ISSN Print: 2518-4261
ISSN Online: 2518-427X
Vol. 58(3), September 2021

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

PAKISTAN ACADEMY OF SCIENCES
ISLAMABAD, PAKISTAN

PAKISTAN ACADEMY OF SCIENCES

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Proceedings of the Pakistan Academy of Sciences B. Life and Environmental Sciences is the official flagship, the peer-reviewed quarterly journal of the Pakistan Academy of Sciences. This journal publishes original research articles and reviews in the field of Agricultural and Biological Sciences (all), Biochemistry, Genetics and Molecular Biology (all), Environmental Science (all), Health Sciences (all). Authors are not required to be Fellows or Members of the Pakistan Academy of Sciences or citizens of Pakistan. The journal is covered by Print and Online ISSN, indexed in Scopus, and distributed to scientific organizations, institutes and universities throughout the country, by subscription and on an exchange basis.

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, Category Y

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

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CONTENTS

Volume 58, No. 3, September 2021 **Page**

Review Articles

Research Articles

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Proceedings of the Pakistan Academy of Sciences: B. Life and Environmental Sciences 58(3): 1-18 (2021)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB (58-3)664

Pakistan Academy of Sciences

Review Article

Nanoparticles in Cancer Treatment: A Narrative Review

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Abstract: Nanoparticles have revolutionized the world with their enormous blessings specifically in cancer treatment. In past, conventional chemotherapy was the primary choice of treatment for patients. However, chemotherapeutics also had several pharmaceutical limitations such as stability, drug-drug interaction, drug resistance, and aqueous solubility. Reciprocally, dose curbing toxicity is significant with non-specific toxicity to healthy cells, loss of appetite, hair loss, peripheral neuropathy, vomiting, muscular fatigue, and diarrhea being the typical adverse effects. The introduction of multi-drug resistance (MDR) also posed a great threat for successful cancer treatment, whereby the tumor cells became resistant to many of the chemotherapeutic agents used. Nanotechnology-based novel chemotherapy opened a new horizon for the treatment of cancer. Particularly, nanoparticle-related medication is a highly potential newcomer for curtailing systemic toxicity via producing functionalized particles for specific treatment. It is also an alternative to circumvent multidrug resistance for possessing an ability to bypass the efflux mechanism correlated with this phenotype. Besides having various advantages in treatment, nanoparticles are also playing a key role in diagnostic entities. This paper aims to specifically outline the role of nanotechnology which it is playing in today's era in the diagnosis and treatment of cancer with contemporary knowledge. To assess the role of nanoparticles in cancer treatment, this review analyzed all articles published from 2002 to 2021 in both Local and foreign journals. The article's inclusion criteria were based on the article which contained relevant data regarding applications of nanoparticles in cancer treatment. Articles with copyright, irrelevant information, and lacking the full text were excluded. This paper will highlight the breakthrough, impediments, and prospects of nanoparticles in cancer treatment with an updated review.

Keywords: Cancer therapy, Cancer treatment, Multidrug resistance, Nanoparticles, Nanotechnology, Nanocarriers, Nanomedicine.

1. INTRODUCTION

Cancer is a lethal disease resulting from uncontrolled cellular proliferation. American Cancer Society opines that men have a 41 % probability of developing cancer while in women this percentage is 38% [1]. The existing therapeutic approaches to treat cancer are chemotherapy, immunotherapy, radiation therapy, hormone therapy, and surgery [2]. The aforementioned therapeutic approaches have dynamically improved patients' survival and treatment outcomes. But still, we have a lot of confusion, limitations regarding the use of these therapies today [3]. Drug targeting and drug delivery is the most challenging limitation because of non-selective tissue intoxication, the presence of organized barriers (Physiological, Physical, Enzymatic) which hinders the drug partitioning and drug distribution to its targeted site [4]. Remarkable advancements in drug targeting and drug delivery talk of the town among research pantheons in recent years [11]. Nano-based drugs delivery system enables the delivery of macromolecules and micromolecules in a targeted or localized manner [5]. Precisely, the development of therapeutic agents in biocompatible nanocomposites like

Received: July 2021; Accepted: September 2021
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drug conjugates, micellar systems, nano-capsules, nanoparticles have gained more focus [6]. Nanotechnology gained the spotlight since the 1980s through the emergence of cluster science, the development of carbon nanotubes and fullerenes, the invention of tunneling microscopes [7]. The development of semiconductor particles widely known as Quantum dots, semiconductor nanocrystals are the edicts of nanotechnology [14]. Nanoparticles are nano-sized colloidal particles, and the therapeutic agent incorporated within the particle-matrix, with the size <100 nm, is partly absorbed through systematic or functional modifications which improve drug efficacy and drug stability [8]. The dimensional resemblance of nano-particles with biomolecules: volume ratio, high-surface, capacity for surface engineering have made them powerful candidates for diagnosis and treatments [9]. Having the advantage of being micro-sized particles, they can deeply penetrate tissues, easily cross epithelial surfaces, easily be taken up by the targeted cells, improves the bioavailability of therapeutic moieties. By manipulating the polymer characteristics, the rate of moiety release can be well-optimized. Bio-specific drug-ligand joint-ventures increase cell targeting and tissue-specific drug delivery [10,11]. Figure 1 is showing the timeline which is representing key moments in the hiostory of biomaterials.

1.1 Nano-Bio Interactions of Nanomedicines

Because of the excellent physicochemical properties, engineered nanomaterials (ENMs) have been created for drug delivery, diagnostics, imaging, and clinical treatment applications. However, the function and final effectiveness of nanomedicines remain inadequate for clinical use, owing to a lack of knowledge of nanomaterial/nanomedicine–biology (nano-bio) interactions. The biological milieu's nonequilibrated, dynamic, and diverse character invariably impacts the dynamic bio identity of nanoformulations at each site of nano-bio interactions (i.e., the interfaces at various biological fluids (biofluids), surroundings, or biological structures). The constant interaction between biological chemicals and nanomedicine and structures in biological settings, for example, might influence cellular absorption or entirely alter the nanomedicine's planned function. As a result, the weak and strong driving forces at the nano-bio interface may cause structural reconfiguration, reduce bioactivity, and promote nanomaterial malfunction and/or redox interactions with biological molecules, all of which may result in undesired and unanticipated biological consequences. These driving factors, on the other hand, may be adjusted to reduce the toxicity of ENMs or increase their targeting abilities [112, 113].

1.2 Advantages of Nanoparticles in Drug-Delivery

The polymeric nanoparticles are the most favorable structures because of their peculiar property of surface modification and can be designed for active and passive drug-targeting [12]. This very

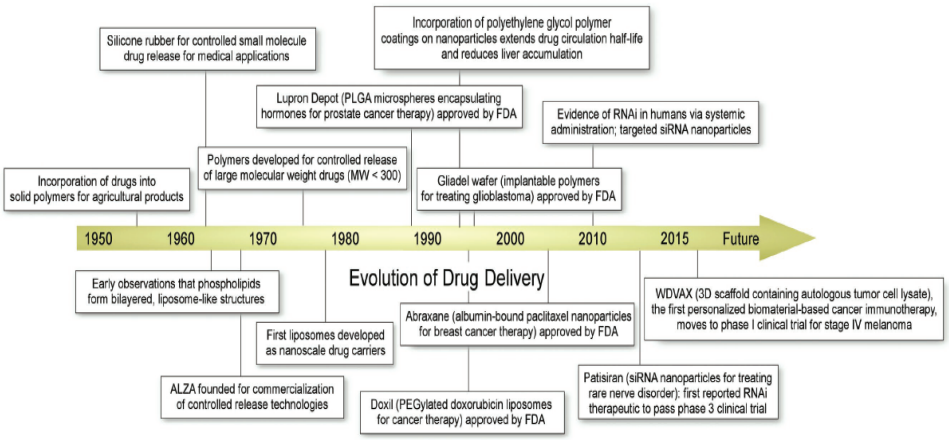

Fig. 1. This figure shows a timeline which is representing key moments in the history of biomaterials [7].

characteristic allows them to be used widely in vivo and *in vitro* sensors targeted therapy and imaging [9]. The steady relation between the nanoparticles and drug equips them for subsequent clearance of the drug, altered organ distribution, and stimulus-triggered site-specific drug release for 'on demand' treatment [13, 14].

These carriers also enhance the stability of the drug by impeding the breakdown of the encapsulated carrier. However, a large number of drugs can be added directly irrespective of chemical reaction, which plays a vital role in drug preserving activity [15]. The development of dry solid dosage forms of drugs is considered a fruitful strategy to optimize the drug's chemical stability [16]. These are considered to be more stable than nano-liquid products [17]. The ionic stabilizers (Sodium dodecyl sulfate, Sodium lauryl sulphate, docusate sodium, and lecithin) or Non-ionic stabilizers (Polyvinylpyrrolidone, Polyethylene glycol, Polyvinyl alcohol, Tween 80) are also used to provide molecular stability to nanoparticles [18]. The production of porous nanoparticles also enhances their stability [19].

Tumors elicit peculiar pathophysiological traits which are different from healthy tissues such as defective vasculature, full-partially impaired lymphatic system, extensive angiogenesis. Nanoparticles vigilantly identify those defected anatomical regions and extensively release the drugs into tumor sites [20, 21]. Because of compromised venous return into tumors, poor lymphatic drainage, nanoparticles are retained in the targeted tumor site for a long time [17]. This process or mechanism is called enhanced permeability and retention [22]. Likewise, the target-specific delivery can be maintained by targeting the tissue which is adjacent to the tumor such as targeting Kupffer cells in the liver [23].

The administration of nanoparticles can be done via several routes like; Oral, parenteral, nasal, instar-ocular, etc [21]. Nanoparticles have a relatively higher surface-to-volume ratio and intracellular uptake as compared to microparticles [23]. Through experiments, it is reported that nanoparticles are more effective for drug delivery than their microparticles sized (>1 μm) counterparts. These characteristics equip them to release the drug in a controlled manner in malignant tissues [24, 25].

1.3 Limitations of Nanoparticles in Drug-Delivery System

While inking on the advantages of nanoparticles in drug delivery, they possess several limitations too which cannot be overlooked. Studies demonstrated that nanoparticles get unstable over a prolonged period [26]. The manufacturing conditions like high pressures, high temperatures, change the crystallinity of drugs. During prolonged storage, crystal growth, sedimentation, and particle agglomeration also destabilize the products [7]. The development of nanoparticles is a quite delicate, challenging process as compared to conventional drug formulations [27]. Precise control of particle size, and surface functionality are uncompromised factors that are required for the successful production of nanoparticles [13].

Nanotechnology is very expensive and costs a lot. Product processing, difficulty in handling molecular structures, high labor costs are the contributing factors for their increased cost [28]. It is quite difficult to develop a formulation in a reproducible manner using nanotechnology [29]. While comparing the costs of drugs containing nanoparticles are described, in 2009, the cost per dose of Paclitaxel was between $90 - $454 as compared to $5,054 for Abraxane (Paclitaxel containing nanoparticles formulations) [34] Similarly, the cost per dose of pure Doxorubicin was between $62 - $162 as compared to $ 5,595 for Doxil® (Doxorubicin containing nanoparticles formulations) [30].

The next limitation is that the physiological response of nano-carriers is yet not very well understood. For example, studies showed that the crystalline silver nanoparticles might cause cytotoxicity in the human fibroblasts, keratinocytes, lesioned skin [31]. Liposomal vesicles can be arrested by the immune system [32]. Various publications have reported the post-treatment accumulation of nanoparticles in the skin. Granite and Dolomite nanoparticles are revealed to be more toxic for lung epithelial cells [29].

2. ADME OF NANOPARTICLES

Several barriers prevent extraneous substances, such as bacteria, viruses from entering the body. These same barriers, which include the pulmonary

system, the gastrointestinal tract, and the skin, regulate nanoparticle access. Previously, only tiny lipophilic compounds (600 Da) and metallic ions (such as cobalt and nickel) could pass through the skin barrier. However, due to their tiny size, nanoparticles may be easily absorbed through the dermis of the skin, as well as the pulmonary and gastrointestinal mucosa, positioning these substances for distribution through the vascular circulation to all tissues in the body. The vascular endothelium, with an average pore size of 5 nm in mammals, provides another possible barrier to nanoparticle absorption and distribution, although nanoparticles smaller than this limit pass readily from the blood over the endothelium and into tissue. Furthermore, because the discontinuous endothelium of these organs includes holes of 50-100 nm in diameter, nanoparticles may be able to translocate efficiently from the blood into the liver, spleen, and bone marrow. As a result, techniques for determining the quantity of total external exposure, absorption effectiveness, and tissue biodistribution of nanoparticles are required. However, the excretion of nanoparticles is majorly done by two ways; from renal filteration through urine and hepatobiliary processing. Choi *et al.* demonstrated that quantum dots are excreted through renal filteration as urine. Another study demonstrated that gold nanoparticles were excreted though hepatobiliary processing [112, 114].

3. IMPEDIMENTS IN CANCER CHEMOTHERAPY

To ensure the success of chemotherapy, a handsome amount of parent drugs should reach the targeted site [33]. At the tumor location, the unpredictable blood flow, abnormal vasculature, are the key factors that prevent the drug penetration into the malignant tissue [29, 33].

A crucial limitation to systematic chemotherapy is peripheral neuropathy, systematic toxicity, loss of appetite, skin damage, hair loss, and diarrhea [34]. Chemotherapy can successively lead towards neutropenia which further instigates infections [65]. These side-effects depend upon several factors like; the duration of treatment, patient-specific characteristics, the prescribed dosage of drugs for treatment [35].

Chemoresistance is a critical phenotype that helps the cancer cells to evade the cytotoxic effects of chemotherapeutic agents [31]. This phenotype results from several mechanisms including decreased apoptosis, increased DNA repair, intracellular sequestration, increased drug detoxification, decreased drug influx, and increased drug efflux [36]. Chemoresistance can either be acquired or intrinsic. Acquisition of an idiosyncratic type of resistance named multi-drug resistance (MDR) is a challenging phenotype in cancer treatments [29]. MDR can appear after exposure to a single anti-cancer agent which results in cross-resistance to a wide array of chemotherapeutic drugs [37].

MDR is strongly associated with overexpression of ATP-dependent multidrug efflux membrane transporters which belong to the ATP- binding cassettes superfamily of which P-glycoprotein is a prototypical relative [31]. The over-expression of the aforementioned transporters helps cellular evasion of cytotoxicity by properly maintaining the sublethal intracellular concentrations of chemotherapeutic drugs. This act leads to the failure of treatment [38].

The genetic accretion of multi-drug resistance has been well studied and recently the spread of MDR through Non-genetic mechanisms like tunneling nanotubes, cell-to-cell contact has been well explained [35]. The spread of MDR in the non-presence of any cellular contact has been discovered, Whereas, the extracellular vesicles (Microparticles (MP's)) facilitate the broad-range and short-range movement of resistance phenotype to hitherto drug-sensitive cells [39, 40].

The microparticles are membrane-derived vesicles whose size ranges 0.1 – 1 μm and shed from many cell types following the calcium-dependent loss of phospholipid asymmetry, cleavage of filaments which attaches cytoskeleton to plasma membranes, and budding of cellular membranes [36]. MP's plays a vital role in extracellular signaling and enhance the dissemination of cellular products via the transfer of vesicle cargo [41].

Including cancer, in many diseases, MP shedding is highly reported and it contributed to the direct progressions of disease [26]. Microparticles

shed from multi-drug resistant cells and have been shown that the MDR phenotype confers to the hitherto drug-sensitive cells in vivo and in vitro [42]. The transfer of these vesicles from MDR allows the proteomic re-templating and transcription of the receipt cells [38]. Taken together the microparticles plays a crucial role in the maintenance of cancer characteristics [43].

4. OVERCOMING THE IMPEDIMENTS IN TUMOR TARGETING

The prime goal of drug delivery is to transport the desired amount of drug into a targeted site for the optimal period [44]. This positive upshot will be achieved with the improved and advanced interactions between the biological barriers and drug carriers. Biological barriers restrict the entry of drugs into tumors [39]. The injectable drug administration route provides minimum barriers so that the drug can reach its target site safely [40]. The small vesicles diffuse along with the biological barriers, hence facilitating the absorption of administered drug [45].

The surface properties of nanoparticles depend upon the nature of the surface component [41]. While talking about the particles which contain amphiphilic copolymers, their hydrophilic part is embedded onto the particle surface because of the hydrophobic moiety of the copolymer [46]. For example; nanoparticles' surface grafted with thiomers have reported well-improved interactions with the intestinal mucosa. Because of their small particle size, nanoparticles mask their recognition by the macrophages and reside in systemic circulation for a longer period [41]. Cyclodextrins and Biotins are widely used surface ligands to optimize nanoparticle-tumor cell interactions [42].

Not long ago, pantheons are giving attention to the delivery of nucleic acids and antibodies for treating cancers in humans [47, 48]. Nucleic acid drugs such as small interfering RNA (siRNA), anti-sense DNA/RNA, aptamers have shown tremendous results in cancer therapy [43]. However, their effectiveness is limited by opsonization and clearance by macrophages, serum nucleases, and lastly by the renal system [28]. These above-mentioned limitations can be overcome using nano-carrier-based drug delivery systems [50]. Nanocarriers have the affinity to be strongly attached to specific cells and other targeting agents like ligands [21].

5. NANOPARTICLES TO OVERCOME THE PROBLEMS CAUSED BY MDR IN CANCER THERAPY

Nanoparticles are receiving attention specifically in cancer treatment because of their ability to co-encapsulate multiple therapeutic agents in targeted specific drug delivery systems [51]. The researchers have reported the co-delivery of Pyrrolidine dithiocarbamate and Doxorubicin using multifunctional Chitosan-folate micellar nanoparticles to gain pH-responsive specific target release of drug to overthrow Doxorubicin MDR [44]. The slow release of these drugs at neutral or alkaline pH, rapid release of both drugs in a weakly acidic medium, and pH-sensitive folate receptor-mediated endocytosis have a high potential to overcome MDR in liver cancers [52].

A group of researchers described a Paclitaxel encapsulated nanocrystal formulation by using D-α-tocopheryl polyethylene glycol 1000 succinate for circumvention of MDR. D-α-tocopheryl polyethylene glycol 1000 succinate works as a surfactant to balance the nanocrystals and meantime it also acts as a P-gp (Pharmacological inhibitor) [45]. These nanocrystals reported controlled release kinetics, and good therapeutic effect in Taxol (A clinical formulation of Paclitaxel formulation – resistant cancer cells. Similarly, intranuclear localization of TAT peptide conjugated doxorubicin encapsulated mesoporous silica nanoparticles have been reported as a strategy to evade cancer MDR [53]. The covered drug is directly released into the nucleus. Advanced nuclear delivery is a favorable strategy to cope-up MDR [46].

The co-delivery of P-gp siRNA along with Doxorubicin has been reported employing mesoporous silica nanoparticles. This dual delivery in KB-V1 cells was proficient in enhancing intranuclear and intracellular drug amounts to levels exceeding that of the free drug [47, 48].

6. MECHANISMS OF CELLULAR TARGETING

For cancer therapy to be more effective, the chosen delivery system should be selective to target cells

without affecting healthy cells [49]. For successful cancer therapy, the anticancer drug reaches the tumor site via two targeting means; either it is active or passive targeting mean [54].

6.1 Passive Targeting of Nanoparticles

The passive tumor targeting highly depends upon certain factors like tumor microenvironment, punctured tumor vasculature, and the direct local application [50]. It is important to note that the presence of tight junctions between the non-malignant tissues, results in resistance to the passage of nanoparticles. Specifically in cancer, the neovasculature is leaky and disorganized [49]. This whole scenario allows the extravasation of nanocarriers in the endothelium of the tumor vessels due to the presence of fenestrations (Figure 2) [51].

The passive drug targeting also depends upon the accumulation of drug at the targeted site and the half-life of the drug carrier. The therapeutic potential of nanoparticles depends upon the surface charge, solubility, biodegradability, and morphology [55]. A hydrophilic biomaterial's (Polyethylene glycol) covering or coating is used to defend nano-formulation against the attack of macrophages and to enhance the circulation time of nano-formulations. In the passive targeting, the nanoparticles conglomerate in the affected tissues because of their retention and permeability effect [52]. The trafficking of nanoparticles over the neoplastic tissues highly depends upon the surface charge, tumor microvasculature, size, and shape [56].

6.2 Active Targeting of Nanoparticles

The active targeting mode of nanoparticles depends upon the utilization of certain ligands like folate and transferrin, which bind to the proteins that are over-expressed or somewhat expressed on the target cellular sites [57]. This instigates the inbound folding of membranes and incorporates the nanoparticles into the cells through a phenomenon named receptor-mediated endocytosis (Figure 3). Under the non-alkaline conditions of the endosome, the encapsulated drug is released from the nanoparticles and sets foot in the cytoplasm after that it acts on the cellular target [58]. The strategies of tumor-targeting are classified into three classes. i) Angiogenesis-associated targeting through the growth factor receptors of vascular endothelial, matrix metalloproteinase receptors, vascular cell adhesion molecule-1 andαvβ3 integrins. ii) Tumor cell targeting for targeting colorectal cancer, for targeting lungs cancer, for targeting breast cancer, for targeting prostate cancer, etc., and iii) the targeting of uncontrolled cellular proliferation through human folate receptors, endothelial receptors, and transferring receptors [56]. Scientists have reported the active targeting of tumor cells by using the multi-functional dendritic nanodevice attached with folic acid which contained Methotrexate as a chemotherapeutic agent. In addition, the Rapamycin-loaded epithelial growth factor antibody-conjugated nanoparticles reported increased efficacy in MCF 7 breast cancer cells

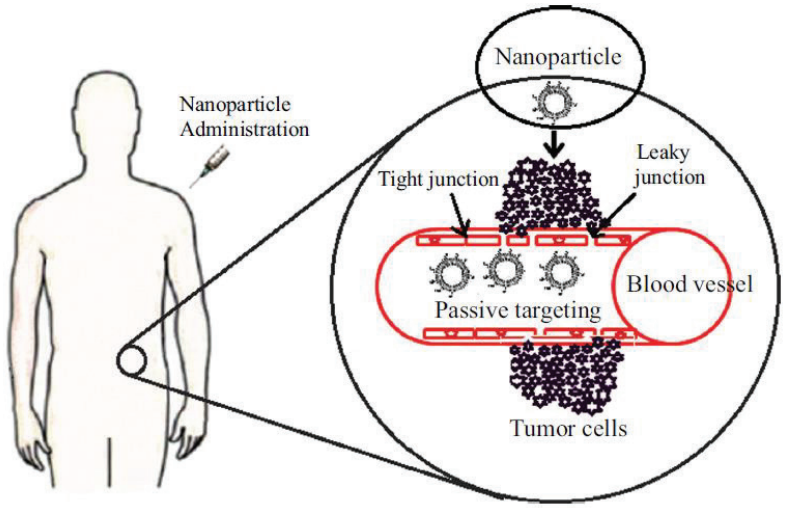

Fig. 2. The figure shows the phenomena of passive tumor targeting by the nanoparticles. The targeting process depends upon the infiltration of nanoparticles of ideal size, shape, and surface charge across a leaky neovasculature [53].

