effectively and immediately addressing needle stick injuries. The initial action in response to a needle stick injury involves expeditiously exposing the wound site where the incident occurred. To delicately address the wound, promoting bleeding and enabling the expulsion of possibly contaminated fluids is recommended. Additionally, it is advised to properly cleanse the area by rinsing it with clean water for at least five minutes. Following the completion of the initial cleaning process, it is imperative to appropriately cover the wound, subsequently proceeding with the correct removal of gloves. Certification of medical surveillance

ought to be conducted after each occurrence of needle stick injury. The act of reporting promptly guarantees that necessary actions are implemented to effectively address the situation at hand and mitigate the likelihood of similar incidents occurring in the future.

The last training session (Station-IV) was about "Experiment Designing, Documentation, and Execution: Hands-on Aspects of Practically Working in an Infectious Disease Laboratory" conducted by Dr. Muhammad Ali. The master trainer guided the participants through important aspects of working in an infectious disease laboratory. The participants were instructed on the need to follow appropriate laboratory entry procedures and maintain sufficient ventilation to establish a secure and regulated workspace. Trainers were advised to thoroughly examine existing literature and acquaint themselves with the experiment's aims and methodologies. The need to check catalog numbers when assessing chemicals was emphasized throughout the presentation to guarantee precise application and safe storage. This procedure keeps the experiment authentic by preventing improper chemical handling. Dr. Ali stressed the need to record every step, observation, and measurement in detail to ensure reliable analysis and experiment replication. The lesson concluded with a reminder to carefully read and understand all necessary equipment manuals before use. Participation in such activities ensures familiarity with equipment types, functions, and potential dangers.

3.2. Session II

Dr. Ibrar Khan gave a talk titled "Decontamination and Infection Control." Dr. Khan discussed the differences between disinfection and sterilization as the two cornerstones of decontamination. Participants were briefed on the need for thorough disinfection in maintaining a safe and clean environment. Attendees learned in depth about disinfection, sterilization, sepsis, medical surveillance, and the value of efficient resource management. Standardized protocols and occupational health programs were highlighted during the event as a means to reduce the spread of disease and protect the health and safety of participants.

Dr. Javed Muhammad gave a talk titled "An Overview of the Institutional Biosafety Committee (IBC)". This event aimed to teach participants everything they needed to know about Institutional Biosafety Committees (IBCs) and their function in biosafety laws. Students gained knowledge about how an IBC works to provide sufficient containment, promote expert evaluation, educate the public about experimental protocols, and open lines of communication between scientists and medical experts. Participants at the workshop discussed the circumstances in which IBCs are required, the requirements for forming an IBC, and the important members of IBC committees. Dr. Javed Muhammad delivered a comprehensive presentation on the proposal flowchart, a graphical representation of the processes required to obtain Institutional Biosafety Committee (IBC) approval.

An exam was conducted after the lectures to assess the understanding of students. Dr. Ali, the workshop's organizer, concluded the day by summarizing the day's events and thanking the keynote lecturers and master trainers for their insightful presentations and useful insights.

4. DAY 3 - SESSION I

Dr. Ibrar Ahmad (Session Chair) and the co-chair Dr. Javed Muhammad, gave introductory remarks on the third day. In the keynote lecture, Dr. Ali Talha Khalil presented different aspects of laboratory quality management, focusing on process optimization and quality indicators. Increasing laboratory precision, dependability, and speed was his main point. Dr. Ali Talha explained the Lab Quality Management System methodology for organizing and improving varied laboratory tasks. His talk also covered internal and external audits, corrective and preventive efforts, and other conventional development tactics. The final portion of the programme consisted of a recap of the most important ideas covered throughout the day as well as an introduction to the WHO's suite of laboratory evaluation instruments.

Prof. Dr. Mushtaq Ahmad in his Keynote lecture discussed the importance of interdisciplinary approaches in the biological sciences. Sustainable applications in areas including alternate biomass energy, food production, and healthcare were

highlighted as important takeaways from the conversation. Academics, businesses, and researchers all work together to improve society, a point driven home in the presentation. Prof. Ahmad emphasized the gap between universities and businesses. The speaker emphasized the importance of fostering collaboration and encouraging innovation as strategies to close the gap and make better use of multidisciplinary research's latent potential. Information about Pakistan's many ecosystems and the rich biodiversity they support was disseminated to the attendees. This makes the country a valuable resource for establishing environmentally friendly policies. Dr. Mushtaq Ahmad has launched studies focusing on preservation, conservation, and revitalization. These initiatives are crucial to ensuring the health of ecosystems and human communities. Prof. Mushtaq Ahmad evaluated many uses of trans-disciplinary research in healthcare. Herbal treatments, medicines, nutraceuticals, and cosmetics were all included in these contexts.

Dr. Shuja ul Mulk focused on risk assessment and management while covering the fundamentals of plant gathering and fieldwork. The protection of biodiversity, labeling techniques, safety, ethics, and other topics were covered in this instructional workshop. Dr. Shujaul Mulk stressed the importance of fieldwork and data collection to collect living specimens from many sites. His focus was on how climate defines terrestrial biome limits. He also addressed herbarium collections, phylogenetic research, phytochemistry analysis, ecological assessments, and disease investigations as reasons for collecting plants. Dr. Shujaul Mulk showed how to efficiently harvest plants with GPS, compasses, sacks, newspapers, blotting paper, and cutters. Fieldwork and plant gathering are complicated, encompassing safety, ethics, and biological variation. The session taught guests how to pick plants appropriately, aiding conservation and sustainability.