[58, 45].

7. NANOPARTICLES IN THE TREATMENT OF CANCER THERAPY

A plethora of nanotechnology-based products have widely been used as drug delivery agents in cancer therapy, [60] these products include liposomes, dendrimers, carbon nanotubes, polymeric micelles, magnetic nanoparticles, Solid Lipid Nanoparticles, Quantum dots, etc (Figure 4) [61, 62].

7.1 Dendrimer-based Nanoparticles

Dendrimers having nano-size (<5 nm diameter) are spherical polymeric particles. They possess a larger area for the incorporation of therapeutic agents [63]. The conceptualization of dendrimers destroys the morphology and characteristics of malignant tumors like; rapid proliferation, specific cell surface antigen expression, and leaky vasculature [61]. The synthesis of dendrimers initiates with an ammonia core which reacts with acrylic acid and fabricates tri-acid molecules [57]. The newly produced tri-

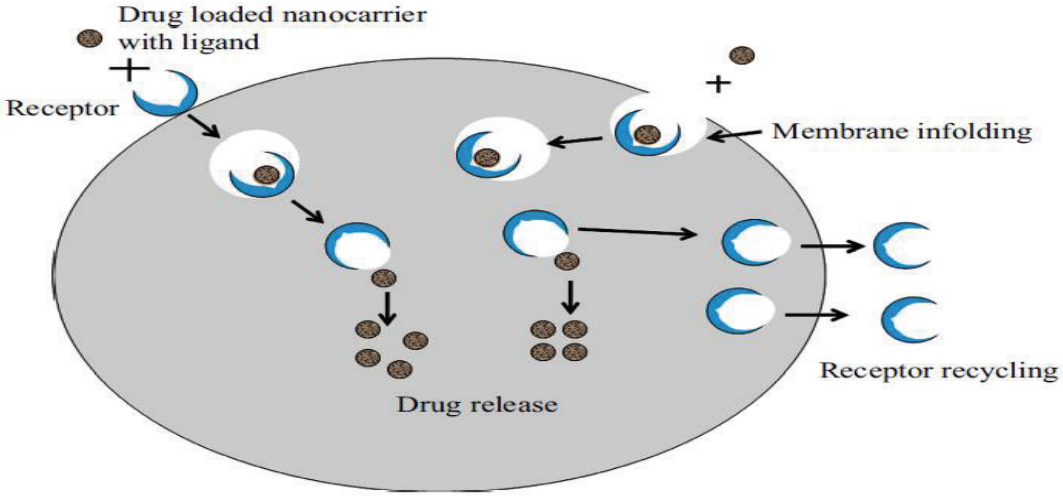

Fig. 3. The process of active tumor targeting by nanoparticles via receptor-mediated endocytosis process. Attaching of nanoparticle surface ligands to the tumor cells leads to membrane inbound folding and incorporation of nanoparticles via receptor-mediated endocytosis. Under the acidic conditions of the endosome, the drug is release from the nanoparticles to the cytoplasm [59].

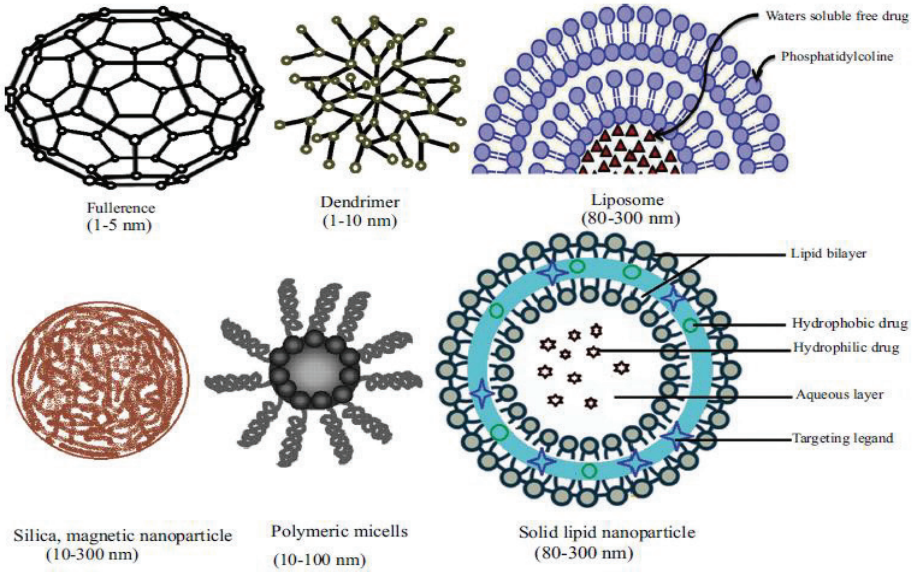

Fig. 4. Different nanoparticles possessing peculiar characteristics, different sizes are being used in cancer therapy [59].

acid molecule then reacts with Ethylenediamine to make a tri-amine molecule generally known as (G0) generation 0 product. This generation 0 product then reacts with acrylic acid to form a Hexa-acid. This Hexa-acid product further reacts with ethylenediamine to form Hexa-amine (G1) the process goes on. Changes in reactions with ethylenediamine and acrylic acid continue until the desired result is not achieved. Polyamidoamine shortly known as PAMAM-based dendrimers is the most investigated and widely accepted for therapeutic applications [64]. The DNA-based polyamidoamine dendrimers for cancer-cell-specific targeting are well described by Choi et al., Folic acid and Fluorescein coupled dendrimers are developed by a mechanism named Acetylation to lessen the drug toxicity [55, 79].

The *in vitro* studies showed specific binding to KB cells expressing the folate receptor [92]. A group of scientists explained the use of paclitaxel-packed multi-functional dendrimers coupled with folic acid and fluorescein isothiocyanate to hit the cancer cells which over-expresses the folate receptors [64]. Multifaceted dendrimers were synthesized from ethylenediamine core whose primary amino acid group has neutralized via partial acetylation [74]. The dendrimer couple investigated the cytotoxic effect on the KB folate receptor. Furthermore, another group of scientists prepared methotrexate-loaded dendrimers for intravenous administration to hit the folate receptors which lie on the surface of cancer cells, these prepared dendrimers tremendously inhibit the growth of epithelial cancer [65].

7.2 Magnetic Nanoparticles

Magnetic nanoparticle-transfection methods follow a principle developed by a group of scientists named Widder and others in the past 1970s for targeting the drug delivery magnetically [74]. The first therapeutic use of these magnetic nanoparticles for transfection was reported in C12S cells in mice by Mah and coworkers [101]. Recombinant single-chain FV antibody fragment-mediated superparamagnetic iron oxide nanoparticles are investigated to be a potential candidate for cancer-specific medical resonance imaging [44]. Superparamagnetic iron oxide nanoparticles conjugated with Luteinizing hormone are shown to be effective for targeting and imaging breast cancers. Roughly, nine or ten magnetic nanoparticle products have been introduced into the market for clinical trials purposes which include Feridex (AMAG pharmaceuticals, Inc) for imaging of liver cancer, Resovist® (Bayer Schering Pharma AG) for imaging of colon cancer and liver metastasis, Ferumoxytol (AMAG pharmaceuticals, Inc) for imaging the Central Nervous System (CNS) cancers [66, 67].

7.3 Calcium Phosphate Nanoparticles

Calcium phosphate nanoparticles alone or in conjugation with non-viral and viral vectors reported tremendous outcomes as drug delivery agents in cellular gene transfer [104]. Calcium phosphate nanoparticles are more advantageous over others because of their low production costs, biocompatibility, reduced microbial degradation, and storage stability. Moreover, it is biodegradable, hence it does not cause any severe damage or side effects at the injection site. It is used as a vehicle to deliver medications like contraceptives, growth factors, antibiotics, and insulin. Their precipitation is used for the delivery of Plasmid DNA and oligonucleotides [49]. A group of researchers investigated that liposomal Nanolipoplex, formulation of glycerol and calcium has decreased cytotoxicity and improved transfection properties of cells [70].

7.4 Polymeric Nanoparticles

Polymeric nanoparticles are the most promising drug delivery agents, despite having many challenges with production, these are the most investigated in nanotechnology for the targeted delivery of anticancer drugs [29]. Polymeric nanoparticles are mainly composed of polylactic acid, polyglycolic acid, acrylates [71]. Scientists find out that nanoparticles that contain anti-human epidermal growth factor receptor 2 and doxorubicin reported nuclear localization of anticancer drug in the human epidermal growth factor 2-overexpressing breast cancer SKBR-3 cells. Another research reported that indomethacin encapsulated nanocapsules shown a significant decrease in the size of the tumor and also reported the increased survival in a xenograft glioma model among rats [72]. Abraxane is an example of polymeric nanoparticle

which is a formulation of Paclitaxel, conjugated to albumin has been approved for metastatic breast cancer treatment [73]. It is another emerging field in medical sciences with more than ten polymeric nanoparticles containing anticancer drugs are currently under clinical trials which includes Paclitaxel poliglumex (Xyotax), PEG- camptothecin (Prothecan), HPMA copolymer-DACH-platinate (AP5346), HPMA copolymerplatinate (AP 5280), HPMA copolymer-doxorubicingalactosamine (PK2), N-(2-hydroxypropyl) methacrylamide (HPMA) copolymer-camptothecin (MAG-CPT), Modified dextran-camptothecin (DE 310), etc. [73, 74].

7.5 Quantum Dots

Quantum dots are semi-conduction nanoparticles. They elicit some unique characteristics like broad absorption spectrum, higher photostability, broad ultraviolet excitations, narrow emission bands, and brighter fluorescence [75]. The narrow emission bands and wider absorption spectrum grant only one wavelength of light to instigate a cluster of quantum dots of many sizes which reciprocally discharge multiplex imaging at different wavelengths [23]. To cope-up with limitations regarding imaging in the visible spectral region, quantum dots that fluoresce in the near-infrared spectral region (700-1000 nm) have been reported [110]. The near-infrared region quantum dots have been experimented with for lymphatic mapping in various animal studies. In 2004, Gao at el. reported that the quantum dots can be effective against cancer targeting in animal models [115]. Another group of scientists Bagalkot et al. investigated quantum dots aptamer – doxorubicin couple for targeting the prostate cancer cells. The prepared nanoparticle couple demonstrated the sensitivity and specificity for cancer therapy and imaging [76]. In the near past, Liu et al. reported a biological activity of conjugated molecules of alyl isothiocyanate and silicon quantum dots, the scientists find out that this conjugation showed identical anticancer properties like alyl isothiocyanate at higher doses by avoiding the lower dose stimulation effect of ayly isothiocyanate on DNA damage and cell migration. Alyl-isothiocyanate coupled silicon quantum dots outlined biphasic anticancer properties in human hepatoma HepG2 cells [77].

7.6 Liposomes

Liposomes are made up of natural phospholipids. Thus, they are biologically inert, elicit low intrinsic toxicity and weak immunogenicity [78]. They are spherical-shaped nanoparticles consisting of the lipid bilayer to encase therapeutic drugs [79]. The presence of lipid bilayer made them prodigious candidates to deliver hydrophilic and hydrophobic drugs. Myocet®, Doxil®, DuanoXomer® are globally approved liposome-based nanoparticles which contain Duanorobucin as an anticancer drug for metastatic breast cancer treatment. MCC-465 (PEG-immunoliposome-doxorubicin) is going through clinical trials for the treatment of stomach cancer, similarly, SPI-077 (Liposomal cisplatin) is also undergoing clinical trials for the treatment of various cancers, OSI-211 (liposomal lurtotecan), Aroplati, (liposomal oxaliplatin), OSI-7904L (liposomal thymidylate synthase inhibitor), LEP ETU (liposomal paclitaxel), LE-SN38 (liposomal SN38 or liposomal irinotecan metabolite) are the products for liposomal-based nanoparticles which are going through clinical trials phase 2 for the treatment of various cancers [91]. A group of scientists has reported the production of the first C60 based slow-release liposomal aerosol to deliver paclitaxel for treating lungs cancer and this product marked a big achievement with promising outcomes [80, 81].

7.7 Gold Nanoparticles

Gold nanoparticles are the intracellular drug delivery agents and possess unique properties, like; their size can be controlled very easily, their surface properties can be modified accordingly, their visible light extinction behavior makes them feasible to encounter nanoparticle trajectories in the cells [82]. To target HER2 positive breast carcinoma, Anti-HER2 functionalized gold-on-silica nano-shells have been prepared, to wipe out the problem of the presence of salt in gold Sodium bromohydride is used [61]. However, sodium bromohydride is unsuitable for target-specific peptides because it lessens the chemical composition of peptides [71]. Hydrazine, dimethyl formamide, sodium bromohydride are the limitations in the therapeutic use of gold nanoparticles [83, 84].

7.8 Silica Nanoparticles

Silica is a prominent component of natural materials such as glass, sand, etc. It has been widely used for thousands of years. Recently, its biomedicine use has been identified [23]. Silica-nanoparticles such as N-(6-aminohexy1) – 3 – aminoproplytrimethoxysilance can effectively result in the transfection of Cos-1 cells with very lower toxicity [85]. A group of scientists Gary-Bobo et al. reported that the anticancer drug camptothecin loaded on mesoporous silica nanoparticles is very effective against colorectal cancer cells [86, 87].

7.9 Carbon Nanotubes

Carbon nanotubes were prepared in the late 1980s [103]. While mentioning their properties they are single and multi-walled tubes and are being used for thermal ablation therapy and used as DNA delivery vectors [88]. Heister et al. investigated that the monoclonal antibody and oxidized single-walled nanotubes which contained fluorescent marker targeted delivery of doxorubicin is effective against the treatment of colon cancer cells [45]. It is also reported that multi-walled carbon nanotube chitosan nanoparticle hybrids are prepared by an inotropic gelatin process has drastically decreased cellular toxicity, improved protein immobilization efficiency as compared to carboxylated multi-walled carbon nanotubes [89].

7.10 Solid Lipid Nanoparticles

These are the colloidal nanocarriers which are composed of phospholipid monolayer coating a solid hydrophobic core and encasing a drug in a high melting point like waxes or glycerides [90]. Anticancer drug mitoxantrone encased in SLN has reported improved bioavailability, drug safety, reduced toxicity. Increased efficacy of doxorubicin and idarubicin being incorporated in SLN's demonstrated better results to treat leukemia cells and murine leukemia in mice models [91, 111].

7.11 Fullerenes

They are big carbon-caged molecules typically known as Buckyballs. They are the most promising anticancer carriers because of their unique physical, electrical, structural (hollow sphere), and chemical properties [92]. Their stability makes them a very choice candidate for effective and safe drug delivery to the tumor cells. Similarly, the existence of π-conjugation, they can absorb light, high triplet yield, and can generate reactive oxygen species upon illumination. These photo properties make them suitable for photodynamic therapy of cancer [93]. Krishna et al. reported the photoacoustic and photothermal properties of polyhydroxy fullerenes for cancer therapy and imaging [94].

7.12 Microbes-mediated nanoparticles

In recent years, there has been a paradigm change toward environmentally friendly, green, and biological production of metal nanoparticles (MNPs) for various nanomedicine applications, including cancer nanotheranostics. Aside from the well-known green synthesis methods of plant materials, the microbial world's (bacteria, fungus, alga, etc.) potential in biofabrication is also realized. Biomolecules and enzymes found in microbial cells can catalyze the biosynthesis process. These microbially generated inorganic nanoparticles have been extensively studied as possible agents in cancer treatments, with promising findings. These microbial-derived nanoparticles have the ability to destroy cancer cells via cellular and molecular mechanisms. Given recent advances in the anticancer uses of microbially generated inorganic MNPs, there is a pressing need to conduct clinical studies [112].

8. APPROVED NANOPARTICLES FOR ONCOLOGICAL APPLICATIONS

In the past few decades, the use of nanoparticles has gained the spotlight. Here are several nanoparticles which are used commercially.

- Doxil® is the first nanoparticle approved by the FDA in 1995 for the treatment of metastatic breast cancer, ovarian cancer, HIV related Kaposi's sarcoma [95].
- DaunoXome® was approved by FDA in 1994 for the treatment of HIV-related Kaposi sarcoma.
- Abraxane® was also approved by FDA in 2005 for the treatment of metastatic breast cancer. Abraxane alone with gemcitabine is effective against pancreatic cancer.

Table. 1. Clinically approved drug-delivery systems [111].

S. No.	Type of Drug Delivery System	Clinically Approved Drugs
1	Nanoparticles	Doxil (Doxorubicin), Abraxane (Paclitarel), DaunoXome (Daunorubicin), Margibo (Vincristine), MEPACT (Mifamurtide), ADYNOVATE (antinemophilic factor (recombinant) PEGylated), Onivyde MM-398 (Irinotecan), Estrasorb (estradiol), Depocyte (cytarabine), AmBisome (amphotericin B), Visudyne (Verteporfin)
2	Microparticle-based depots	Decapeptyl/Trelstar (Triptorelin), Zmax (Azithromycin), Vivitrol (Naltrexone), Risperdal/Consta (Risperidone), Sand-ostatin LAR Depot (Octreotide), Arestin (Minocycline), Nutropin Depot (Somatropin), Lupron Depot (Leuprolide), DepoDur (Morphine), Bydureon (Exenatide), DepoCyt (Cytarabine), Somatuline LA (Lanreotide), Suprefact Depot (Buselerin), Zoladex (Goselerin), Signifor (Pasireotide)
3	Transdermal materials and devices	Nitro-Dur (Nitroglycerin), Transderm-Scop (Scopolamine), Catapres TTS (Clonidine), Estraderm (Estradiol), Duragesic (Fentanyl), Combipatch (Estradiol with norethindrone), Androderm (Testosterone), Lidoderm (Lidocaine), Climara Pro (Estradiol with levonorgestrel), Synera (Lidocaine and tetracaine), Daytrana (Methylphenidate), Oxytrol (Oxybutynin), Emsam (Selegiline), Neupro (Rotigotine), Exelon (Rivastigmine), Sancuso (Granisetron), Butrans (Buprenorphine), Ortho Evra (Estradiol and norelgestromin), Flector (Diclofenac epolamine), NicoDerm/Habitrol/ProStep (Nicotine), Qutenza (Capsaicin), Retin-A (Tretinoin), IONSYS (Fentanyl), SonoPrep (Lidocaine via ultra-sound), LidoSite (Lidocaine and epinephrine via iontophoresis), lontocaine (Lidocaine and epinephrine via iontophoresis)
4	Oral	Ditropan XL (Oxybutynin), Concerta (Methylphenidate), Teczem (Enalapril Diltiazem), Dilacor XR (Diltiazem), Covera-HS (Verapamil), Minipress XL (Prazosin), Procardia XL (Nifedipine), DynaCire CR (Isradipine), Fortamet (Metformin), Altoprev (Lovastatin), Glucotrol XL (Glipizide), Tegretol-XL (Carbamazepine), Allegra D (Pseudoephedrine and Fexofenadine), Invega (Paliperidone), Efdac/24 (Pseudoephedrine and Brompheniramine or Chlorphenir-amine), Volmax (Albuterol), Orenitram (Treprostinil), Sudafed 24 h (Pseudoephedrine), Exalgo (Hydromorphone), Vesanoid (Tretinoin), Venclexta (venetoclax), Farydak (panobinostat), Syndros (Dronabinol), Renagel (Sevelamer)
5	Pulmonary	Proventil HFA (Albuterol), Tudorza/Pressair (Aclidinium), Ventolin HFA (Albuterol), ProAir HFA (Albuterol), Combivent Respimat (Albuterol and ipratropium), Brovana (Arformoterol), QVAR (Beclomethasone), DuoNeb (Albuterol and ipratropium), Pulmicort Flexhaler (Budesonide), Symbicort (Budesonide and Formoterol), Alvesco (Ciclesonide), Bro/Ellipta (Fluticasone and vilanterol), Flovent/Diskus (Fluticasone), Flovent HFA (Fluticasone), Foradil/Aerolizer (Formoterol), Perforomist (Formoterol), Arcapta Neohaler (Indacaterol), Atrovent HFA (Ipratropium), Xopenex HFA (Levalbuterol), Asmanex/Twisthaler (Mometasone), Dulera (Mometasone and Formoterol), ADVAIR Diskus (Salmeterol Fluticasone), Serevent/Diskus (Salmeterol), ADVAIR HFA (Salmeterol Fluticasone), Spiriva/Handihaler (Tiotropium), Cayston (Aztreonam), Ventavis (lloprost), Tyvaso (Treprostinil), TOBI Podhaler (Tobramycin), Afrezza (human insulin).
6	Implants	Retisert (Fluocinolone), Vitrasert (Ganciclovir), Ozurdex (Dexamethasone), Gliadel (Prolifeprosan and Carmustine), Zoladex (Goserelin), Vantas/Supprelin LA (Histrelin), Viadur (Leuprolide), NuvaRing (Etonogestrel and ethinyl estradiol), Nexplanon (Etonogestrel), Mirena/Norplant (Levonorgestrel), Paragard (Copper).

- Myocet® was approved in Europe and Canada for the treatment of metastatic breast cancer [96].
- Depocyt® was approved in 1996 for treating Lymphomatous Meningitis
- Genexol PM® a South-Korean approved nanoparticle is used to treat metastatic cancer; it is under clinical phase 2 study for Pancreatic cancer treatment [97].
- Oncaspar® was approved by FDA in 2006 to treat Lymphoblastic Leukemia.

9. TOXICITIES OF NANOPARTICLES

The toxicity concerns about nanoparticles are a non-negligible act and this should be addressed too [98]. The particle size, particle shape, aggregation solubility, drug release, nanoparticle-drug interactions, surface area are the major concerns. The small size makes them more vulnerable to health hazards [99]. These nanoparticles are more prone to lung deposition and are responsible for rapid systemic translocation having several cytotoxic, oxidative, inflammatory effects as compared to larger particles [100]. Hussain et al. conducted a study on the toxicity of metal-based nanoparticles and investigated that silver was highly toxic for human lungs, whereas, molybdenum, iron oxide, aluminum, manganese oxide, and tungsten were reported less toxicity. However, it is still unclear that how nanoparticles induce toxicity, it might be because of oxidative stress [101]. Lam et al. investigated that rats and mice showed a higher degree of pulmonary toxicity being treated with carbon nanotubes as compared to the treatment with carbonyl ion particles and carbon black. It is also investigated that surface modification in quantum dots with N-acetylcysteine lowers the issues of toxicity [102]. Therefore, it is recommended that the screening of nanoparticles should be made while considering their chemical, physical, properties, cellular, and tissue interactions in animal testing [103].

10. CONCLUSION AND FUTURE PROSPECTS

It is worth mentioning that nanotechnology has given us tremendous outcomes for cancer diagnosis, detection, therapy, and circumventing multi-drug resistance [104]. They provide a wide range of opportunities to improve therapeutic outcomes. While forecasting the prospects of nanomedicines and drug delivery systems, there is no ambiguity in saying that this emerging field has revolutionized the world with its contemporary research dynamics [104,105]. The science of nanomedicine is right now among the top-notch research areas. Much of the research in this domain has been conducted in the past two decades, thousands of patents have been completed and hundreds of clinical trials have been conducted [106]. Tumor cells therapy is still the point of discussion among scientists, there is a lot more is still to be done. It is also a point of focus that nanoparticle development is challenging due to the lack of suitable in vitro models which are masking accurately the in vivo state. Contemporary therapeutic applications of the nano-formulations are prepared on in vitro evaluation while using cell lines which fails to capture the peculiarity and complexity of nanoparticle-cell interactions in vivo [107,108]. But still, there is no ambiguity in saying that it is an emerging area and it remained very helpful in treating cancers [109]. For a logical nanotechnology outline, we need an enhanced understanding of cellular, pharmaceutical, physiological constituents regulating nanotechnology-based drug delivery [110, 111].

11. ACKNOWLEDGMENTS

The authors would like to acknowledge Prof. Deng Linhong and Prof. Chen Xn for their worthy guidance and support.

12. 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 58(3): 19-28 (2021)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(58-3)663

Pakistan Academy of Sciences

Review Article

Single Cell RNA Sequencing (scRNA-Seq) as an Emerging Technology in Cancer Research

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Abstract: RNA sequencing (RNA-seq) has revolutionized basic biomedical research by studying the transcriptome at high resolution, and thus it has been proved to be very successful for understanding the molecular mechanisms of cancers. For example, RNA-seq has facilitated a comprehensive and multidimensional mapping of the key genomic changes that lead to various types of cancers. Nevertheless, the heterogeneous nature of cancer tissues has always been a problem. To overcome this challenge, single-cell RNA sequencing (scRNA-seq) has emerged as the most powerful tool to characterize cancer tissues by enhancing our knowledge of transcriptome at a single-cell resolution. In addition to disentangling the heterogeneity problem, scRNA-seq has other applications such as determining the molecular mechanisms of cellular differentiation, characterizing gene expression levels, and determining rare cell types found within cancer tissue. scRNA-Seq is used, as an emerging diagnostic tool, in tertiary healthcare settings with diverse clinical applications. Thus, the utility of scRNA-Seq in a healthcare system not only provides compelling evidence about understanding cancer biology but also points towards the development of therapeutic options in the future. The purpose of this review is to educate readers about the applications of scRNA-seq in cancer research in a wider context..

Keywords: Soybean; Cancer, Gene Expression, Single Cell RNA Sequencing, Transcriptome Profiling.

1. INTRODUCTION

RNA sequencing (RNA-seq) is a powerful and important technology for understanding the underlying molecular mechanisms of cancer, thus revolutionizing basic biomedical research. By profiling gene expression levels across the entire transcriptome, RNA-seq allows cancer researchers to detect diverse genetic changes such as translocations, deletions, insertions, alternative splicing, and gene fusions. As cancer tissues are mostly characterized by different kinds of genomic changes, RNA-Seq has proven valuable for diagnosing tumors, and for further characterizing them [1]. Besides, RNA-Seq has also revolutionized other fields of biology such as research involving vision, autoimmune diseases, cardiovascular diseases, developmental/evolutionary relationships among brain cells by delineating biological processes at an unprecedented scale [2-6].

The Cancer Genome Atlas (TCGA) has been a notable example of RNA-seq application in cancer research [7]. By using genome sequencing, TCGA has quantified gene expression levels in 33 types of cancers and generated a comprehensive and multidimensional map of the key genomic changes that drive these cancers [7]. Similarly, The Encyclopaedia of DNA Elements (ENCODE) is another example of genome analysis based on RNA sequencing. ENCODE aims to identify all functional elements in human and mouse genomes through various functional genomic assays by studying transcription factors and their target genes,

Received: June 2021; Accepted: September 2021
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chromatin structures, and histone modifications [8].

These two datasets strongly improved and accelerated our understanding of genomic and molecular characteristics of cancers, which further helps to improve the prevention, diagnosis, and treatment of cancer. However, until recently, all transcriptomic studies were typically conducted on a bulk level, averaging the variable transcriptomes from millions of cells [9], even though cancer tissues are highly heterogeneous. In addition to cancer cells themselves, cancer contains other cell types including immune cells and other kinds of stromal cells. Furthermore, due to a variety of factors including genomic instability, epigenetic alterations, environmental differences, even individual cancer cells within a single tumor may be highly plastic in their molecular signatures during the development of tumor progression.

Given the extreme heterogeneity within a cancer tissue, it is not surprising that cancer can develop resistance to cancer treatments. Thus, understanding the underlying mechanisms that drive heterogeneity in cancer tissues is becoming more urgent. The development of sing-cell RNA sequencing (scRNA-seq) is a leap towards this challenge. With scRNA-seq, cancer researchers could further dissect the diversity of tumors into different molecular states [10], and once fully explored, scRNA-seq will finally help to tailor cancer treatments and to develop personalized medicine [11].

2. RNA-SEQ TOOLS IN CANCER RESEARCH

Several analytical tools have been developed to further enhance the contribution of RNA-seq to cancer research and to simplify the massive data that are being produced in RNA sequencing (Table 1). These tools have been promising in the field of cancer research as intra-tumor heterogeneity in a single cancer sample has always been a problem. Among them, CIBERSORT [12] is well-known and considered to be the most popular tool used by various cancer research groups. Essentially, CIBERSORT stands out from the other tools in being highly accurate, resistant to noise, and can characterize the cellular composition in a cancer sample. Its predecessor, ESTIMATE [13], as its name indicate, can estimate the fraction of stromal and immune cells in tumor tissue. This fraction is important in determining the tumor purity. But as time passed by, other tools such as MCP-Counter [14], xCELL [15], and most recently ICeD-T [16], were introduced to answer other questions that were previously unresolved by CIBERSORT and ESTIMATE. MCP-Counter, for example, is a tool used mainly to identify multiple immune cells in a tumor microenvironment that are involved in the prognosis of certain cancers, such as adenocarcinoma and breast cancer [14]. Similarly, xCELL is used to paint a picture of the tumor microenvironment of the disease. This includes analyzing 64 different cell types based solely on their gene signatures [15]. Lastly, ICeD-T uses tumor purity information to effectively discriminate between the aberrant group of genes from the consistent group [16].

In addition to tools that use immune cells as the quantitative parameter, there are tools designed to target or evaluate the outcome of certain treatments. For example, QuanTISeq works by quantifying the tumor immune contexture. Contexture used in this analysis is the absolute fraction based on five immune cells. The information can then be derived to determine the optimum pharmacological therapies which include a combination of pharmacological therapies [17]. Other examples include TIMER, which is an analytical tool that is based on the estimation of abundance of six tumor-infiltrating immune cells. These parameters can then be derived to determine the antitumor therapy on the disease [18]. Altogether, these analytical tools have undeniably elevated the contribution of RNA-seq in the field of cancer. Among the challenges, however, include predicting cell types that have a very low fraction and determining the gene expression of specific cell types. To this end, scRNA-seq emerged as a promising tool thus enabling scientists and clinicians to analyze transcriptomic data at a single-cell resolution.

3. APPLICATIONS OF scRNA-SEQ IN THE FIELD OF CANCER RESEARCH

A range of applications of scRNA-Seq in the field of cancer research is summarized in Figure 1 and discussed below.

3.1 Intra-tumour Heterogeneity in Cancer

Cancer is generally associated with genetic modifications (also called “mutations”) such as

Table 1. Various RNA-seq analytical tools

Analytical Tool	Application	Output Data	Advantages	References
CIBERSORT	Characterization of cell heterogeneity using RNA mixtures from nearly any tissue	Cell composition in complex tissues	High accuracy in analysis of mixture with noise and closely related cell types	[12]
ESTIMATE	Estimate the fraction of stromal and immune cells in tumor tissues	Determine the fraction of infiltrating stromal and immune cell in tumor tissues Predicting tumor purity	Able to determine the stromal score and immune score separately Has the potential to be used in other type of cancer which were not validated in the study	[13]
MCP-Counter	Robust quantification of the absolute abundance of multiple immune and stromal cell populations in the heterogeneous tissues	Quantitative analysis of the microenvironment of normal and cancer tissues via eight immune and two stromal cell populations Prognostic value associated with MCP-counter estimation	Able to quantify more than two immune cell populations Quantitatively validated Has been used in studies involving lung adenocarcinoma, colorectal, and breast cancer prognosis	[14]
TIMER	Explorations of the disease-specific clinical impact of different immune infiltrates in the tumor microenvironment	Estimation of the abundance of six tumor infiltrating immune cell types The impact of antitumor immunity on cancer immunotherapies	Do not suffer from biased estimations due to statistical co-linearity Able to analyze data derived from TCGA RNA-seq data Has been used in studies involving 23 different types of cancer	[19]
EPIC	Robust approach to determine the immune/cancer cell profile in bulk tumors	The proportion of immune and cancer cells	Applicable to most solid tumors	[20]
QuanTISeq	Quantification of the tumor immune contexture	Quantitative analysis of immune cell proportion in the form of absolute fractions based on five immune cells Provides mechanistic rationale in designing combination of pharmacological therapies	Provide prognostic values from immunoscore Has been validated in three different types of cancer Potential to be used in inflammatory and infectious diseases	[17]
xCELL	Cell type enrichment analysis from gene expression data for multiple immune and stroma cell types	Portrays the tumor microenvironment through gene signature-based method	Analyze gene signatures for 64 cell types (adaptive and innate immunity cell, hematopoietic progenitors, epithelial cells, and extracellular matrix cells) Does not discriminate between closely related cell types	[15]
Iced-T	Asist users with immune cell expression deconvolution within tumor tissues Identifies the genes whose expression in tumor samples inconsistent with reference profiles	Quantitative visualization of immune cell expression within tumor tissues Estimates cell types abundance through the use of tumor purity information	Able to perform deconvolution o the linear-scale and incorporate the beneficial properties of the log-transformation at the same time Able to differentiate genes into two different groups: aberrant group and consistent group	[16]
Slingshot	Identifies multiple lineage trajectories based on noisy sir	To model branching lineage trajectories using scRNA-seq data	Slingshot is flexible to the type dimensionality reduction, normalization, and clustering procedures	[21]

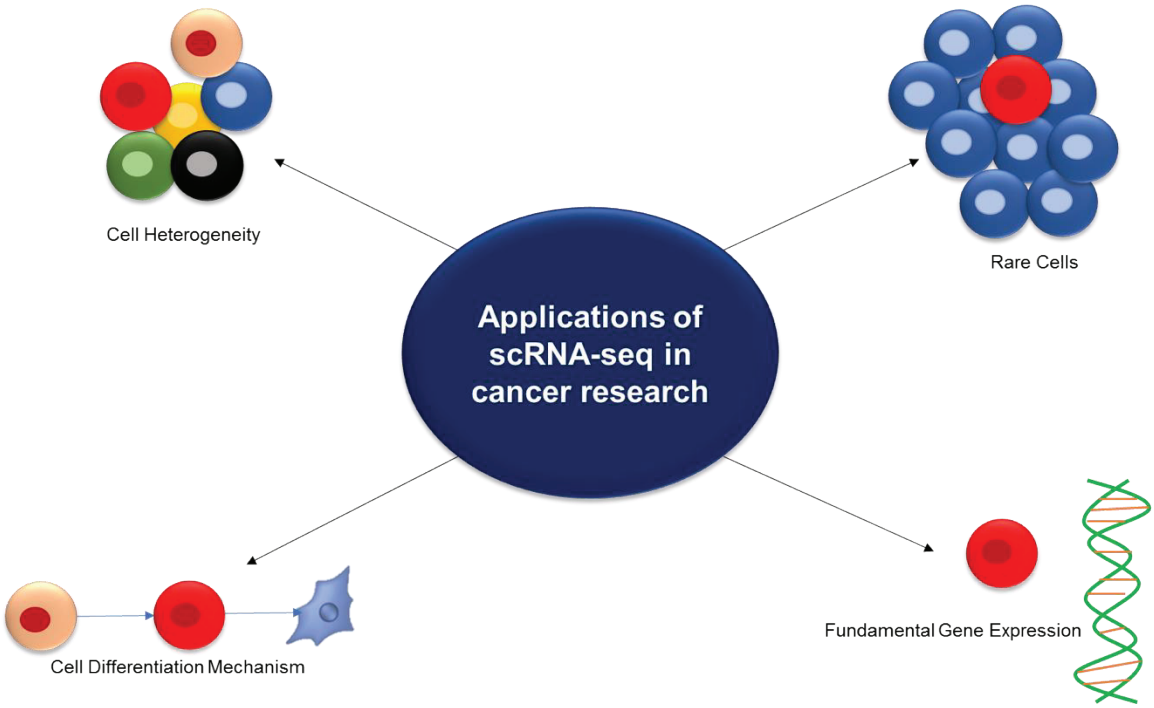

Fig.1. Applications of scRNA-seq in cancer research

the alteration of chromosome number and/or structural chromosomal abnormalities [22]. Such modifications of the genome can arise in different organs and lead to different forms of cancers, although the known mechanisms (or hallmarks) of cancers seem to be recurrent [23]. To add complexity to the situation, tumors are not uniform masses of cells presenting the same characteristics in all situations. Rather, their composition varies from one patient to another [24], and thus comprises an array of cellular types, many of them potentially contributing to the progression of cancer (Pietras and Ostman, 2010) [25]. This intratumoral heterogeneity is a real challenge from a research standpoint because it raises the possibility that every cell type could, in principle, behave differently and express specific genes. It is not clear yet how the interaction between these cell types affects cancer progression, however stromal cells, for example, seem to be involved in the process [26]. It was also proposed that some normal cells could be recruited to provide a micro-environment for cancer cells [27].

Analyses of the tumors transcriptome are usually performed in bulk. Bulk analysis performs well to differentiate through different types of tumors, such methods are, however, not informative enough keeping in view the intratumoral heterogeneity [28]. This is because the observed signal is only the compilation of profiles from all cell types, meaning that the transcription of a specific cell type is lost. Thanks to the recent advances in single-cell RNA sequencing that have allowed a very fine detection of gene expression profiles at a single-cell resolution. With scRNA-seq, it is now possible to characterize gene expression levels of virtually all cell types, with an exception for those genes whose expression rates are extremely low. Many variants of the scRNA-seq have been developed to better study cellular subpopulations. Most of these variants have been compiled in Table 2. An example of the potential application of these methods is the work performed by Klein and colleagues [29], who developed the droplet barcoding method as a way of identifying the transcriptome of individual mouse embryonic stem cells. With this method, the cells are initially captured individually in a droplet followed by barcoding and sequencing. The barcoding allows for the easy association of a cDNA to a cell in particular.

3.2. Identification of Rare Cell Types in Cancer

Among the variety of cellular types in cancer tissue, particular cells play an important role concerning cancer though their number is limited. These include rare cell types such as cancer stem cells from which

the tumor originates [30]. These cells are essential for the proliferation and the maintenance of the tumor and compared to thousands of other cellular types that could not lead to the formation of a tumor, the number of these rare tumorigenic cells remains to be in hundreds [31]. As long as specific markers for detection of these rare cell types are integrated into the analysis, traditional bulk sequencing remains to be inadequate in this scenario because the signal of a handful of rare cells would most likely be lost among the ones from all other cells types. Even if the signal was noticeable for the tumor as a whole, it would still be impossible to trace their particular lineage.

Instead, scRNA-seq could effectively discriminate among rare cell types based upon their transcriptional activity. Since these rare cells are anticipated to be most challenging in terms of treatment, understanding their correct transcriptomic profiling and their underlying molecular mechanisms would possibly pave the way for the development of cancer therapy in the future. As an example, Cao and colleagues have successfully developed single-cell combinatorial indexing RNA sequencing (sci-RNA-seq, Figure 1), a technique that allowed them to cluster all the cell types in *C. elegans*, a multicellular organism [32]. Among these cell types, Cao and colleagues recovered rare neuronal cells in the L2 larval stage. Thus, the application of this method for early detection of rare tumorigenic cell types such as stem cells are very promising and could lead to a better understanding of the initiation of cancers.

3.3 Cellular Differentiation and Developmental Mechanisms

Many tissues continuously grow and regenerate through a coordinated differentiation activity of stem cells. Yet, understanding the molecular mechanisms that control cellular differentiation and fate determination in stem cells is still one of the fundamental questions in developmental and stem cell biology [33]. To answer this question, it is imperative not only to trace the lineage of a cell or a group of cells that underlie these processes but to fully dissect all the intermediate stages as well as the endpoints along the path of differentiation. In other words, understanding relationships among these lineages will illuminate the fundamental mechanisms underlying normal development, and can provide insight into the development of different pathologies including cancer. In general, lineage relationships are experimentally revealed through fate mapping methods, and once fate mapping is carried out at a single-cell resolution, it is known as lineage tracing [34]. Traditionally, this goal was achieved by: (1) direct examination of dividing cells in a transparent embryo, (2) through staining cells with dyes in opaque embryos, and (3) via distinct pigmentation patterns or cellular appearance [33]. Though versatile, these methods had certain inherent limitations. For instance, these methods were providing sufficient information about fate maps of the labeled cells but failed to explain the lineage trajectories, transient intermediates, and lineage branch points during the whole differentiation process.

With the advent of high-throughput sequencing approaches, it is now possible to define the molecular status of cells and to fine map their path along a differentiation axis. One such technique is scRNA-seq that allows for discrimination cellular heterogeneity. Based on single-cell transcriptomic data, scRNA-seq could be used efficiently in stem cells research to trace the lineage trajectories, cellular intermediates, and branch points along the axis of differentiation [35-38]. To achieve these goals, the only requirement for researchers would be to collect cell samples at different time points. Interestingly, by integrating scRNA-seq with more recent approaches like CRISPR- or transposon-mediated modification of DNA barcodes, it is possible to fine map the lineage hierarchies and to identify endpoint cells based on their transcriptomic profiles without previous knowledge about cell types [39-42]. Taking advantage of a single cell resolution, this integrative approach now provides a robust method for deconstructing how individual stem cells maintain tissues. Finally, by integrating single-cell RNA sequencing with clonal lineage tracing, it is possible to delineate the molecular mechanisms that control the differentiation of stem cells into tissues, organs, and ultimately an organism.

3.4 Fundamental Characteristics of Gene Expression

Growing evidence suggests that gene transcription

Table 2. Various techniques developed for scRNA-seq

Protocols	Method	Throughput	Cost	Reference
Indrop	Droplet microfluidic-based	High throughput (4,000-12,000s cells per hour) Low noise Low cell bias	High cost Low efficiency in cells of genes with transcript abundance lower than 20-50 transcripts Low detection rate	[29, 49]
Drop-seq	Droplet-based	Relatively high throughput (10,000 cells per day for 6.5 cents per cell) High efficiency	Venerable to impurities in the cell isolation process Low detection rate	[49]
CytoSeq	Microwell-based	High throughput (10,000 - 100,000s cells per day) High detection rate	High cost Restricted to pre-defined set of genes	[50, 51]
Sci-RNA-Seq	Combinatorial indexing	High detection rate High throughput (50,000 cells) Reduced batch effect	Relatively high cost Limited to copy number variant	[32]
Smartseq2	PCR-plate based (Full-length RNA sequencing)	Low cost	Relatively low throughput (100s cells) Labor intensive	[49, 52]
CEL-seq and CEL-seq2	Tag-based sequencing	Strand specificity High barcoding efficiency Low cost	Relatively low throughput Low sensitivity for low expressed transcripts	[53]
MARS-seq	Tag-based sequencing	Molecular counting High degree of multiplexing Low cost	Requires enrichment to detect rare cell population	[54]
Fluidigm C1	Microfluidic chips	High detection rates Highly-accessible and affordable	High cost-efficiency Low throughput (96 cells) Sub-optimal performance on primary cells	[55]

does not follow a continuous path. Rather, it completes in several short steps called "transcriptional bursts". Under the control of various stochastic processes, these transcriptional bursts finally lead to extreme transcriptional heterogeneity as seen between cells. Recent studies also suggest that cells preferentially express a single allele or a single splice isoform resulting in a process known as differential gene expression. Additionally, a single genetic locus can undergo diverse splicing patterns resulting in multiple isoforms, each having different transcriptional start sites or polyadenylation sites [43]. Moreover, processes such as dosage compensation or X-chromosome inactivation in most diploid female animals ensure expression of only one allele at a time, either maternal or paternal [44]. Historically, much of our understanding of the transcriptome is based on bulk studies conducted on cell populations. It is now, however, well established that the homogeneous cell populations *in vitro* or *in vivo* are quite heterogeneous in terms of expression patterns owing to both intrinsic and extrinsic factors [45].

Understanding the gene expression pattern at a single cell level is important for two main reasons. First, recent findings suggest that the expression of a transcript in all individual cells of a particular tissue or organ is only facultative rather than obligatory. This was previously not anticipated. Similarly, the adjoining cells that share the same microenvironment are deemed to differentially

express a transcript for unknown reasons. Studying gene expression patterns at a single-cell resolution is therefore important for the identification of co-regulated gene modules and to define gene-regulatory networks [46]. Though several methods have been previously used to achieve these goals, scRNA-seq has been exceptional in this respect. This method allows genome-wide profiling of both coding and non-coding cellular transcriptome with higher efficiency. Thus, even minor changes in gene expression could be detected with greater sensitivity. Moreover, scRNA-seq has the potential to identify previously uncharacterized transcript isoforms, novel exons, SNPs, or mutations [11]. This method has specific applications when allele-specific expression of either autosomal or X-linked genes is required [47]. Finally, Single-cell RNA sequencing has greatly revolutionized the fields of medicine and biology by delineating biological processes at an unprecedented scale and resolution in the recent past.

4. TECHNIQUES DEVELOPED FOR scRNA-SEQ

With such a wide range of applications and impact on the field of cancer research, various methods and protocols for scRNA-seq have been developed (Table 2). These techniques are used to serve a range of purposes: from understanding the tumor architecture to identifying specific cell types in the tumor microenvironment. These methods range from a high throughput method, which can easily sequence 10,000 cells to more affordable methods which sequence 100 cells in a single run. Some can complete a run in a matter of hours, while others might take a whole day. Also, some of the methods need a few days to prepare while others which has a higher cost might have a less hands-on approach. A recent study has suggested Drop-seq as the most cost-efficient method among its contemporaries for quantification of the transcriptome at a large scale [48]. With so many options already in place, it is intriguing to see how the field of cancer research is going to grow soon, especially with the help of scRNA-seq.