4.1. Field Trip

Participants were led by Prof. Dr. Ghulam Mujtaba Shah, Prof. Dr. Mushtaq Ahmad, and Dr. Shujaul Mulk Khan on a fascinating and thought-provoking field trip. The excursion showcased various plant species with important practical uses, including those having medicinal, dietary, and other medical applications.

- The potential of *Bistorta amplexicaulis* (D. Don) Greene in Sharbat-e-Anjbar, was introduced to the audience and its vitality was emphasized because of the plant's historical role in a variety of popular cuisines and drinks.
- The field trip served as an excellent platform from which to talk about the role that *Indigofera tinctoria* L. plays in the indigo production process, with special emphasis on its use in the textile dyeing process. Participants learned about the application of *Plantago ovata* Forssk. seeds in the processing of "Ispaghol," a traditional remedy for gastrointestinal health. It is also an important remedy for lessening the cholesterol level. *Butea monosperma* (Lam.) Kuntze a key ingredient in "Kamarkas," a medicine used to relieve back pain, was demonstrated.
- *Viburnum grandiflorum* Wall. ex DC. nutritional value as a rich source of vitamin C was underlined.
- Botanical specimen *Polygonatum* has been found to have a substantial number of alkaloids, indicating promising medical applications.
- The ability of *Isodon rugosus* (Wall.) Codd to relieve toothache and its antiseptic potential was explored.
- *Geranium wallichianum* D. Don was highlighted for its ability to alleviate joint pain and backache.
- The uses of green tea and *Origanum vulgare* L. in various types of drinks and medicines were discussed.

Participants were able to get an up-close and personal look at how various plant species are used in disciplines like medicine, nutrition, and more on this field trip. The field visit led by the Experts highlighted the importance of plants in their position as providers of long-term solutions in a wide range of fields.

4.2. Concluding Session

After the field visit, Dr. Ghulam Mujtaba Shah (Dean, of Hazara University) gave his remarks to the audience. "*The fields we see in the wild medicine and the subject of our interest, the endangered species or the loss of the greenery, we ought to*

Fig. 3. Participants of Concluding Session of ANSO-PAS-QAU Workshop on "Ensuring Biosafety: Empowering Trainers in Risk Management and Biosecurity" with Prof. Dr. Shafiq ur Rehman (Vice Chairman Haripur University), Dr. Ghulam Mujtaba Shah (Dean, Hazara University), Prof. Dr. Mushtaq Ahmad, Prof. Dr. Shjaul Mulk Khan (Associate Professor, Quaid-i-Azam University, Islamabad), Dr. Muhammad Ali (PI ANSO Project, Assistant Professor, QAU, Islamabad, Member, PAS), Dr. Javed Muhammad (General Secretary of PBSA and Assistant Professor. Department of Microbiology University of Haripur), Dr. Ali Talha Khalil (Assistant Professor & Consultant Molecular Biologist at LRH-MTI, Peshawar), and Dr. Ikram Ullah (Assistant Professor, Hazara University, Mansehra) organized by the Pakistan Academy of Sciences (PAS), Quaid-i-Azam University (QAU) and University of Peshawar; and sponsored by the Pakistan Academy of Sciences (PAS) and Alliance of International Science Organization (ANSO).

collect the plant. The students don't care what they are collecting or what will be the effect of their collection on plant species. Most of the time, they don't know that this is an endangered species, how to collect it, or which part to collect. So, we have to not only do our work but also give the rest of the work to the people in the field. We have to give them a place and culture to live in. The diseases that we see are more or less resistant to microbes. Every year, we study different diseases related to the climate change. So, we have to do research in the environment, where you are working, and where you are growing."

The chief guest of the concluding session, Prof. Dr. Shafiq ur Rehman (Vice Chairman of Haripur University) discussed leadership, responsibility, and critical thinking. A moving illustration is Quaid-e-Azam, whose inspirational leadership gave birth to our beloved country. The success of individuals, aided by their mentors, improves the prospects for everyone. *"You are the leaders of the biosafety. You got the training. Now, skies are the limits. You can excel in this field and become a biosafety expert in this country. The world will also welcome you with open arms."*

Instead of placing blame, let's embrace responsibility, look to the future rather than lingering on the past, and make use of AI's potential to advance society. Our exclusive job is to protect populations from bio-hazards while innovating successful waste management techniques. Unity is the key to our power. As guardians of this land, our joint will determine Pakistan's future.

At the end of the conference, all of the master trainers, speakers, focal persons, organizers, and attendees were presented with shields and certificates.

5. ACKNOWLEDGEMENTS

We are thankful to all the master trainers, guests, participants, facilitators, and coordinators for contributing to the event. Authors are also grateful to the Alliance of International Science Organizations (ANSO) and the Pakistan Academy of Sciences (PAS) for their support and financial assistance.

6. CONFLICT OF INTEREST

The authors declared no conflict of interest.

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