5. UTILITY OF scRNA-SEQ IN HEALTHCARE SETTINGS

scRNA-Seq is used, as an emerging diagnostic tool, in tertiary healthcare settings with diverse clinical applications. For example, scRNA-Seq has been crucial in the discovery of differentially expressed genes in different cancer types [56], and has helped track the intra-tumoral heterogeneity and in visualizing tumor microenvironment [57]. In addition, studies suggest that scRNA-Seq has been key to the detection of many cellular and molecular therapeutic targets [58], and for the identification of biological features representing as biomarkers of clinical outcomes [59]. Indeed, scRNA-Seq has also enhanced our understanding of the immune cell heterogeneity and the infiltrating T Cells [60-61]. Thus, the utility of scRNA-Seq in a healthcare system not only provides compelling evidence about understanding cancer biology but also points towards the development of therapeutic options in the future.

6. LIMITATIONS OF scRNA-SEQ

scRNA-Seq has numerous limitations, and they are beyond the scope of this review. However, a few of them include excessive noise and complexity due to technical or biological reasons, transcript coverage bias, also known as high dropouts or low capture efficiency, high variation in data output, and computationally challenging data analysis [62]. For an in-depth insight on the subject, Lähnemann and colleagues [63] have recently highlighted eleven major challenges encountered while dealing with scRNA-Seq data.

7. CONCLUSION

In summary, big advancements such as high throughput transcriptome profiling coupled with the availability of robust analytical tools have greatly revolutionized the field of cancer research in the recent past. The sole purpose was (1) to fully characterize cellular components in a tumor microenvironment (2) to understand the molecular pathophysiology of cancer, and (3) to mitigate cancer-related morbidity and mortality through the development of novel therapeutics. Among them, scRNA-seq have been developed to suit any scale of research in cancer, whether it is in clinical settings, institutional or private research.

8. 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 58(3): 29-42 (2021)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(58-3)665

Pakistan Academy of Sciences

Review Article

Biosynthetic Gene Clusters in Bacteria: A Review

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Abstract: Soil is a nutrient-rich environment that harbors billions of microbial species. The diversity of microbes in an environment varies with the change in edaphic factors. To survive these environmental changes, microbes produce secondary metabolites which are not directly associated with their growth and reproduction. Bacterial genomes possess biosynthetic gene clusters (BGC) which regulate the synthesis of these secondary metabolites. These BGCs encode for megasynthases such as nonribosomal peptide synthases (NRPS) and polyketide synthases (PKS) which produce metabolites such as antimicrobial compounds, which are the most common metabolites produced by these megasynthases. They help bacteria to survive in the competitive environment by killing surrounding microbes. As chemical drugs may pose immense damage to human health and the environment, so antibiotics produced by natural sources are of major attention these days. The extraction of antimicrobial compounds from bacterial sources also provides scaffolds for new synthetic drugs. Bacteria maintain strict genetic control over antibiotic production. They have particular quorum sensing pathways that help to trigger the surrounding cells to produce antibiotics when required. Biosynthetic gene clusters need to be explored widely under various culture conditions so that more useful products can be extracted from a single type of bacterium. This review focuses on the secondary metabolite production, extraction, and biosynthetic gene clusters which encode megasynthases responsible for the production of antimicrobial compounds.

Keywords: Secondary metabolites, Biosynthetic gene clusters, Megasynthases, Antimicrobial Compounds, Quorum Sensing.

1. INTRODUCTION

Soil is a rich source of microbes as per gram of soil harbors billions of species of various microorganisms [1]. The growth of a bacterium requires certain compounds to be metabolized for fulfilling its needs. The starting, intermediate, or end products of the metabolism of bacteria are known as metabolites. These metabolites can be of two types i.e. primary and secondary metabolites [2]. Primary metabolites are those metabolites that are essential for the normal growth, development, and reproduction of a bacterium. While secondary metabolite is the type of metabolite that is not directly involved in any of such activities but it has some other ecological functions [3]. The uncountable interactions between microbes or amongst microbes and plants including, mutualism, predation, competition occur within the soil ecosystem due to the presence of secondary metabolites secreted by these microbes [4].

Bacteria are useful to mankind due to their ability to produce diverse nature of metabolites. Bacteria are the catalytic factories that possess the potential to undergo evolutionary changes in their genome over a short period and thus introducing a new diversity of metabolites having specialized applications. These metabolites get structurally optimized when bacteria use them against different targets for their defense mechanism. Thus, bacterial metabolite production potential, evolutionary adaptation, and the aptitude of response to external stimuli enhance their potential to be used in industries, agriculture, and medicines [5].

Single bacterial strain can yield different types of secondary metabolites depending upon their

Received: July 2021; Accepted: September 2021
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nature. Secondary metabolites are produced by different biosynthetic pathways which are regulated by biosynthetic gene clusters. These biosynthetic gene clusters encode various megasynthases such as Polyketide Synthases and Non-Ribosomal Peptide Synthases which yield polyketides and non-ribosomal peptides respectively. These are the most copious families of secondary metabolites consisting of diverse compounds with numerous functions. The functions of polyketides and non-ribosomal peptides include protection against stress factors by pigments, iron scavenging by siderophores, antimicrobials, and communication molecules [6].

These secondary metabolites are produced in great quantities by bacteria but the major issue arises when they cannot be extracted properly from the bacterial growth medium or from inside the cells. Bader et al. [7] have reported a supercritical fluid extraction method by using various co-solvents to extract various secondary metabolites from bacteria. Nevertheless, to identify novel compounds plenty of different solvents will have to be used. Hence, there is a need to explore more convenient culture-dependent and culture-independent methods to identify and extract secondary metabolites.

A major class of secondary metabolites produced by bacteria is antimicrobial drugs. The need to explore natural antibiotics increased due to the over and misuse of antibiotics leading to the persistence of antibiotic-resistant bacteria and antibiotic resistance genes in our environment. These antibiotics are released in wastewater of healthcare services, industrial plants, agriculture, and the general population. Even after treating this wastewater by its treatment plants, these antibiotics remain in the plants, and as soon as this treated water is released these antibiotics also get released into the environment. Some of the antibiotics like sulfonamides, fluoroquinalones, and tetracyclines get attached to the soil particles and thus hinder the process of their biodegradation. These issues urge scientists to discover natural products to avoid health and environmental problems [8].

This study focuses on the culture-dependent and independent methods for the extraction of secondary metabolites from bacteria, and the overview of PKS and NRPS megasynthases involved in the production of these metabolites. The most common category of compounds being produced by these megasynthases i.e. antimicrobial compounds will also be discussed.

2. SECONDARY METABOLITE BIOSYNTHETIC GENE CLUSTERSS

Secondary metabolites are of great concern as a specific type of secondary metabolite is produced by only a narrow range of species. Comprehensive analytical techniques result in a better understanding of the complex secondary metabolome of species. Besides, it can help in evaluating the deviations in the metabolite profile and their morphological changes under variable culture conditions [9]. Microorganisms possess secondary metabolite biosynthetic gene clusters (BGC) in their genomes. Biosynthetic gene clusters (BGCs) are a locally clustered group of two or more genes that function together to encode a biosynthetic pathway for particular secondary metabolite production [10]. For instance, various BGCs present in Bacillus include polyketide synthases (PKS), non-ribosomal peptide synthases (NRPS), siderophores, phosphonates, ectoines, terpenes, thiopeptides, lanthipeptides, bacteriocins, and other non-traditional, hybrid BGCs [11]. Conventionally, bacterial strains are focused to obtain a particular novel metabolite from them which is being produced in large quantities. But, this metabolite is generally a low-hanging fruit of that particular bacteria so, for getting a better fruit it must be explored further by modifying the culture conditions in which the strain is being grown. This technique is known as the “one strain many compounds” (OSMAC) approach where one strain is studied on a deeper level to investigate various compounds being produced by it [12].

This hunt for more compounds from a single strain was stimulated by the rise in genome sequencing and BGC annotation. This advancement revealed the presence of more biosynthetic gene clusters in strains which shows that the particular strain is explored for characterization of lesser biosynthetic pathways as compared to the biosynthetic genes present in it. This variation in characterization and presence of biosynthetic gene clusters can be because many of the BGCs either become cryptic (un-expressed) or even if they are expressed the expression is so low that it

cannot be detected in the growth conditions being analyzed [13]. As studied by a group of researchers, *Streptomyces albus* has genes to produce about 14 anticancerous and antifungal compounds but they remain silent until a specific elicitor is added to the culture [14].

These BGCs are responsible for encoding megasynthases which are large multi-enzymatic proteins involved in the production of various important natural compounds. These megasynthases use the precursor units and do their condensations and modifications to get diversified natural products [15]. Examples of megasynthases include fatty acid synthases, polyketide synthases, non-ribosomal peptide synthases, etc. These metabolites are valuable in various industries as well because they are known to provide aid as competitive weapons against other organisms like bacteria, fungi, plants, or animals; metal transporters; symbiotic agents between bacteria and plants, insects, nematodes, and other higher animals; differentiation effectors and as sexual hormones [16]. The identification of these secondary metabolites can be done by culture-based and culture-independent methods. Culture-based methods give us the idea of metabolites that can be produced by various bacteria under different culture conditions while culture-independent methods help to identify the potential of bacteria to produce secondary metabolites based upon the biosynthetic gene clusters present in them. These metabolites may not be identified by culture-based methods because of the lesser production or unsuitable conditions for better yield.

2.1 Culture-Based Methods for Identifying Secondary Metabolites

For decades, scientists have isolated bacteria and optimized them for their better growth and secondary metabolite production by changing temperature, time of incubation, pH, and nutritional requirements i.e. carbon, nitrogen, iron, and trace elements sources [17]. If the growth conditions in which bacteria are surviving are not met by the artificial media, or there occurs any competition for the nutrients in bacteria, it hinders the proper growth. Other than growth medium, the incubation conditions may vary for bacteria or some bacteriocins might be produced in the medium by other bacteria thus inhibiting the growth of some important bacterial strains [18]. According to a study by Bode *et al. Streptomyces* sp. experience a significant shift in streptazoline production by the addition of supplements, like $CaCO_3$ and Al_2O_3, in the medium. Production of rubromycin was also affected by changes in pH. Rubromycins were produced only in slightly acidic pH conditions while under neutral pH i.e. 7.3, different compounds were observed [19].

Sometimes bacteria are thriving in nutrient-deprived environments thus when they are provided with nutrient-rich culture media they do not grow. This limitation can be breached by diluting the nutrient medium. Researchers have used this technique to isolate bacteria from aquatic and terrestrial habitats [20]. Other important techniques may involve a physical reduction in the number of mixed bacterial colonies by filtration methods, density-gradient centrifugation, elutriation, and extinction dilution whereby samples are diluted to isolate single colonies [21].

Diffusion chambers are the devices in which semi-permeable membranes are enclosed, to ensure the flow of external nutrients into the chamber. These devices were developed about 20 years ago for the isolation of unknown novel marine isolates [22]. Researchers took the marine sediment samples and serially diluted them and inoculated them in diffusion chambers containing agar. The natural marine environment was mimicked by reinoculating it in marine aquaria. This experiment ended up in 300 times increased growth of known as well as novel bacterial taxa as compared to the standard plate methods. Some other groups of scientists have also used this approach to cultivate bacteria of soil and forests that could not be isolated by previous traditional methods [23]. Other than diffusion chambers, difficult to culture bacteria that can also be isolated by developing growth media that imitate soil conditions. As methanol can be used to extract metabolites from the soil that can be supplemented in growth media to ensure better growth of uncultured microbes. This culture media is known as Intensive Soil Extract Medium (ISEM) [24]. According to a study on *Streptomyces* spp. it was observed that by using soil enriched medium about 4 novel secondary metabolites were obtained which were otherwise not produced in the medium [19]. This approach is a better choice for

the cultivation of unknown bacteria because it is inexpensive and easy to perform in the laboratory. This approach does not require technical expertise or special equipment that's why it can be utilized for an extensive range of applications. This growth media adjustment can be used to activate cryptic or silent BCGs. Most of the unexpressed genes in cryptic or silent BCGs help to avoid unnecessary energy costs as the secondary metabolites produced by these genes are not a prerequisite for the survival of bacteria [25]. This concept was demonstrated by a group of researchers that when *Streptomyces* sp. is co-cultured with Bacillus subtilis or Methicillin-resistant *Staphylococcus aureus*, it causes the activation of Granaticin cluster in *Streptomyces* thus enabling the production of granaticin, granatomycin D, and dihydrogranaticin B [26].

Development in culture-based methods for natural compounds' isolation has been very modest. While contrary to this, culture-independent methods are getting major concerns of scientists. This shift in isolation methods is mainly because of the challenges associated with *in vitro* and in vivo environment differences. Sometimes, it becomes very difficult to imitate in-vivo conditions in a laboratory where traditional culture media are used to isolate bacteria from samples. It results in the rediscovery of the same natural products, repeatedly. It can be attributed to the fact that typical growth media comprise a limited range of nutrient concentrations, carbon sources, oxygen saturation, and pH. Besides, these media favor the growth of fast-growing bacteria only, and metabolic interactions between bacteria are not considered [18]. Bioprospecting, (systematic search for natural products from bioresources) novel compounds has urged the development of unconventional culturing techniques known as culturomics which is the culturing and identification of unknown bacteria employing non-conventional and novel approaches. This technique circumvents many of the restrictions posed due to culture-based techniques by simulating nutritional or environmental conditions that are found in soil [27]. Advances in research have also helped to express silent biosynthetic gene clusters, those were unable to express under normal laboratory conditions, and thus, the innovation of novel products in known bacteria has become easier [25].

2.2 Culture-Independent Methods for Secondary Metabolites

Culture-independent methods for the identification of biosynthetic gene clusters emerged with the development of high throughput sequencing techniques. This development has facilitated the discovery of most of the BGC's that remain unexplored due to the inability to culture certain bacterial strains. Bioinformatics tools use various algorithms that are a necessity for investigating different domains and clusters of genes within sequenced datasets. These algorithms are also used for the identification of regulatory sequences, or to predict molecular structures of synthesized chemicals and discover homologous structures [28]. To access these bioinformatics tools, researchers have developed a web portal that comprises various databases and tools. It is known as the Secondary Metabolites Bioinformatics Portal (SMBP, www.secondarymetabolites.org) [29].

Mining tools use the curated reference data fingerprints of metagenomes and assembled genomes to analyze gene clusters in sequenced data. AntiSMASH is the most commonly used for this analysis. AntiSMASH utilizes Hidden Markov Models (HMM) to detect secondary metabolite coding gene clusters in the bacterial and fungal genomes [30]. Other commonly used tools for the prediction of BGC include Cluster Finder and Prediction Informatics for Secondary Metabolome (PRISM). Cluster finder identifies the BGC based on statistical analysis. Decisions are based on the probability of each converted nucleotide sequence domain to be part of a gene cluster, depending on the frequencies at which this domain occurs in BGC and non-BGC reference training sets, and the identities of neighboring domains [31]. PRISM also works on the principle of AntiSMASH i.e. it uses HMM and reference genomes to identify biosynthetic gene clusters but it focuses on the structural identification of metabolites being produced by the clusters [32].

These tools are used for the already available genomes which are well assembled. But for the case of unassembled short reads or PCR products these tools are not suitable. For such samples, another web portal has been designed which is known as "Environmental Surveyor of Natural Product Diversity (eSNaPD)". This platform has made it

easier to access functions of short PCR amplicons having sequence tags of an adenylation (AD) and ketosynthase (KS) domain by relating them to the reference dataset of gene clusters. Other tools such as Natural Products Domain Seeker (NaPDoS) and SBSPKS v2 are also helpful for short sequences that focus on pathways and their chemical products; and sequence and structure-based analysis of secondary metabolites respectively [33]. These approaches have led scientists to discover BGC for nargenicin macrolides which are anti-Staphylococcus aureus antibiotics, first isolated in the 1970s. Its BGC was rediscovered by genome sequencing in Nocardia species after being hidden for about 40 years [13]. To store these numerous data of BCG tools there are 3 major repositories including MIBiG (Minimum Information about Biosynthetic Gene cluster), ABC (Atlas of Biosynthetic Gene Clusters), and antiSMASH-DB [33]. Where, MIBiG contains data of about 1700 gene clusters, AntiSMASH contains 7800 NRPS and 4500 PKS clusters while ABC consists of 2400 experimentally validated secondary metabolites and 1,000,000 non-verified entries [17]. These databases help researchers worldwide to upload or access the data submitted in these repositories.

3. MEGASYNTHASES INVOLVED IN SECONDARY METABOLITE PRODUCTION

Secondary metabolites in bacteria are produced by multi enzymatic and multi-domain megasynthases. These megasynthases are the multienzyme protein products of biosynthetic gene clusters. They include various structural types but here nonribosomal peptide synthases (NRPSs) and polyketide synthases (PKSs) are being considered which are responsible for the production of nonribosomal peptides and polyketides. These secondary metabolites are a great source of valuable biological activities as well as clinical applications such as antifungal, antimicrobial, antitumor, antiparasitic, and immunosuppressive agents. These domains further have their core catalytic domains that aid in the biosynthesis of polyketide or nonribosomal backbone moieties. These catalytic domains are adenylation (A), an acyl carrier protein (ACP), acyltransferase (AT), condensation (C), dehydratase (DH), enoylreductase (ER), ketoreductase (KR), ketosynthase (KS), and thiolation (T) [34]. Other than these catalytic domains, several auxiliary functional domains called tailoring domains also support providing a diverse range of chemical alterations to the backbone moieties of these secondary metabolites to enhance their structural diversity [34]. The scheme of these megasynthases is shown in figure 1 while the various types of compounds being synthesized from these domains are given in table 1.

3.1 Nonribosomal Peptide Synthetases (NRPSs)

NRPSs are the megasynthases that are recognized to be the largest known enzymes having a

Table 1. Examples of Non-ribosomal peptides and Polyketides derived from bacteria

Compound	Known Function
Non-ribosomal Peptides [38]	
Pyochelin	Antibacterial
Bactobolins	Antibacterial
Xylocandin	Antifungal
Glidobactins	Anticancer
Polyketides [40]	
Actinorhodin	Antibacterial
Tetracycline	Antibacterial
Amphotericin	Antifungal
Anthracycline	Anticancer

molecular weight of up to 2.3MDa. They consist of several units each of which is a set of enzymatic domains that determines the primary structure of the corresponding peptide product in terms of their specificity, number, and organization [35]. To get a final peptide a cascade of reactions takes place along a particular line of direction. NRPSs provide this assembly line so that proper functional peptides can be produced. In the first step, the primary sequence of the peptide is defined by the arrangements of recurring units of an NRPS. This minimal repetitive module comprises three domains known as adenylation domain (A-domain), condensation domain (C-domain), and peptidyl carrier domain (PCP-domain). All three domains are responsible for different tasks. A domain is involved in the recruitment of amino acids that are to be incorporated in the final product. Each type of amino acid is recruited by a particular A-domain substrate because there are several hundred A-Domain substrates with their specific specialties. A domain activates the amino acids for peptide synthesis. PCP domain then takes these activated amino acids and acts as a scaffold for adding amino acids and establishing covalent bonds in them. The condensation domain is responsible for amide bond formation between nascent peptides and the amino acids it adds to the chain [36, 37]. Some examples of NRPs include lipopeptides (xylocandin, cepacidin, occidiofungin, and burkholdins, etc), siderophores (ornibactin, malleobactin, pyochelin, and cepaciachelin, etc), and hybrid PKS-NRPS (thailandamides, thailanstatins, bactobolins, glidobactins, rhizoxin, and rhizonin, etc) [38].

3.2 Polyketide Synthases

Polyketides are a group of natural products which contain diverse carbon skeletons that comprise enediynes, macrolides, polyenes, polyethers, and polyphenols. The exact function of these compounds is still to be known but their estimated

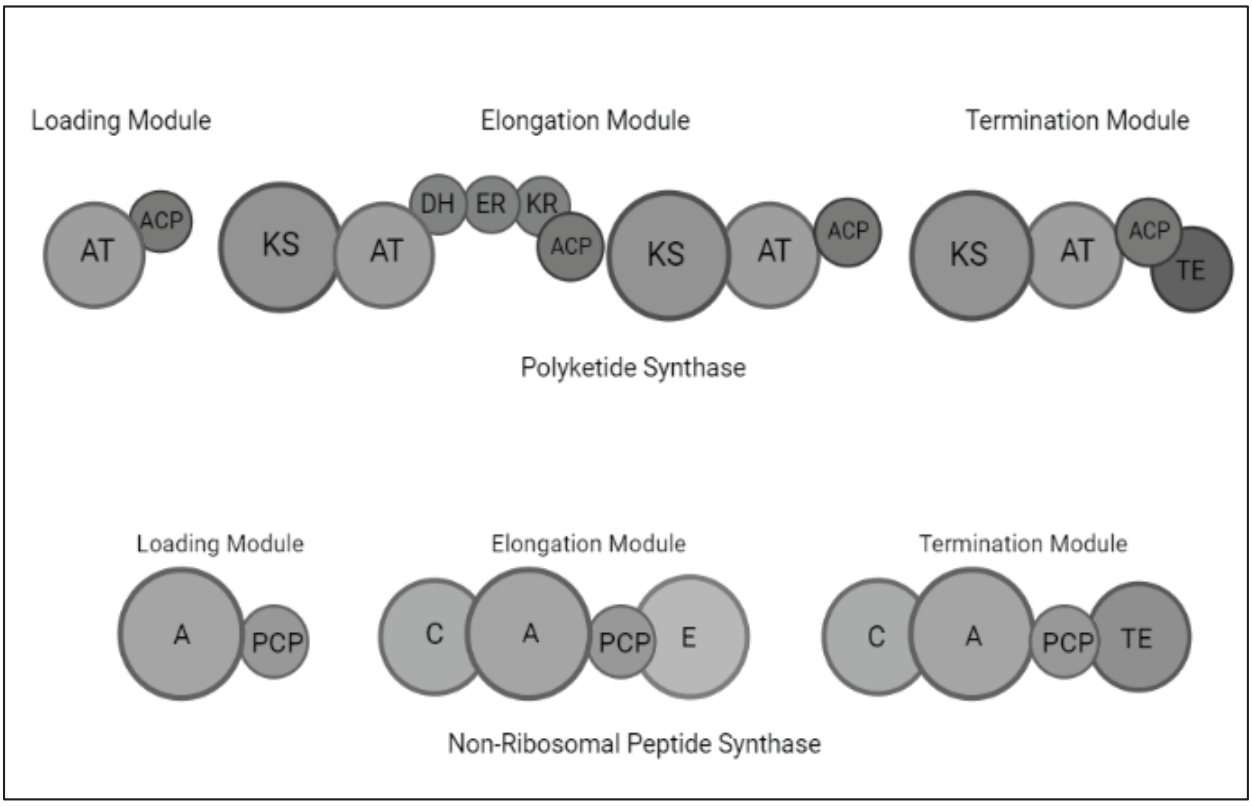

Fig. 1. Schematic representation of PKS and NRPS megasynthases. In Polyketide synthase, a minimal module is made up of Ketosynthase (KS), Acyltransferase (AT) and Acyl Carrier Protein (ACP) domains. DH (dehydrogenase), ER (enoyl reductase) and KR (ketoreductase) are additional domains these are optional. PKS proteins may contain more than one module. These interactions occur between the C terminal of ACP and N terminal of KS. In Non- ribosomal peptide synthases, C (condensation), A (Adenylation) and PCP (Peptidyl Carrier Protein) domains make the minimal module. E (epimerization) domain is optional here.

functions include virulence factors, infochemicals, pigments, or defense mechanisms. Polyketides are known as a potential source of novel therapeutic drugs, in pharmacology. In medicines, they can be used as an immunosuppressant, antibiotics, antiparasitic, antitumor, and cholesterol-lowering agents. Polyketides are classified based on their biosynthetic enzyme products. A minimal module in them is made up of different domains including the ketoacyl synthase (KS) domain, an acyltransferase (AT) domain, and an acyl carrier protein (ACP) domain [39].

There are three kinds of polyketide synthases. Multimodular PKSs contain either one or more multidomain polypeptides. In this type of synthase, the polyketide chain passes serially from one active site to another. With the change in catalytic domains of these megasynthases the variety of chemicals being produced by them and their complexity also alters in a stepwise manner [36]. While the other class of polyketides that is, iterative PKSs consists of a single set of catalysts that aims at assembling a polyketide of controlled chain length by consuming active sites repetitively. The third type of polyketide synthases which is called type III PKSs is not the same as the rest of the two types of PKSs. In this synthase, the growing polypeptide chain is not directly involved with a protein [40]. Bacteria are an abundant source of polyketides that act as an antibiotic. The major example of these polyketide antibiotics is erythromycins, tylosin, tetracyclines, monensin, tiacumicin, rifamycin, and streptogramins [41]. Antimicrobial compounds being produced by bacteria are of major concern these days.

4. ANTIMICROBIAL COMPOUNDS

According to the definition of the English dictionary, antibiotics are defined as "a substance produced by a microorganism and able, in dilute solution, to inhibit or kill another microorganism." Lietman altered this definition by adding that human beings are also a producer of antibiotics as they modify the drugs by chemical changes. According to another group of researchers "antibiotics encompass a chemically heterogeneous group of organic, low-molecular-weight compounds produced by microorganisms that are deleterious to the growth or metabolic activities of other microorganisms" [42].

The need for the discovery of new antimicrobials is increasing tremendously due to the increasing antimicrobial resistance by bacteria which may lead to the insufficiency of the already discovered antimicrobials. The majority of the compounds being used for anti-infective purposes are the derivatives of naturally occurring compounds. Antimicrobial production is a general phenomenon of most bacteria. Bacteria are well-known to yield a diverse range of antimicrobials that can be formed either by a specific bacterial group while others may be produced by a broad range of species. Major antibiotic categories such as tetracyclines, beta-lactams, and macrolides depend on the natural product scaffold [43]. Both gram-negative and gram-positive bacteria produce secondary metabolites. Among gram-positive strains, *Streptomyces* produces cypemycin, bottromycins, eomycin, grisemycin, and chloramphenicol while *Bacillus* species are known to produce four non-ribosomal antibiotics including bacilysin, bacitracin, surfactin, and plipastatin; three ribosomal TasA, sublancin, and subtilosin, antibiotics [44]. While, among gram-negative strains, Proteobacteria such as *Burkholderiales, Myxobacteria, Photorhabdus, Pseudomonads*, and *Xenorhabdus* produce a large and underexploited variety of secondary metabolites [45]. Different types of antimicrobials produced by bacteria include classical broad-spectrum antibiotics, bacteriocin, protein exotoxins, metallic by-products, and other lytic agents.

Bacterial secondary metabolites can be a potential source of leads for new drugs such as cytostatic and antibiotics. These compounds are synthesized in a stepwise manner. The process begins with the synthesis of building blocks by using primary metabolites such as amino acids or acyl CoA derivatives as educts. The use of primary metabolites is dependent on the structure and class of the metabolites being produced. In the next step, precursor molecules are gathered either by modular mega enzymes like polyketide synthases or nonribosomal synthases. While in the final step, the assembled molecules are further altered by extremely specific reactions such as hydroxylation, ring formation, or glycosylation [46].

More than 75 % of all antibacterial while about

50 % of anticancer drugs are being produced from natural products [47]. As apratoxin A, produced by *Lyngbya boulloni* is used for the treatment of cancer [48]. The rate of antibiotic resistance is getting increased with the widespread use of antibiotics. This increase in resistance does not have any limitation because of emerging mutations and genetic transfers among pathogens thus making them least prone to the available antibiotics [49]. The fight for new drug discovery must be continued at all times by the pharmacies but because of the difficulty and expense of this process scientists rely on synthetic chemicals to treat humans. For this reason, the interest in finding new lead structures for drug discovery has reduced in past years [50]. This decrease in interest can be attributed to various disadvantages that a natural product discovery possesses over synthetic chemicals:

- The discovery of new drugs is a laborious task.
- It requires much more time compared to the synthesis of new chemical drugs.
- Natural products are produced by some biological agents which require proper handling by experts and specialized conditions and equipment for growth and maintenance [51]

But the advantages that we get from these natural compounds can always set back the disadvantages posed by them. One of the major advantages is that natural compounds possess a wider range of chemical space as compared to synthetic compounds. Both of the compounds are aimed at producing biological matter that can help to treat the ailment but the synthesis process is different for both of the products [52]. Naturally, in the biosynthetic process, a very limited amount of building blocks is exploited while in the case of chemical synthesis we have tens of thousands of chemicals available for use. Consequently, we get a massive number of different products just by changing the input. While in the case of natural products limited building blocks are fed into different pathways to achieve diversity of products. Other than the difference in building blocks the other difference lies in the synthetic transformation of the products. The biosynthetic process easily accomplishes site-selective C_H activation to introduce oxygen and distinguish between various functional groups while chemical synthesis involves nitrogen or sometimes uses additional atoms such as sulfur and halogens which are infrequent. A final difference in these pathways is their stereochemistry. The second major advantage of natural products over chemical compounds is their ability to be amended into other useful compounds. It is obvious from the fact that natural product extracts or the pure form extracted from these compounds are further modified to be used. In some other cases, it can be used in its crude or pure form as well [53].

This finding made scientists think about "natural products like" compounds synthesis strategies. They focus on finding new lead compounds by identifying the natural product scaffolds or by synthesizing the analogs of these compounds. This approach leads to the fusion of natural product research and combinatorial chemistry. Both of these disciplines now support, fertilize and rely on each other [54].

Bacteria have developed multiple strategies to defend themselves against predators and competitors in soil microhabitat. Among all of the defense strategies being adopted by these bacteria, antibiotics act as a weapon in numerous conflicts as shown in Figure 2 [42]. Many bacterial species and genera involved in antibiotic production had been isolated from diverse soil environments. Streptomycetes that belong to the family of Actinomycetes are a primary source for clinical antibiotics scaffolds. Other than *Streptomyces*, Myxobacteria are also a potential source of antibiotic discovery. These antibiotic makers arise from the soil but can originate in microbial accumulations in or on plants and insects, as well [55].

In nature, antibiotics act as a weapon or a shield but this potential of antibiotics is not the sole benefit of antibiotics being produced by bacteria in the soil. This notion came from the fact that antibiotic resistance occurs in bacteria but this resistance is also counteracted by these bacteria. So this concept leads to the discovery of a new phenomenon that is "hormesis". Hormesis refers to the ability of metabolites to act differently at varying concentrations [56]. According to various research groups antibiotics act in a concentration-dependent manner i.e. they act as an inhibitor at high concentrations while at low concentrations they function as a mediator of intracellular cell responses [57].

4.1 Antibiotics Produced in Secondary Metabolism

As discussed earlier, the soil is best known to possess organisms that produce antimicrobial agents. Among these diverse species *Streptomyces*, bacilli, and myxococci are known to be the best producer of antibiotics [58]. Antibiotics are mostly produced by secondary metabolic pathways in various growth conditions. *Streptomyces* species produce griseofulvin, erythromycin, anthracyclines, tetracyclines, nystatin, and curvularin, etc as a product in their secondary metabolism. While other organisms are known to produce mycotoxins, alkaloids, terpenes, glycosides, steroids, and other secondary metabolic products that act as antibiotics [59]. Antibiotics can be different in their structure and activity, based upon their production pathway [60]. Growth dynamics of bacterial cells indicate the metabolic activity of strains at various stages. For example, at the high nutrient level, the production of proteins, nucleic acids, and other macromolecules that are necessary for survival as well as the exponential growth of the bacterial population increases. While at a low concentration of nutrients, cells stop dividing and enter into the stationary phase. This limitation in nutrient concentration causes the metabolic routes to open which leads to the synthesis of secondary metabolites. These antibiotics may possess activities ranging from killing or inhibiting competitors to controlling cell growth or modulating colony morphology [57].

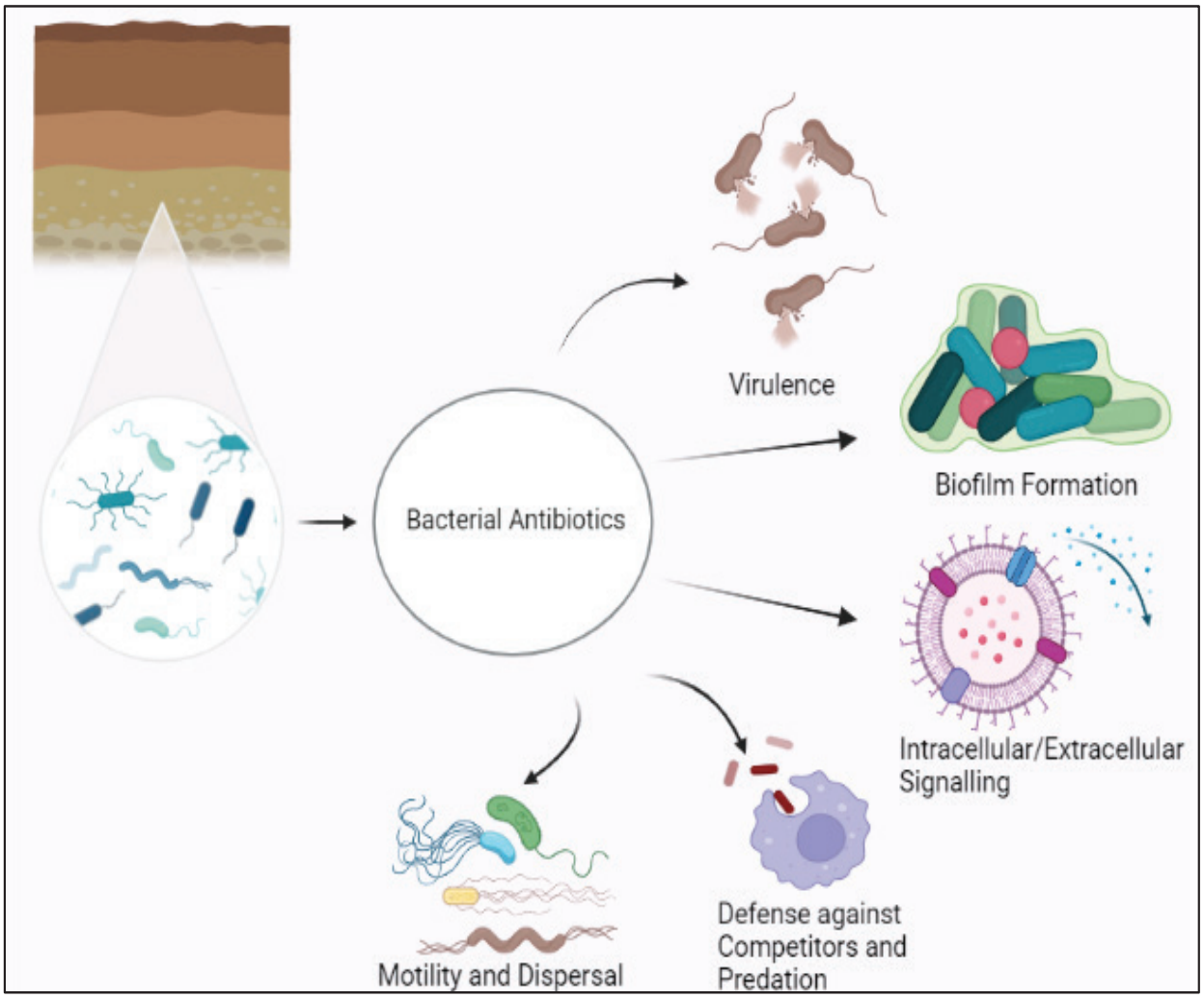

Fig. 2. Functions of antibiotics produced by bacteria. Bacteria isolated from soil produce various antibiotics which not only help to kill the bacteria in their surroundings for survival, but also aid in biofilm formation, intracellular or extracellular signaling, defense against competitors and predators and in motility and dispersal of cells.

4.2 Quorum Sensing: Antibiotic Production Regulation

Antibiotic production is under stringent genetic control. It usually starts in the stationary phase of bacterial cell growth. By various bacterial systems, we get information regarding the presence of these complicated signaling routes which pave the way for communication between the cells of the same or different species [61]. This kind of signaling makes it necessary for a transduction system to be present in bacteria so that the external information could induce the production of antibiotics at an accurate time and in proper quantity by a particular subpopulation of cells. This phenomenon is known as "Quorum Sensing". Quorum means the least number of board members required to make a decision. Here, microbiologists, refer to this term as the decision-making ability of bacteria to regulate their expression of genes based upon the population size. This process helps bacteria to count population members in culture to control the production of antibiotics. Self-produced signaling molecules or auto-inducers are released in the medium. When these molecules reach the threshold value, it induces the quorum-sensing response. The molecule which is triggering the response determines the specificity of the receptor and guarantees the appropriate recognition and genetic response in that particular population [62]. As shown in Figure 3, gram-positive and gram-negative strains have different signaling pathways. In gram-positive bacteria, the signal transduction system is a two-component system, which comprises small and post-translationally modified peptide signal molecules. An ABC exporter system (Ex) secretes these peptides which bind to the receptor and in return trigger the auto-phosphorylation of the sensor kinase (SK). Then the expression of the gene is modified by the response regulator (RR) which is activated by the transfer of the phosphate group. While in gram-negative bacteria, quorum sensing usually consists of the LuxI-LuxR system. LuxI acts to synthesize and export N-acyl homoserine lactone (AHL). When the threshold value of AHL is reached, it gets bound to the LuxR, a transcription regulator, which consequently modifies the expression of genes. [63].

Pseudomonas aeruginosa contains two kinds of quorum system AHLs i.e. Las and Rhl. Both of these systems produce their specific AHLs i.e. LasI and RhlI respectively. These AHL signal molecules bind to their cognate receptors LasR and RhlR respectively and trigger the gene expression. These genes express and perform various physiological

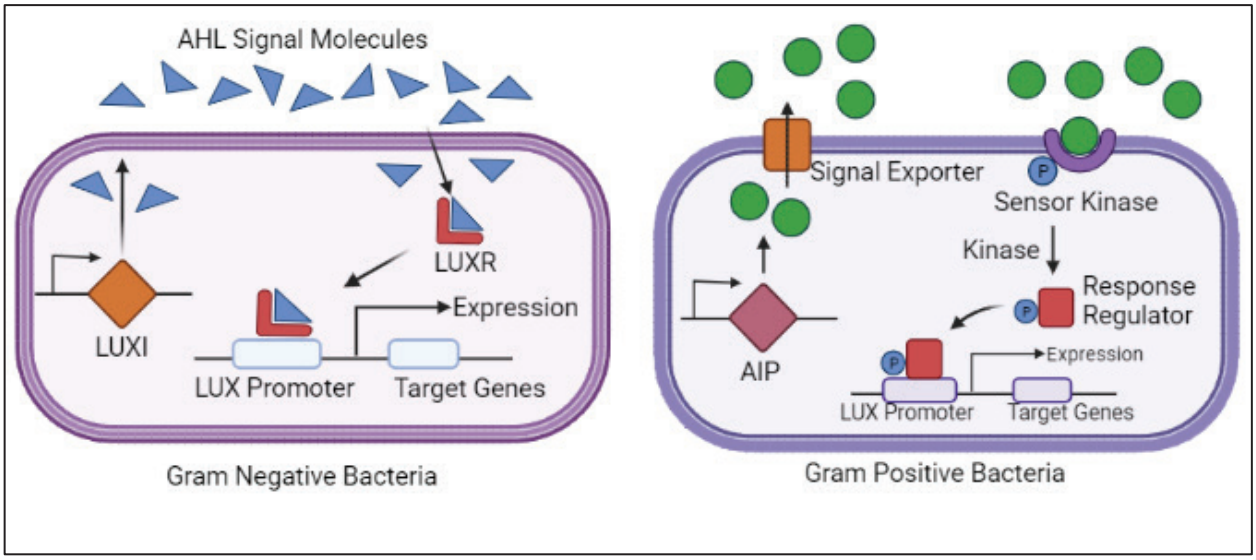

Fig. 3. Quorum sensing in gram positive and gram negative bacteria. In gram negative bacteria, LUXI produces N-acyl Homoserine Lactone (AHL) signal molecules. When threshold level of these molecules is reached, they attach to the LUXR receptors which ultimately binds to the LUX promoter and starts the expression of target genes such as biofilm formation. While in gram positive bacteria, Auto-inducer peptides are secreted by signal exporter (EX). These peptides get bound to the receptor sensor kinase and cause its auto-phosphorylation. It then helps to phosphorylate response regulator which ultimately enhances the expression of target genes such as virulence processes.

processes like biofilm formation or virulence [64]. In gram-positive bacteria, different signal molecules which are altered by post-translational changes are used for quorum sensing. *Bacillus subtilis* uses ComX or CSF and CSP in *Streptococcus pneumoniae* to control the gene's expression which is associated with different processes such as competence, sporulation, or virulence [65].

5. CONCLUSION

Production and extraction of biomolecules from bacteria have been an area of tremendous research for scientists. Researchers have utilized bioactivity-based, culture-based, metabolome based and genome-based strategies for the production and isolation of bacterial secondary metabolites Use of culture-dependent methods usually provides only the low-hanging fruit produced by these bacteria. The major challenges associated with these methods include the use of excessive chemicals or nutrients to identify a variety of metabolites being produced. Thus, mining the bacterial genome for secondary metabolite biosynthetic gene clusters provides rapid and limited resources using the approach to determine most of the metabolites producing potential in one go. In the future, studies should be targeted towards novel methods for isolations of secondary metabolites.

6. ACKNOWLEDGMENTS

This is a part of the Ph.D. thesis of author Rimsha Dilshad. The authors are thankful to the University of the Punjab and the Higher Education Commission of Pakistan for the funding under HEC indigenous fellowship program 5000 (Phase II, Batch 5) Pin No. 518-74646-2BS5-025.

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 58(3): 43-54 (2021)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB (58-3)661

Pakistan Academy of Sciences

Research Article

Pakistan has the 2nd Highest Growth Rate in Medical Sciences Research Publications for the Year 2019-2020: Comparison with 46 Countries

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Abstract: In 2020, Forty-Six (n=46) countries have published at least three thousand research documents in the field of Medical Sciences. Based on the number of publications, the top three countries are United States (202373/26.65 %), China (116155/15.30 %), and United Kingdom (58892/7.75 %). We also calculated the relative growth rate (GR) (for the year 2019-2020) of 46-countries. The highest GR was recorded for Saudi Arabia (n=44.31), Pakistan (n=37.58), and Nigeria (n=37.36). We extended the idea and explored the publication details of Pakistan. From 1947 to 2000, Pakistan published 4378 research documents, while from 2001 to 2020, it published 41889 research documents. Based on the number of publications, the list of top 50 researchers, universities, and sources are described. Some of the prominent features for this astonishing increase in research publication output are the establishment of the higher education commission (HEC) Islamabad, an increase in the number of research scholars, faculty, universities, national educational budget, funding, and international collaboration. Although based on the number of publications Pakistan holds 42nd position in the world. But it has a meager share of 0.19 % in the total global production (n=23905729). This confirms that strong policies, planning, and management are required to improve the overall research progress.

Keywords: Scopus, Medical Sciences, Research Growth Rate, Pakistan.

1. INTRODUCTION

Numerous studies reported the research publication and productivity of various countries in different disciplines like nursing, transplantation, pharmacology, and biomedical sciences, etc. For example, United States was found to be the most prolific and productive country in research contributions in almost every field of science. The bibliometric analysis of the US has been extensively reported in different areas, such as neuroimaging [1], natural hazards [2], cocaine intoxication [3], and tuberculosis [4], etc. In the current scenario of rapidly increasing research publications, Australia was ranked as one of the most productive countries. This has been confirmed by the bibliometric reports of Australia's involvement in nonspecific low back pain [5], and medicine [6]. In Europe, Italy was found to be one of the top productive countries. There are several bibliometric studies for Italy in different special topics including COVID-19 [7] and pathology [8]. While Neudí *et al.* [9] highlighted that Brazil has one of the highest counts of publications. Additionally, various bibliometric studies have been reported on malocclusion, imaging, and morphology, on dental material, leishmaniasis [10], economics [11]. In Asian countries, India is one of the top-ranked countries in research publication and productivity in the past decade. The role of India in engineering and technology [12], oncology [13], etc. has been confirmed by bibliometric studies. Similarly, numerous studies have shown the contribution of Turkey in neuroscience [14], mathematics [15], and breast reduction [16]. A holistic summary through bibliometric analysis for Canada is also reported, where the top publications in forensic sciences [17], spinal cord injury [18] are reported.

Similarly, there are a few bibliometric studies

Received: July 2021; Accepted: September 2021
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about different fields in Pakistan. For example, Bajwa and Yaldram reported the history and development of biotechnology research in Pakistan. The authors covered the publication history from 1980 to 2011. The authors reported that in 1980, there were only 15 publications in 1980 which increased to 3273, with an average growth rate of 22 %. The authors extracted publications data from Scopus and they reported the growth rate and doubling time of publications [19]. While, Siddique et al., reported the 62 years (from 1957–2018) of research in library and information science in Pakistan [20]. The authors provided details about the most productive organizations, authorship, collaboration pattern, and the most preferred journals. In the same vein, Ikram et al. performed the bibliometric analysis of social sciences in Pakistan from 1961 to 2019 [21]. The authors found 9,292 papers on social sciences with an average of 157.49 papers per year in the Scopus database. The most productive authors, departments, and sources are described in detail. There is another study, where the author reported bibliometric analysis of computer science literature of Pakistan from 2000 to 2017. They also highlighted the top contributing authors and departments [22].

The present research study is designed to explore the relative growth of the rate of the world (for the year 2019-2020). We will also highlight the contribution of Pakistan and its top-ranked researchers and institutions.

2. MATERIALS AND METHODS

2.1 Ethics Statement

The study did not involve human or non-human subjects. Therefore, neither approval by the institutional review board nor informed consent was required.

2.2 Study Design

This was a bibliometric study of a specific topic from a literature database.

2.3 Data Sources/Measurement

On 11th May, the data was retrieved from Scopus, one of the largest databases in the world. In the advanced search field, we selected the subject of medicine. In other words, the code was SUBJAREA(MEDI). According to Scopus, the following subjects are classified under it.

Medicine (all), medicine (miscellaneous), anatomy, anesthesiology and pain medicine, cardiology and cardiovascular medicine, critical care and intensive care medicine, complementary and alternative medicine, dermatology, drug guides, embryology, emergency medicine, endocrinology, diabetes, and metabolism, epidemiology, family practice, gastroenterology, genetics (clinical), geriatrics and gerontology, health informatics, health policy, hematology, hepatology, histology, immunology and allergy, internal medicine, infectious disease, microbiology (medical), nephrology, neurology (clinical), obstetrics and gynaecology, oncology, ophthalmology, orthopedics and sports medicine, otorhinolaryngology, pathology and forensic medicine, pediatrics, perinatology and child health, pharmacology (medical), physiology (medical), psychiatry and mental health, public health, environmental and occupational health, pulmonary and respiratory medicine, radiology nuclear medicine and imaging, rehabilitation, reproductive medicine, reviews and references (medical), rheumatology, surgery, transplantation, and urology. It is important to note that we only focused on research articles and reviews. The publications data of 2021 was also ignored in the analysis.

3. RESULTS AND DISCUSSION

In 2019, the world has produced 638026 research publications. Based on the number of publications and % share, the top ten countries are United States (177048/27.8 %), China (86580/13.6 %), United Kingdom (50730/7.9 %), Germany (38401/6 %), Italy (30942/4.8 %), Japan (31938/5 %), Canada (30549/4.7 %), Australia (27221/4.2 %), France (24998/2.9 %) and India (23575/2.6 %).

While in 2020, total publications were found to be 759420. The top ten countries are United States (202373/26.65 %), China (116155/ 15.30 %), United Kingdom (58892/7.75 %), Germany (43747/5.76 %), Italy (39966/5.26 %), Japan (36093/4.75 %), Canada (34395/4.53 %), Australia (31194/4.11%), France (28827/3.80 %)

and India (28365/3.74 %).

For growth rate calculations, we selected those countries which have published at least three thousand research documents. Forty-six (n=46) meet the threshold. The top ten countries are, Saudi Arabia (n=44.31), Pakistan (n=37.58), Nigeria (n=37.36), China (n=34.16), Colombia (n=32.62), Egypt (n=31.73), Mexico (n-29.95), Italy (n=29.16), Singapore (n=27.79) and Malaysia (n=25.04). The growth rates of all 46-countries with the number of publications (for the year 2019 and 2020) is described in Table 1.

3.1 Focus on Pakistan

Since Pakistan occupied 2nd Position, this further motivated us to explore its entire publication

Table 1. The number of research publications (NoP) and relative growth rates (RGR) of 46 countries.

S#	Country	NoP 2019	NoP 2020	2019-2020 RGR
1.	United States	177048	202373	14.30
2.	China	86580	116155	34.16
3.	United Kingdom	50730	58892	16.09
4.	Germany	38401	43747	13.92
5.	Italy	30942	39966	29.16
6.	Japan	31938	36093	13.01
7.	Canada	30549	34395	12.59
8.	Australia	27221	31194	14.60
9.	France	24998	28827	15.32
10.	India	23575	28365	20.32
11.	Spain	22612	27488	21.56
12.	Netherlands	20012	22978	14.82
13.	Brazil	17230	21409	24.25
14.	South Korea	18264	20660	13.12
15.	Turkey	13682	17057	24.67
16.	Iran	13902	16809	20.91
17.	Switzerland	13770	16139	17.20
18.	Russian Federation	12008	13841	15.26
19.	Sweden	11766	13286	12.92
20.	Belgium	9225	10961	18.82
21.	Denmark	8715	10050	15.32
22.	Poland	8400	9273	10.39
23.	Taiwan	7468	8531	14.23
24.	Austria	6284	7557	20.26
25.	Egypt	5272	6945	31.73
26.	Israel	5568	6710	20.51
27.	Saudi Arabia	4617	6663	44.31
28.	Mexico	5081	6603	29.95
29.	Norway	5961	6562	10.08
30.	South Africa	5439	6519	19.86
31.	Portugal	5111	6312	23.50
32.	Greece	5016	5939	18.40
33.	Singapore	4156	5311	27.79
34.	Pakistan	3763	5177	37.58
35.	Ireland	4195	5173	23.31
36.	Thailand	4064	4912	20.87
37.	Malaysia	3886	4859	25.04
38.	Finland	4306	4788	11.19
39.	New Zealand	3951	4587	16.10
40.	Hong Kong	3686	4502	22.14
41.	Indonesia	3749	4478	19.45
42.	Czech Republic	3888	4205	8.15
43.	Chile	2918	3614	23.85
44.	Colombia	2707	3590	32.62
45.	Argentina	2905	3573	22.99
46.	Nigeria	2385	3276	37.36

history since independence i.e. 1947. To the best of our knowledge, this is the 1st report which has described the research growth of Pakistan in medicine (for 2019-2020). Pakistan has produced 52333 research documents majorly comprising of articles (n=42946), reviews (n=3356), letters (n-2373), conference papers (n-1145), editorials (n=1095), notes (n=545), book chapters (n=498), short surveys (n=183), errata (n=154), books (n=24), retracted documents (n=5), abstract reports (n=1) and undefined documents (n=8).

For further data acquisition, we focused on research articles and reviews (n=46302). For simplicity, we divided the total years into two eras.

From 1947 to 2000, Pakistan published 4378 research documents. It also includes those documents (n=35) which are published before 1947. Based on the number of publications, the top ten authors in this era were; Zuberi, S.J. (n=74), Bhutta, Z.A. (n=68), Qureshi, H. (n=48), Qureshi, I.H. (n=46), Khurshid, M. (n=36), Ahmad, V.U. (n=35), Malik, I.A. (n=35), Khan, H.A. (n=34), Ahmed, W. (n=32), and Alam, S.E. (n=32). Institutionally, Jinnah Postgraduate Medical Centre, Karachi, published the highest number of documents (n=456), followed by The Aga Khan University Hospital (n=450), The Aga Khan University (n=401), University of Karachi (N=282), Pakistan Institute of Nuclear Science and Technology (n=238), Dow Medical College Pakistan (n=179), King Edward Medical University Lahore (n=163), University of Punjab, Lahore (n=113), Mayo Hospital Lahore (n=104), and Liaquat National Hospital (n=102).

From 2001 to 2020, Pakistan published 41889 research documents. The highest documents are published by Bhutta, Z.A. (n=573), followed by Ahmad, W. (n=188), Jafri, W. (n=168), Abbas, Z. (n=155), Pervez, S. (n=124), Goldenberg, R.L. (n=118), Choudhary, M.I. (n=114), Kayani, N. (n=112), Hasan, R. (n=111) and Saleem, S. (n=110). The top ten institutes in this era are The Aga Khan University (n=4340), The Aga Khan University Hospital (n=3883), Dow University of Health Sciences Pakistan (N=1599), Quaid-i-Azam University (N=1458), University of Punjab, Lahore (N-1249), Liaquat National Hospital (N=1192), University of Karachi (N=1102), King Edward Medical University Lahore (n=1016), Khyber Medical College (N=973), and Mayo Hospital Lahore (n=962), or collectively (from 1947 to 2020) based on the number of publications, the top ten authors are Bhutta, Z.A. (n=641), Ahmad, W. (N=191), Jafri, W. (n=188), Abbas, Z. (n=183), Pervez, S. (n=150), Choudhary, M.I. (N=142), Kayani, N. (n=136), Goldenberg, R.L. (n=118), Hasan, R. (n=118), and Hamid, S. (n=117). The list of the top 50-authors is provided in Table 2. While the highest affiliations were recorded for The Aga Khan University (n=4741), The Aga Khan University Hospital (n=4333), Dow University of Health Sciences Pakistan (n=1599), Quaid-i-Azam University (n=1525), University of Karachi (n=1384), University of Punjab, Lahore (n=1362), Liaquat National Hospital (n=1294), Jinnah Postgraduate Medical Centre, Karachi (n=1227), King Edward Medical University Lahore (n=1179) and Mayo Hospital Lahore (n=1066). The list of top 50-universities is provided in Table 2. While

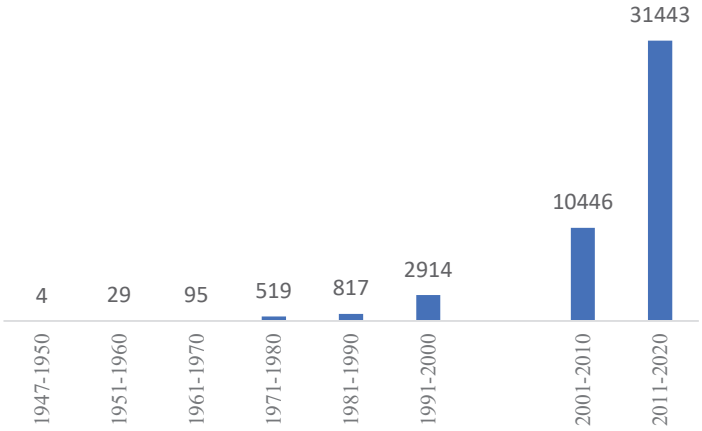

Fig. 1. List of publications for each decade (from 1947 to 2020).

Table 2. The list of top 50 authors and institutes of Pakistan. The names of the universities do not describe the affiliations of the authors.

Top Authors			Top Institutions		
S#	**Author Name**	**NoP***	S#	**Affiliations**	**NoP***
1.	Bhutta, Z.A.	641	1.	The Aga Khan University	4741
2.	Ahmad, W.	191	2.	The Aga Khan University Hospital	4333
3.	Jafri, W.	188	3.	Dow University of Health Sciences Pakistan	1599
4.	Abbas, Z.	183	4.	Quaid-i-Azam University	1525
5.	Pervez, S.	150	5.	University of Karachi	1384
6.	Choudhary, M.I.	142	6.	University of the Punjab, Lahore	1362
7.	Kayani, N.	136	7.	Liaquat National Hospital	1294
8.	Goldenberg, R.L.	118	8.	Jinnah Postgraduate Medical Centre, Karachi	1227
9.	Hasan, R.	118	9.	King Edward Medical University Lahore	1179
10.	Hamid, S.	117	10.	Mayo Hospital Lahore	1066
11.	Khurshid, M.	113	11.	Khyber Medical College	1058
12.	McClure, E.M.	110	12.	Liaquat University of Medical and Health Sciences	938
13.	Saleem, S.	110	13.	COMSATS University Islamabad	937
14.	Wasay, M.	108	14.	Lady Reading Hospital	830
15.	Idrees, M.	107	15.	Dow Medical College Pakistan	713
16.	Riazuddin, S.	106	16.	University of Lahore	673
17.	Ahmad, Z.	104	17.	University of Agriculture, Faisalabad	652
18.	Basit, A.	104	18.	Pakistan Institute of Medical Sciences	633
19.	Shaikh, M.A.	102	19.	University of Veterinary and Animal Sciences Lahore	610
20.	Ahmad, M.	101	20.	National University of Sciences and Technology Pakistan	609
21.	Qureshi, H.	101	21.	Services Institute of Medical Sciences Lahore	580
22.	Rehman, R.	100	22.	Combined Military Hospital, Multan	580
23.	Shamim, M.S.	99	23.	Islamia University	558
24.	Kazi, T.G.	97	24.	University of Peshawar	555
25.	Das, J.K.	96	25.	University of Health Sciences Lahore	539
26.	Afridi, H.I.	95	26.	Shaukat Khanum Memorial Cancer Hospital and Research Centre	530
27.	Jabbar, A.	94	27.	King Saud University	528
28.	Zafar, A.	93	28.	Allama Iqbal Medical College	523
29.	Zuberi, S.J.	93	29.	Pakistan Institute of Nuclear Science and Technology	510
30.	Khan, M.S.	91	30.	Children's Hospital Lahore	494
31.	Zaidi, A.K.M.	90	31.	Baqai Medical University	471
32.	Gupta, R.	88	32.	Government College University Faisalabad	470
33.	Mubarak, M.	88	33.	Shifa International Hospital	443
34.	Ather, M.H.	87	34.	Jinnah Hospital Lahore	442
35.	Hussain, I.	87	35.	Organisation Mondiale de la Santé	439
36.	Gilani, A.H.	86	36.	London School of Hygiene and Tropical Medicine	428
37.	Abid, S.	85	37.	Sindh Institute of Urology and Transplantation	427
38.	Islam, M.	85	38.	Nishtar Medical College and Hospital	426
39.	Pasha, O.	83	39.	Bahauddin Zakariya University	419
40.	Carlo, W.A.	82	40.	Armed Forces Institute of Pathology Rawalpindi	418
41.	Hasan, S.H.	82	41.	Jinnah Sindh Medical University	381

S#	Author Name	NoP*	S#	Affiliations	NoP*
42.	Chomba, E.	81	42.	Ziauddin Medical University	378
43.	Malekzadeh, R.	81	43.	Ayub Medical College	376
44.	Khan, T.M.	80	44.	Army Medical College Pakistan	373
45.	Lassi, Z.S.	79	45.	Hospital for Sick Children University of Toronto	370
46.	Rahman, A.	79	46.	Khyber Medical University	367
47.	Riazuddin, S.	79	47.	Ziauddin University	351
48.	Ahmad, V.U.	78	48.	Shifa College of Medicine	336
49.	Bhutta, Z.	78	49.	Riphah International University	334
50.	Haroon, T.S.	78	50.	Johns Hopkins Bloomberg School of Public Health	323

*NoP: Number of Publications.

the per-year publications are described in Figure 1.

Mostly these documents are published in Journal of the Pakistan Medical Association (n=4380), Journal of the College of Physicians and Surgeons Pakistan (n=4116), Pakistan Journal of Medical and Health Sciences (n=3974), Medical Forum Monthly (n=3086), Pakistan Journal of Medical Sciences (n=1752), Journal of Ayub Medical College Abbottabad JAMC (n=1573), JPMA the Journal of the Pakistan Medical Association (n=1282), Journal of Postgraduate Medical Institute (n=749), Journal of Pakistan Association of Dermatologists (n=679) and Journal of the Liaquat University of Medical and Health Sciences (n=550). The details about the top 50 sources are provided in table 3.

It is worthy to note that Pakistan produced 90.54 % (or 41889) documents after 2001. Or in other words, only 9.46 % (4378) were published from 1947 to 2000. One of the fundamental questions is, why such an enormous rise in research output. This could be explained by some of the following reasons.

In 1947 there was only one University i.e. University of Punjab. According to Isani & Usman Ali, the 1st official education policy was introduced in 1956. In 1972, a new education policy was announced and in 1974, University Grants Commission was officially established by the act of parliament. Later some other major reforms and policies were introduced, for example, The National Education Policies (in 1979 and 1992) and in 1993 in eighth five-year plans for the national economy of Pakistan, the education was also focused [23-25].

The Higher Education Commission (HEC), Islamabad is a statutory body formed by the government of Pakistan in 2002. Initially, it was known as University Grants Commission (UGC), which was established in 1974. The HEC revolutionized science and higher education under the leadership of Prof. Atta-ur-Rahman when he was the Federal Minister of science & technology and later chairman of the Higher Education Commission with the status of Federal Minister. HEC introduced various and reforms which helped in improving the overall research culture and productivity. For example, In 2001-02, there were 74-universities, 5160-faculty members, and 276000-students. In 2017-18, the number of universities substantially increased to 186, with 56885 teachers, and 1575000 students. While in 2019-20 the total number of universities are 211. The total number of institutions are 317,323 with 50,292,570 students and 1,836,584 teachers. This shows a remarkable development.

This also significantly helped in research output. For example, from 1970 to 1999, there was only 1034 thesis in all subjects. The thesis production enormously increased and from 2000 to Jan 2020, 10,096 theses in all subjects are archived in the Pakistan research repository (PRR).

In 1975, the national education budget was 11.3 billion which increased to 72.3 billion in 2001. Later on, the budget augmented from 83.2 billion in 2003 to 315 billion in 2018.

After the establishment of HEC, the higher education budget for the years (2005-06, 2006-07, 2007-08, 2008-9 and 2009-10) also enormously increased from 21.38 to 28.74, 27.92, 32.18, and 44.00 billion, respectively.

From 1947 to 2000, only six (n=6) funding bodies have been acknowledged in at least ten

Table 3. The list of top 50 sources

S#	Source Title	NoP*
1.	Journal of the Pakistan Medical Association	4380
2.	Journal of the College of Physicians And Surgeons Pakistan	4116
3.	Pakistan Journal of Medical and Health Sciences	3974
4.	Medical Forum Monthly	3086
5.	Pakistan Journal of Medical Sciences	1752
6.	Journal of Ayub Medical College Abbottabad JAMC	1573
7.	JPMA the Journal of the Pakistan Medical Association	1282
8.	Journal of Postgraduate Medical Institute	749
9.	Journal of Pakistan Association of Dermatologists	679
10.	Journal of the Liaquat University of Medical and Health Sciences	550
11.	Journal of Medical Sciences Peshawar	523
12.	Pakistan Paediatric Journal	484
13.	Asian Pacific Journal of Cancer Prevention	334
14.	Pakistan Journal of Nutrition	274
15.	Tropical Journal of Pharmaceutical Research	248
16.	Journal of Radioanalytical and Nuclear Chemistry	233
17.	Anaesthesia Pain and Intensive Care	216
18.	BMJ Case Reports	208
19.	Eastern Mediterranean Health Journal	196
20.	Lancet	190
21.	Journal of Natural Products	182
22.	Jpmi Journal of Postgraduate Medical Institute	162
23.	BMC Complementary and Alternative Medicine	160
24.	Ecotoxicology and Environmental Safety	158
25.	BMC Public Health	156
26.	Medical Channel	152
27.	Microbial Pathogenesis	147
28.	Biological Trace Element Research	141
29.	Journal of Medical Sciences	136
30.	Virology Journal	132
31.	Microscopy Research and Technique	129
32.	International Journal of Environmental Research and Public Health	123
33.	Computer Methods and Programs in Biomedicine	115
34.	Frontiers in Microbiology	110
35.	Specialist	110
36.	Journal of the College of Physicians and Surgeons Pakistan JCPSP	106
37.	Reproductive Health	102
38.	Journal of Photochemistry and Photobiology B. Biology	94
39.	Analytical Letters	88
40.	BMJ Open	87
41.	Frontiers in Pharmacology	87
42.	American Journal of Human Genetics	86

S#	Source Title	NoP*
43.	Journal of Asian Natural Products Research	86
44.	International Journal of Environmental Analytical Chemistry	84
45.	Journal of Medicinal Plants Research	83
46.	Natural Product Communications	80
47.	Pharmaceutical Biology	80
48.	Journal of Radioanalytical And Nuclear Chemistry Articles	78
49.	Lipids in Health And Disease	76
50.	Cochrane Database of Systematic Reviews	75

*NoP: Number of Publications.

publications. They are the U.S. Department of Health and Human Services (n=63), National Institutes of Health (n-61), National Institute of Allergy and Infectious Diseases (N=18), United States Agency for International Development (n=12), International Atomic Energy Agency (N=11), and U.S. Department of Health, Education and Welfare (N=10).

After 2001, the trend in financial sponsorship and grants dramatically increased. National Institutes of Health and the U.S. Department of Health and Human Services have financially acknowledged in more than one thousand (n=1000) publications. Some other worthy donors are the National Natural Science Foundation of China (n=408), National Natural Science Foundation of China (n=408), UK Research and Innovation (n=375), Medical Research Council (n=328), Bill and Melinda Gates Foundation (n=310), Fogarty International Center (N=257), Eunice Kennedy Shriver National Institute of Child Health and Human Development (n=207), European Commission (n=197), Wellcome Trust (n=184), Alabama Commission on Higher Education (N=166), King Saud University N=163), Ministry of Science and Technology of the People's Republic of China (N=143), National Institute on Deafness and Other Communication Disorders (n=135), National Heart, Lung, and Blood Institute (n=120), Deanship of Scientific Research, King Saud University (N=118), World Health Organization (n=113), National Institute for Health Research (N=105), Ministry of Education of the People's Republic of China (N=101) and Government of Canada (n=100), to name a few.

Furthermore, thirty (n=30) national and global sponsors are acknowledged in fifty to ninety-nine (50 to 99) publications. Last but not the least, seventy-four (n=74) organizations have been acknowledged in twenty to forty-nine publications. In table 4, the list of the top 50 funding sources is described for both eras (1947 to 2000 and from 2001 to 2020).

There is no doubt that national and international collaboration plays a pivotal role in research development. It can enable the researchers to access additional infrastructure, laboratory facilities, and gain new perspectives in research. It may involve bilateral and multilateral relationships and collaborations in different fields of science. It has an integral place in the careers of researchers.

In Pakistan, from 1947 to 2000 the international collaboration was very low. From 1947 to 2000, eighty-fours (n=84) countries affiliations are noticed in publications. The highest collaboration in publications was noted with United States (n=274), followed by United Kingdom (n=216), Sweden (N=50), Germany (n=38), Japan (N=32), Switzerland (n=30), China (n=27), India (N=23), Netherlands (n=22), Thailand (N=22), and Turkey (n-22). Ten countries were noticed in ten to twenty publications (10 to 20), while 63 countries collaborated in less than nine (n=9) publications.

From 2001-onwards the collaboration completely changed. One hundred and forty-eight countries' affiliations were noted in at least ten publications. The top ten countries in this list are United States (n=3962), United Kingdom (n=2557), China (N=1793), Saudi Arabia (N=1736), Canada (N=1311), India (N=1228), Germany (N=892), Australia (n=880), Malaysia (N=874), and Switzerland (n=589).

In Table 5, the list of the top 50 collaborating

Table 4. The list of top 50 funding sponsors for both eras.

S#	From 1947 to 2000	NoP*	S#	From 2001 to 2020	NoP*
1.	U.S. Department of Health and Human Services	63	1.	National Institutes of Health	1062
2.	National Institutes of Health	61	2.	U.S. Department of Health and Human Services	1050
3.	National Institute of Allergy and Infectious Diseases	18	3.	Higher Education Commission, Pakistan	615
4.	United States Agency for International Development	12	4.	National Natural Science Foundation of China	408
5.	International Atomic Energy Agency	11	5.	UK Research and Innovation	375
6.	U.S. Department of Health, Education and Welfare	10	6.	Higher Education Commision, Pakistan	345
7.	World Health Organization	9	7.	Medical Research Council	328
8.	U.S. Public Health Service	8	8.	Bill and Melinda Gates Foundation	310
9.	Medicinska Forskningsrådet	6	9.	Fogarty International Center	257
10.	National Cancer Institute	6	10.	Eunice Kennedy Shriver National Institute of Child Health and Human Development	207
11.	National Institute of Neurological Disorders and Stroke	6	11.	European Commission	197
12.	U.S. Department of State	6	12.	Wellcome Trust	184
13.	UK Research and Innovation	5	13.	Alabama Commission on Higher Education	166
14.	European Commission	4	14.	King Saud University	163
15.	Medical Research Council	4	15.	Ministry of Science and Technology of the People's Republic of China	143
16.	U.S. Department of Defense	4	16.	National Institute on Deafness and Other Communication Disorders	135
17.	Center for Communicable Disease Dynamics	3	17.	National Heart, Lung, and Blood Institute	120
18.	Department for International Development, UK Government	3	18.	Deanship of Scientific Research, King Saud University	118
19.	Harvard T.H. Chan School of Public Health	3	19.	World Health Organization	113
20.	Pakistan Science Foundation	3	20.	National Institute for Health Research	105
21.	United Nations High Commissioner for Refugees	3	21.	Ministry of Education of the People's Republic of China	101
22.	Wellcome Trust	3	22.	Government of Canada	100
23.	Bangladesh Council of Scientific and Industrial Research	2	23.	Canadian Institutes of Health Research	93
24.	British Heart Foundation	2	24.	National Human Genome Research Institute	89
25.	Engineer Research and Development Center	2	25.	Ministry of Education, Culture, Sports, Science and Technology	88
26.	Epilepsy Foundation	2	26.	National Institute of Allergy and Infectious Diseases	86
27.	International Research Office	2	27.	Seventh Framework Programme	86
28.	Karolinska Institutet	2	28.	National Institute of Diabetes and Digestive and Kidney Diseases	84
29.	National Academies of Sciences, Engineering, and Medicine	2	29.	GlaxoSmithKline	82
30.	National Academy of Sciences	2	30.	Deutsche Forschungsgemeinschaft	80
31.	National Heart, Lung, and Blood Institute	2	31.	National Health and Medical Research Council	79

S#	From 1947 to 2000	NoP*	S#	From 2001 to 2020	NoP*
32.	National Institute of Arthritis and Musculoskeletal and Skin Diseases	2	32.	Sanofi	77
33.	National Institute of Diabetes and Digestive and Kidney Diseases	2	33.	Department of Health, Australian Government	76
34.	Scottish Office Home and Health Department	2	34.	National Cancer Institute	75
35.	U.S. Army	2	35.	Fundamental Research Funds for the Central Universities	74
36.	U.S. Army Corps of Engineers	2	36.	Ministry of Finance	72
37.	U.S. Navy	2	37.	National Research Foundation of Korea	70
38.	United Nations	2	38.	Pfizer	69
39.	United Nations Development Programme	2	39.	United States Agency for International Development	69
40.	Universiteit Utrecht	2	40.	Japan Society for the Promotion of Science	68
41.	University of Maryland	2	41.	National Eye Institute	67
42.	University of Maryland School of Public Health	2	42.	National Key Research and Development Program of China	67
43.	Yorkshire Cancer Research	2	43.	Pakistan Science Foundation	66
44.	Alexander von Humboldt-Stiftung	1	44.	Boehringer Ingelheim	63
45.	Atomic Energy Council	1	45.	National Institute of General Medical Sciences	63
46.	Biotechnology and Biological Sciences Research Council	1	46.	Novartis	63
47.	British Dietetic Association	1	47.	National Institute of Mental Health	61
48.	Canadian International Development Agency	1	48.	Quaid-i-Azam University	59
49.	Chinese Arctic and Antarctic Administration	1	49.	National Institute on Aging	56
50.	Chinese Center for Disease Control and Prevention	1	50.	AstraZeneca	54

*NoP: Number of Publications.

Table 5. The list of top 50 collaborating countries for both eras.

S#	From 1947 to 2000	NoP*	S#	From 2001 to 2020	NoP*
1.	United States	274	1.	United States	3962
2.	United Kingdom	216	2.	United Kingdom	2557
3.	Sweden	50	3.	China	1793
4.	Germany	38	4.	Saudi Arabia	1736
5.	Japan	32	5.	Canada	1311
6.	Switzerland	30	6.	India	1228
7.	China	27	7.	Germany	892
8.	India	23	8.	Australia	880
9.	Netherlands	22	9.	Malaysia	874
10.	Thailand	22	10.	Switzerland	589
11.	Turkey	22	11.	Italy	581
12.	Australia	19	12.	South Korea	558
13.	Singapore	19	13.	South Africa	549
14.	Saudi Arabia	18	14.	Bangladesh	544
15.	Canada	17	15.	Sweden	522
16.	Italy	17	16.	Turkey	515
17.	France	16	17.	France	511
18.	Egypt	15	18.	Brazil	510
19.	Brazil	14	19.	Egypt	487
20.	Austria	12	20.	Iran	478

S#	From 1947 to 2000	NoP*	S#	From 2001 to 2020	NoP*
21.	Philippines	10	21.	Netherlands	474
22.	Denmark	9	22.	Japan	471
23.	South Africa	9	23.	Spain	383
24.	Bangladesh	8	24.	United Arab Emirates	380
25.	Indonesia	8	25.	Singapore	334
26.	Mexico	8	26.	Thailand	326
27.	Russian Federation	8	27.	Nigeria	324
28.	United Arab Emirates	8	28.	Kenya	299
29.	Poland	7	29.	Austria	290
30.	Zambia	7	30.	Belgium	290
31.	Chile	6	31.	Norway	273
32.	Colombia	6	32.	Philippines	270
33.	Cuba	6	33.	Denmark	267
34.	Jamaica	6	34.	Argentina	266
35.	Jordan	6	35.	Nepal	260
36.	Belgium	5	36.	Poland	255
37.	Kenya	5	37.	New Zealand	252
38.	Tunisia	5	38.	Indonesia	248
39.	Finland	4	39.	Mexico	247
40.	Greece	4	40.	Colombia	238
41.	Hong Kong	4	41.	Oman	236
42.	Hungary	4	42.	Taiwan	235
43.	Kuwait	4	43.	Finland	231
44.	Malaysia	4	44.	Vietnam	230
45.	Nigeria	4	45.	Hong Kong	217
46.	Spain	4	46.	Sri Lanka	217
47.	Sri Lanka	4	47.	Ireland	199
48.	Czech Republic	3	48.	Russian Federation	198
49.	Guatemala	3	49.	Peru	192
50.	Ireland	3	50.	Chile	191

*NoP: Number of Publications.

countries is described for both eras (1947 to 2000 and from 2001 to 2020).

4. LIMITATIONS

The major limitation is, we only analyzed the Scopus data. Other databases were not explored.

5. ACKNOWLEDGEMENTS

The hard work of all authors, institutes, funding and collaborative countries in all research publications is sincerely appreciated.

6. CONFLICT OF INTEREST

There is no conflict of interest.

7. FUNDING

The author received no financial support for this article.

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Proceedings of the Pakistan Academy of Sciences:
B. Life and Environmental Sciences 58(3): 55-63 (2021)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB (58-3)662

Pakistan Academy of Sciences

Research Article

Characterization of Selected Soybean Germplasm through Fatty Acid and Oil Composition using Near-Infrared Spectroscopy

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Abstract: Soybean belongs to legumes and is said to be one of the best sources of oil and fats. The objective of this research was to identify specific and individual accession among the fifty soybean accessions collected from Plants Genetics Research Institute (PGRI) Islamabad for oil contents and fatty acids profile using near-infrared reflectance spectroscopy. The amount of oil content in 50 soybean accessions was recorded in the range of 13.816-23.40 % among which accessions 017421, 17423, 17430, 17435, and 17459 were found with the highest percentage of 23.4 %, 22.80 %, 22.214 %, 22.105 %, and 22.08 % respectively. The values of fatty acid content in these accessions for palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2) and linolenic acid (18:3) ranged between 10.077-18.48%, 1.95-5.88 %, 18.204-30.12 %, 22.756-53.879 % and 4.714-9.96 % respectively. Soybean accessions observed higher for oil content are recommended for cultivation; where it is grown for oil production. Based on essential fatty acids contents, accessions 017418 and 017420 are recommended for a future breeding program to improve human health. Due to the variability identified in the present research, it is critical to select single Soybean accession having all the traits for further breeding programs until a specific trait is chosen.

Keywords: Soybean; Glycine Max (L).; Oil; fatty acid; NIRS; ANOVA.

1. INTRODUCTION

Soybean *Glycine max* L. is recognized as a "miracle" bean plant and 4th most important crop of the 20th century, because of its multiple uses, least expensive source of best quality protein, phytochemicals, and dietary fiber [1–4]. The crop also plays a vital role in the economy as well as an important source of protein used by a large population of the world and constituent for various biochemicals including isoflavone which makes it unique among the other legumes [5, 6]. It supplies about 20 % of fats and oil to the world which has a pivotal role in the production of various chemicals like soap, medicines, paints, varnishes, and lubricants [7, 8]. Moreover, its help in the prevention of diabetes, cardiovascular diseases, and weight loss cannot be neglected [9, 10]. Soybean was first introduced to Pakistan in 1969 and is grown during the spring and autumn seasons for the production of oil [11, 12]. The vast areas of Sindh, Khyber Pakhtunkhwa and Punjab provinces of Pakistan were found ideal for the cultivation of soybean [13]. Chemically, a fatty acid is known as an organic acid that carries both acidic and methyl groups to each end [13]. Fatty acids are typically categorized based on their omega (ω) groups like ω, 3, 6, 7, and 9, their specific double bonds location and are therefore very important to human health except ω7 [15]. It is reported that the fatty acid composition of the oil is dependent on the climate of the area where the crop is grown [16]. Cooler the climates higher will be its linoleic acid and n-6 polyunsaturated fatty acid (PUFA) concentrations while, if the climate is hot it will produce monounsaturated fatty acid (MUFA) and oleic acid in higher concentrations

Received: June 2021; Accepted: September 2021
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and will therefore have different properties to affect human health [16]. With the advancement of electronics, near-infrared reflectance spectroscopy (NIRS) is said to be one of the best tools for the estimation and evaluation of food products due to its fast and non-destructive properties [18].

The application of NIRS for the chemical characterizations of food products has been confirmed previously by many researchers i.e. Lopez (2013) and his co-workers have successfully analyzed potato products for their fats and moistures [19]. Another study also used NIRS measurement to find out the concentration of fatty acid content using 262 winter oilseed rap while, keeping the calibration for this experiment in such a way that it will only calculate the amount of linoleic acid, oleic acid, and linolenic acid with different composition during screening [20]. Topas, pactol, screw 4, screw 6, and silvo of rapeseed cultivars were also used for total tocopherol, glucosinolate, phenolic content, amino acids, and fatty acids profile among which significant variation was found in fatty acids contents [21]. The contents of fatty acids as published in 2010 by El-Beltagi and Mohamed in their research article revealed the percentage of linolenic acid from 8.83 %-10.32 %, linoleic acid 10.52-13.74 %, erucic acid 0.15 % - 0.9 %, and oleic acid 56.31 % - 85.24 % respectively [22]. The oils of corn, peanut, and cotton revealed a greater value of linoleic acids and oleic acid. Oleic acid will also effectively enhance soybean oil uses in cosmetics, medical and industrial goods like biodiesel and lubricants [23]. The essential fatty acids (EFAs) are used by our body for the development of well-built cell membranes, the proper growth and working of the brain and nervous system, and for the making of hormone-like chemicals called eicosanoids (thromboxanes, leukotrienes, prostaglandins). These substances control various body functions such as hypertension, the viscosity of blood, inflammation, immune mechanism, and vasoconstriction [23, 24].

As the previous studies revealed that the composition of fatty acids and the variability found in their concentrations play a key role in terms of human health and industries, therefore, the present study was proposed to examine the fatty acid composition of soybean and to select those specific accessions which will be best fit for future breeding program, health care management and in industries.

2. MATERIALS AND METHODS

Fifty (50) accessions of Soybean *Glycine max* (L.) were collected from NARC, Islamabad Pakistan for oil and fatty acids contents analysis. NIRS reflectance spectra, expressed in the form of log (1/R), were collected from 5gm of seed sample using NIRS model 6500 spectrometer. NIRS spectra were measured for each and individual seed samples with the help of a spinning cup that has a diameter of 3.8 cm and depth of 0.9 cm, for the collection of radiation reflected from the overall surface of the seeds. The instrument evaluates diffuse reflectance within the range of 400 nm-2500 nm at 2 nm resolutions. Twenty-five monochromatic scans were averaged from each seed sample and simultaneous data was recorded for oil content, stearic acid, linolenic acid, palmitic acid, linoleic acid, and oleic acid as described by Choung *et al.* [25], Kovalenko *et al.* [26], Roberts *et al.* [27] with a little modification. ANOVA with LSD was performed on the obtained data using SPSS version 21.

3. RESULTS AND DISCUSSION

Significant results were obtained using analysis of variance with LSD values ranging from 0.2170 to 0.6148 respectively (Table 1). The lowest LSD value was found in Olic Acid while the highest LSD value was found in Stearic Acid.

Percent oil contents were determined for 50 soybean accessions and the results obtained were found with different values for oil contents as mentioned in Table 2 and Fig 1. The results depicted that accession 017421 gave the highest oil contents of 23 % which was followed by accessions 17423, 17430, 17435, and 17459 with the values of 22 % while, the lowest oil contents were obtained by the accession 017445 (13.816 %) (Table 2 and Fig 1). It has been reported that soybean is grown for its oil production and those having their values greater than 22 % are said to be better therefore, the accessions having more or equal values within this range should be selected for oil production. Esmaeili *et al.* [28], Chowdhury *et al.* [29] Messina [30] reported similar findings for oil contents as obtained in this study.

Table 1. Analysis of variance (ANOVA) and least significant difference (LSD) in 50 Accessions of *Glycine max* L.

S. No.	Traits	Sum of square	Degree of freedom	Mean Square	F value	P value	LSD value
1	Oil contents	1214.67	49	24.7092	376.66	0.0000	0.415
2	Palmitic Acid	826.907	49	16.8757	884.17	0.0000	0.2239
3	Stearic Acid	110.030	49	2.2455	15.60	0.0000	0.6148
4	Oleic Acid	1427.65	49	29.1356	1624.00	0.0000	0.2170
5	Linoleic Acid	5199.35	49	106.109	2027.05	0.0000	0.3707
6	Linolenic Acid	222.841	49	4.5478	64.62	0.0000	0.4298

Table 2. Contents of fatty acids composition in 50 *Glycine max* L. based on NIRS

Accession	Oil contents %	Palmitic Acid C16:0 (%)	Stearic Acid C18:0 (%)	Oleic Acid C18:1%	Linoleic Acid C18:2 (%)	Linolenic Acid C18:3 (%)
17415	17.100	13.500	4.500	22.050	42.300	7.380
17416	20.880	16.800	4.800	20.700	41.130	6.930
17417	21.720	17.040	5.040	20.790	42.660	5.850
17418	16.200	13.140	4.140	21.365	53.879	7.200
17419	15.300	13.500	4.500	22.487	50.123	8.880
17420	18.720	10.258	3.060	26.357	49.654	9.960
17421	23.400	15.120	3.120	22.860	41.400	7.920
17422	21.600	17.760	4.800	21.600	42.300	7.560
17423	22.800	17.160	5.880	21.780	43.650	5.760
17424	17.640	12.330	2.790	30.120	44.235	9.840
17425	16.020	13.680	4.320	22.456	46.568	8.760
17426	18.000	13.140	3.870	22.547	50.326	9.120
17427	21.240	15.720	3.600	20.340	41.040	7.290
17428	20.400	18.480	4.800	22.500	41.400	6.480
17429	20.400	18.000	5.760	20.700	42.210	6.300
17430	22.080	15.600	4.680	22.230	40.770	7.110
17431	15.441	11.784	3.820	18.773	37.953	6.502
17432	14.791	10.565	3.088	20.155	38.685	6.177
17433	15.360	10.646	2.926	19.749	37.222	4.714
17434	14.060	11.784	3.657	19.992	39.010	5.689
17435	22.214	14.087	5.635	25.573	45.698	8.019
17436	15.035	11.053	3.738	26.006	47.889	9.211
17437	15.116	11.378	3.413	25.681	49.195	6.718
17438	15.441	12.028	1.950	27.307	49.521	8.019
17439	14.385	11.297	2.926	26.657	48.762	7.694
17440	14.629	12.272	2.438	27.090	48.762	9.319
17441	14.791	10.403	3.657	25.356	50.062	6.935
17442	15.197	11.378	2.438	27.090	48.256	8.019
17444	15.929	11.134	2.519	27.198	49.412	8.886
17445	13.816	12.191	4.064	24.578	48.99	8.019
17446	16.904	10.890	2.763	25.898	50.279	8.994
17447	15.441	12.191	4.064	19.911	38.197	6.664
17448	18.855	15.170	4.334	18.692	37.140	6.258
17449	20.588	15.495	5.310	19.667	39.416	5.201

Accession	Oil contents %	Palmitic Acid C16:0 (%)	Stearic Acid C18:0 (%)	Oleic Acid C18:1%	Linoleic Acid C18:2 (%)	Linolenic Acid C18:3 (%)
17450	19.180	14.195	3.251	18.367	37.059	6.583
17451	18.421	16.687	4.334	20.318	37.384	5.851
17452	16.660	11.540	2.519	19.667	37.384	5.201
17453	14.304	10.077	4.389	18.204	37.547	6.420
17454	15.766	11.053	4.064	19.180	37.791	5.201
17455	14.629	10.565	2.438	18.692	37.872	5.283
17456	19.722	15.929	5.526	19.342	22.756	6.502
17457	21.455	14.954	3.034	19.505	37.465	6.827
17458	19.071	15.170	4.334	20.236	38.603	5.526
17459	22.105	16.146	4.009	18.692	37.384	6.827

The 50 Soybean accessions were also examined for palmitic acid content and found variation in their results as indicated in table 2 and Fig 2. The accessions 017428, 017429, 017422, and 017423 were found highest for palmitic acid content with the values of 18.48 %, 18 %, 17.76 %, and 17.16 % respectively while; the least among them was obtained in the accession 017453 with the value of 10.077 %. Accessions with higher palmitic acid are called more desirable as compared to those having lower palmitic acid contents. The same results (18 % - 19.5 %) for palmitic acid were also reported by Erawati *et al.* [31] Asghar and Majeed [32].

According to stearic acid content analyzed in the present study, the highest percentage of stearic acid was found in the accession 017423 (5.88 %) followed by accessions 017429 (5.760 %), 017435 (5.635 %), 017456 (5.526 %) while, its least content was obtained in accessions 017438, 017455 and 017442 ranging from 1.95%-2.44 % respectively (Table 2 and Fig 3). These results are nearly similar to the results described previously [2, 32]. As the stearic acid of soybean has no Side effect on the level of blood cholesterol and hence may not increase threats for cardiovascular disease [33]. It is hence revealed that the accessions with higher stearic acid contents are said to be more desirable as compared to those with lower content of stearic acid for human development.

In the present study fifty soybean accessions, the results for oleic acid contents were found varied between 18.204 % - 30.12 % respectively, among which the highest (30.12 %) was found in accession# 017424 followed by the accessions 017438 (27.31), 017444 (27.2 %), 017440 and 017442 (27.10 %) as described in Table 2 and Fig 4. The lowest content of oleic acid was found in the accession 017453 (18.20 %) followed by 017459, 017455, and 017448 with the values of 18.69 % (Table 2; Fig 4). Our results are in accordance with those obtained by Prabakaran *et al.* [2] and El-Beltagi and Mohamed [21].

Similarly, contents of linoleic acid in these 50 accessions of soybean were found with different concentrations ranging from 22.756 % - 53. 879 % among which the highest was found in the accession 017418 with the values of 53.87 % followed by the accessions 017462 (51.22 %), 017426 (50.23 %), and 017446 (50.27 %) while, the lowest contents of linoleic acid were found in accessions 017456 (22.756 %) followed by 017450 (37.059 %) (Table 2 and Fig 5). The results obtained for linoleic acid during this study are in agreement with the results reported previously [21, 30, 34].

Linoleic acid has the potential of precursor activities with many positive benefits for health i.e. to improved cognitive function and to reduce cardio vascular diseases [35–37]. Linolenic acid is vital meant for appropriate composition, function and structure of several body systems for example eyes and nervous system [24]. Table 2 and Fig 6 indicates that highest value (9.960 %) of linolenic acid was found in accession # 017420 followed by accessions 017424 (9.840 %), 017440 (9.319 %) and 017436 (9.319 %), as the minimum content for linolenic acid was found in accessions 017433 (4.714 %), followed by 017449, 017452 and 017454 with the same value of 5.201 % respectively. These highest values presented in our study are much

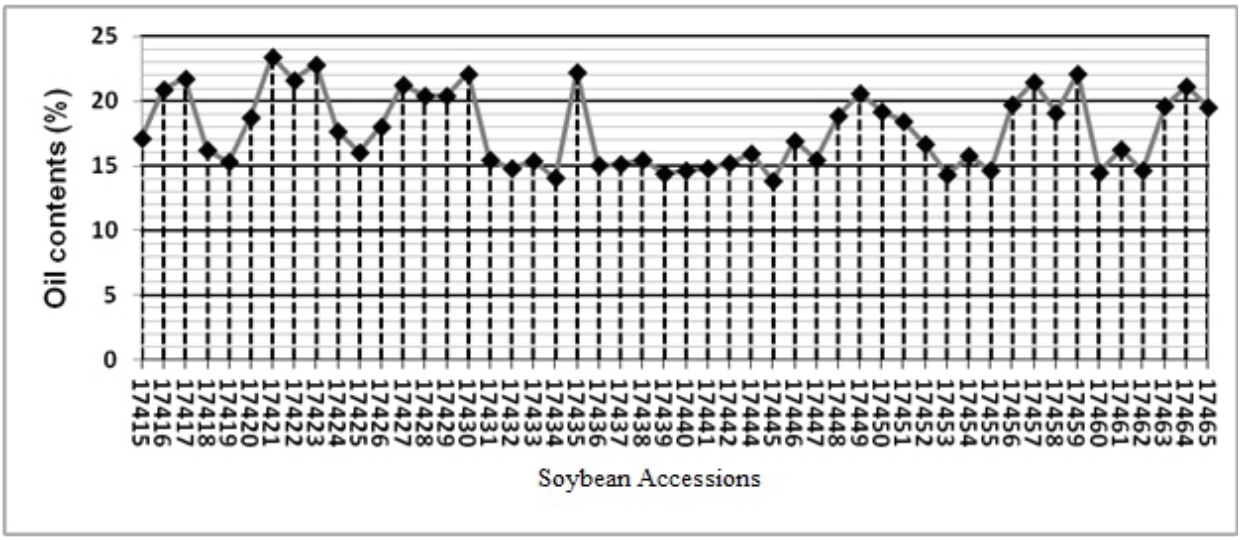

Fig. 1. The concentration of oil content in 50 accessions of soybean

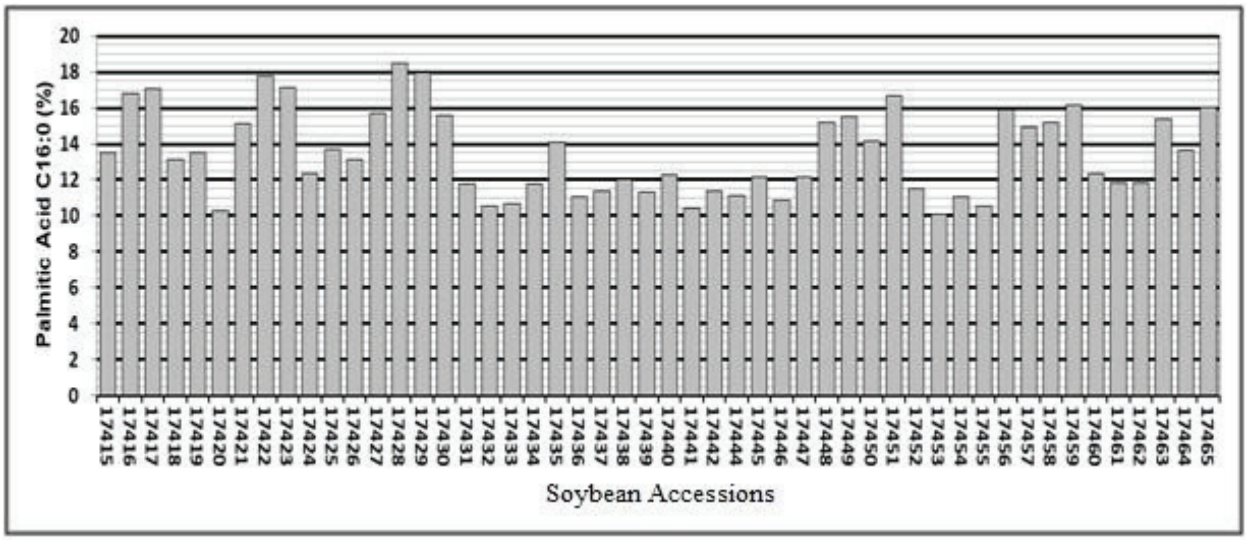

Fig. 2. The concentration of Palmatic acid in 50 Soybean accessions.

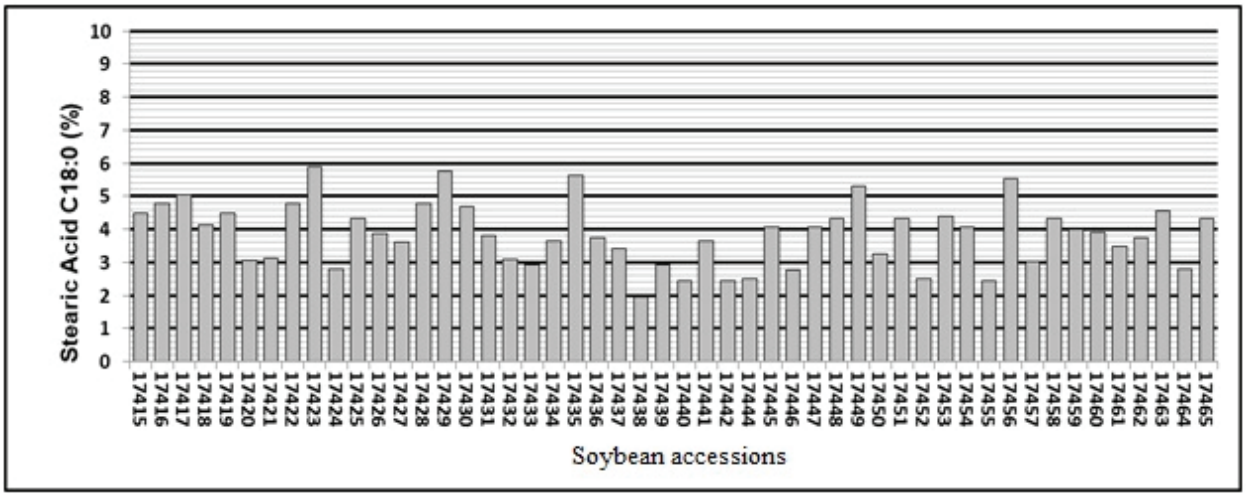

Fig. 3. The concentrations of Stearic acid in 50 Soybean accessions.

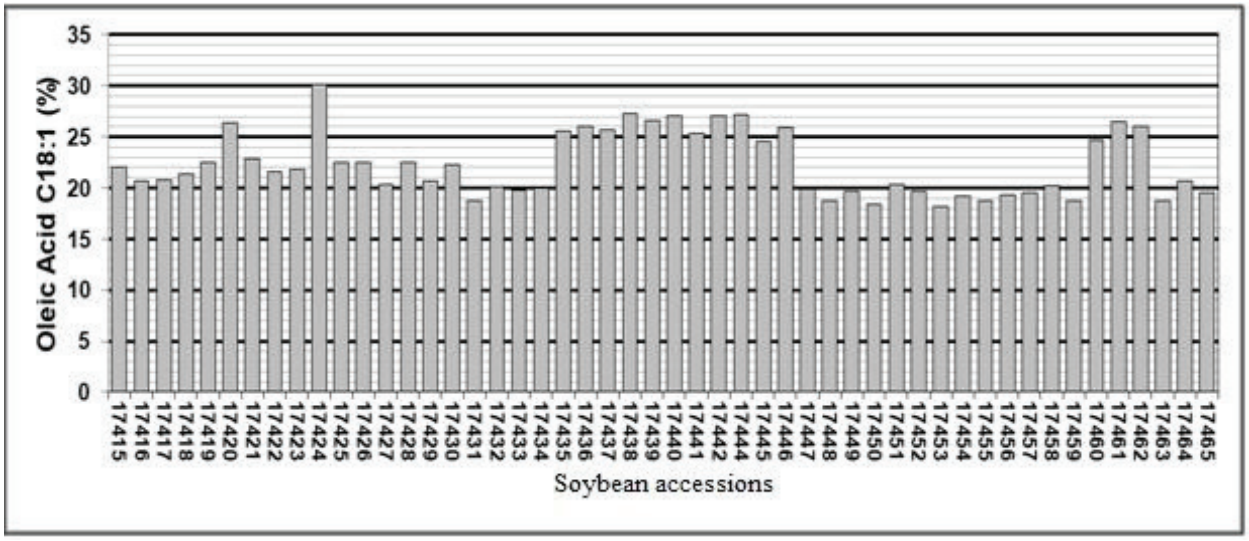

Fig. 4. The concentrations of Oleic acid in 50 Soybean accessions.

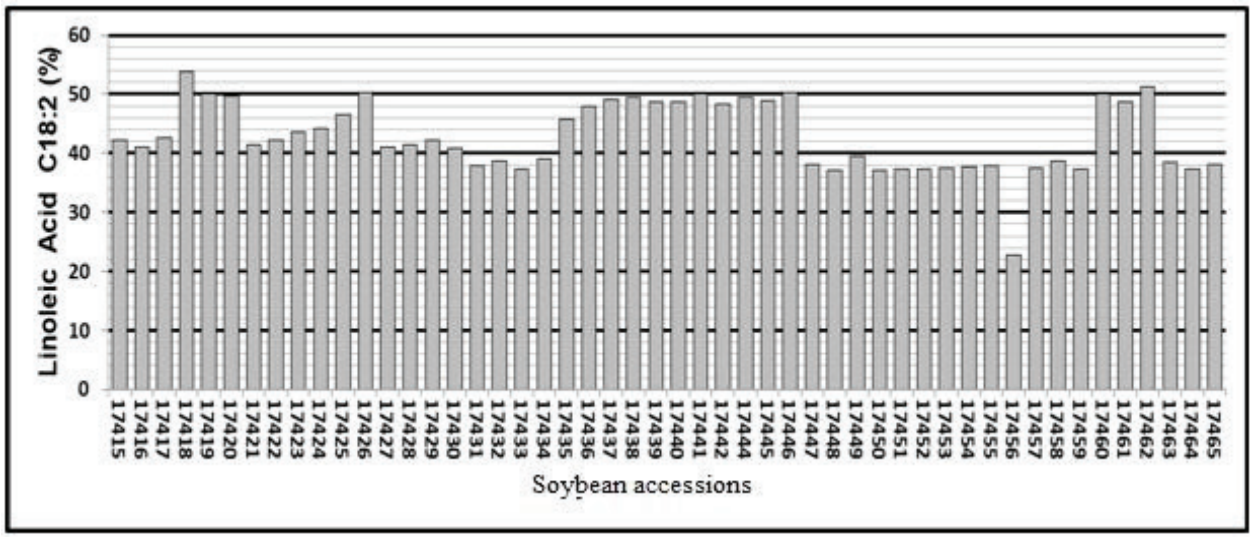

Fig. 5. The concentrations of Linoleic acid in 50 Soybean accessions.

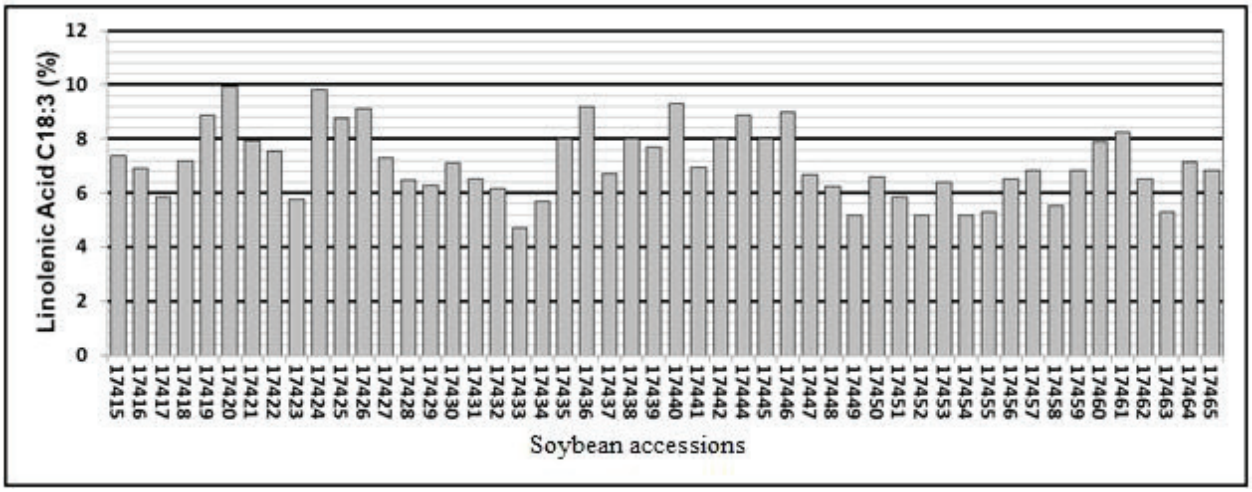

Fig. 6. The concentration of Linolenic acid in 50 Soybean accessions.

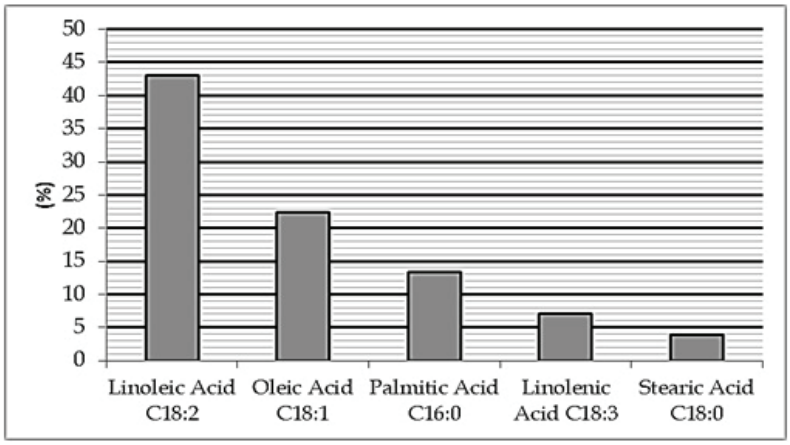

Fig. 7. Average concentrations of the five fatty acids contents in 50 Soybean accessions

related to those observed by [21, 30].

When the average values of the present results were calculated for the five major fatty acid components i.e. 13.4 % palmitic acid (16:0), 3.9 % stearic acid (18:0), 22.3 % oleic acid (18:1), 43 % linoleic acid (18:2) and 7.1 % linolenic acid (18:3) (Fig 7), it was found that these average values are nearly identical to those reported by the other researchers [38, 39].

4. CONCLUSION AND RECOMMENDATIONS

Accessions of soybean having a high amount of essential fatty acid and oil are considered more advantageous in contrast to those with a lesser amount of fatty acid and oil. In the present study, none of the accessions was found consistent for oil and fatty acid composition. The composition of oil in accessions exceeding 22 % is regarded as best because it is mainly cultivated for the production of oil therefore we recommend accessions 017421, 17423, 17435, 17430, and 17459 for the improvement of oil production. Palmitic acid contents are an important trait of soybean as their low contents are desirable for human consumption while higher contents are required for lubrication and other mechanical purposes. In the current research work, the highest palmitic acid content was observed within two accessions 017428 and 017429 and hence can be recommended for lubricant production while, the accessions 17453, 17420, 17441, and 17432 for human consumption. Stearic acid found in the fatty acids of soybeans is important for the prevention of cardiovascular disorders in humans as its high level has no negative effects on blood and LDL cholesterol levels. In the present study accessions, 017417, 017423, 01729, 017435, 017449, and 017456 are found with the highest stearic acid content and are therefore suitable for breeding to improve stearic acid production. Oleic acid is called an important fatty acid that helps to improve the shelf life of oil hence, accessions 017424, 017438, 017444, 017440, and 017442 and may be selected to improve the shelf life of soybean oil. Linoleic acid concentration is very important in both lower and high concentrations because its lower concentration is helpful for flavor stability while its high concentration is important for the human diet therefore its recommendations depend on its need. Similarly, the accessions 017420, 017424, 017440, and 017436 were found with high concentrations for linolenic acid and are therefore recommended for the high yield of linolenic acid to overcome brain and eye diseases.

5. ACKNOWLEDGEMENTS

The authors are highly thankful to Nuclear Institute for Food and Agriculture (NIFA), Peshawar for providing all facilities for the experiment and laboratory analysis. The authors are also thankful to Plant Genetic Resources Program, National Agriculture Research Center Islamabad (NARC) for providing 50 soybean accessions.

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 58(3): 65-73 (2021)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(58-3)668

Pakistan Academy of Sciences

Research Article

Assessing Public Opinion Regarding COVID-19 Vaccinations in Pakistan: Knowledge, and Perceptions of General Public

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Abstract: The COVID-19 infodemic can be counteracted by clear and consistent communication of scientific evidence and improved health literacy between the public and informants. For complete eradication of COVID-19, several vaccines are approved in various countries for public use by regulatory authorities. Assessing public perception regarding COVID-19 vaccination is an important area of research. In the current study, we aim to evaluate the opinions of individuals from multiple localities about COVID-19 and its vaccination through an online survey. Participants of the study were divided into different groups based on age, profession, demography, and income, and their opinions were calculated in percentage. In age group analysis we reported the highest willingness, 62.8 % (n=22) in age group 30-40, followed by 60 % (n=3) in age group >50, 58.6 % (n=244) in age group 20-30, 57.95 % (n=51) was in age group 15-20 and the least willingness, 33.33 % (n=4) in age group 40-50. The highest disagreement regarding vaccination of 60 % was found in age group >50, followed by 33.3 % in the age group 40-50, 14.7 % in the age group 20-30, 11.4 % in the age group 15-20 and 30-40. Similarly based on profession, maximum acceptability, 59.1 % (n=262) was reported in students, followed by a businessman (68.7 %, n=11), professional workers (3.5 %, n=20). Likewise, in demographic analysis, individuals from Khyber Pakhtunkhwa (KP) (61.3 %, n=200) were found more enthusiastic for vaccination, followed by AJK (58.33 %, n=7) and Islamabad (58.1 %, n=32). In the same way, people with income range $435 - $621/month showed the highest willingness (65.7 %, n=69) regarding vaccination, followed by income group ($621 or more $s/month). Surprisingly, individuals from the low-income group were found more interested in vaccination as compared to the higher-income group. Comparatively low interest of high-income group individuals may be due to more exposure to conspiracy theories shared on social media.

Keywords: COVID-19, vaccination, survey, perception, demography, willingness, infodemics

1. INTRODUCTION

Coronavirus belongs to the family of zoonotic viruses that cause illnesses ranging from the common cold to severe respiratory conditions like breathing difficulties, dry cough, high fever, etc [1]. In some serious cases, it can cause pneumonia, severe respiratory syndrome, kidney or lungs failure, or even death [2]. It can transmit from one person to another by respiratory droplets and personal contact [3]. It may take 2-14 days for a normal person to show its symptoms or it can be asymptomatic altogether [4].

The first case was documented in Wuhan, China in December 2019, COVID-19 was declared as a global pandemic in March 2020 by WHO. The early form of the virus has symptoms similar to the influenza virus and common cold but the death rate is much higher in covid-19 [5]. Soon this viral infection gained attention globally due to the high rate of transmission in humans and it crossed the borders of China and take over the whole world in a few days [6]. Research centers around the world started to find anti-viral treatments to cure the infection and the priority was to make a vaccine against this deadly virus. So phylogenetic studies showed COVID-19's relation with SARS, severe acute respiratory syndrome-like viruses [7]. But the origin of SARS-CoV-2 is still unknown [8].

Received: May 2021; Accepted: September 2021
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After primary researches on the virus genetics and structural biology, pharmaceutical and biotech companies started producing vaccines, and after production, clinical trials began [9]. Now talking about human attitudes towards this disastrous biological calamity, the behavior which was dominated all over the world that was taking no serious measure to overcome the viral transmission [10]. A part of the population attributed this virus to race and blamed their eating habits [11].

According to WHO the novel coronavirus has infected more than 209 countries around the globe. The first coronavirus case in Pakistan was reported in Karachi on February 26, 2020, and confirmed by the Pakistan ministry of health, Islamabad [12, 13]. The government of Pakistan took all the necessary measures to control the spread of SARS-CoV-2 and ensure the health and safety of its people [12]. The SOP's were followed according to the WHO standards and the complete lockdown was imposed by the government. But Pakistan, being a 3rd world country and facing economic instabilities, this pandemic proved to be causing a more alarming condition and have a huge negative impact on the economy and people [14]. So the government has to make the difficult decision to ease the restrictions of lockdown to keep the economic wheel of the country running, that's why it is very necessary to vaccinate the people as soon as possible [15].

The pandemic situation and the imposed lockdown appeared to have a role in the uncertainty, substantial public anxiety, and distrust in the people and most of them started blaming the government. [16]. Half of the population wanted to get rid of this lockdown due to poverty as the majority Pakistani population works on daily wages [17]. All the reasons for the population's insensitivity towards government measures come due to underestimating the danger of COVID-19 [18].

Many companies around the globe have launched the vaccine for the common public after 3rd phase of the clinical trial and a few companies are still in clinical trials [19]. But unfortunately, people have lots of concerns about the long-term effects of the vaccine. There is a lack of trust in vaccination from humans [20]. Illiteracy in Pakistan can be the reason but response towards vaccination is also the same in the educated population [18]. Now people are more concerned about the coronavirus rather than vaccination. The main reason for such concerns is overthinking due to the infodemics and misconceptions about COVID-19 being shared without any investigation on social media [21]. Also, the reason for this type of attitude is the elevated death rate in the last few months [22]. As this natural calamity proved to be disastrous for more than a year, it affected mentally everyone and made them confused about the vaccination [20]. The purpose of our current survey is to evaluate the perception and acceptance of the COVID-19 vaccine among people in Pakistan. The results of our study may help the government policymakers and health care professionals to formulate the best possible approaches to implement the mass immunization programs against COVID-19 in Pakistan.

2. METHODOLOGY

2.1 Study design

This was an online study that was conducted from 15th November 2020 to 10th December 2020 after the ease of lockdown in the country. We choose the online platform to survey because it was difficult to perform a community-based survey at that time, as nowadays almost every individual has internet access.

2.2 Survey instrument

The survey instrument or questionnaire was designed in an online Google forum in such a way that the people of any community and age group can easily understand it, a thorough literature survey of other vaccine-based studies was done. Once the survey conceptualization was done it was rechecked and reviewed by senior experts with a background in biotechnology. Some changes were made as suggested by the experts for a better understanding of the participants.

The questionnaire had 31 questions that had the demographic information of the participants, such as gender, age, marital status, education, occupation, and current residence. To investigate the knowledge of the participants, the questions were asked in the second part with three options: "yes", "no", and "maybe". These knowledge-based

questions were based on the presence of the virus, family individuals being infected, and strategies to prevent the transmission of COVID-19. The third part had questions focused on the attitude toward the COVID-19 vaccine and the perception of the people towards the SOPs after the COVID vaccine intake. The last part mainly focused on the misconceptions and infodemic-related views of the participants. The answers to the question of the third and fourth part comprised of five options: "strongly agree", "agree", "disagree", "strongly disagree", "don't know".

The questionnaire was shared through social media platforms like WhatsApp groups, Facebook, Twitter, Instagram, and through emails with friends, family, and colleagues. The participants were requested to share the questionnaire with their friends and family and they were requested to fill the form honestly by clicking the options on the link. Moreover, the main objective of the study was mentioned on the first page, and the sharing of individual data, confidentiality, and consent was also provided. Participants above the age of 15 years old living in Pakistan were added to the study and no incentives were provided to the participants.

2.3 Data analysis

The data was collected from 580 participants of which 556 participants' data was considered correct and the exclusion was made based on incomplete information and it was analyzed and arranged through Microsoft excel and was distributed among different age groups, financial status, province, education level, and professions.

3. RESULTS AND DISCUSSION

Assessing public perception regarding COVID-19 vaccination is an important area of research. In the current study, we had evaluated the opinions of individuals from multiple localities about multiple aspects including vaccination. Participants consisted of males and females comprising 46.2 % (n=257) males and 53.8 % (n=299) were females (Figure 1) belonging to multiple localities throughout Pakistan, 60.3 % (n=335) were from rural areas and 39.7 % (n=221) from urban areas (Figure 2). The majority of the participants that took part in this survey were of graduation level that was 53.6 % (n=298) followed by postgraduate level 34 % (n=34) and high school level were 12.4 % (n= 69) (Figure 3). They were further divided into different age groups and their opinions were calculated in percentage.

In the age group 15-20, 57.9 % (n=51) individuals were willing to be vaccinated, 11.4 % (n=10) individuals were not keen to be vaccinated and 30.7 % (n=27) individuals were not sure about being vaccinated. In age group 20-30, 58.6 % (n=244) people want to get vaccinated, 26.7 % (n=111) might get vaccinated and 14.7 % (n=61) people will not get the vaccines for COVID-19. Overall the highest percentage (62.8 %, n=22) of individuals who agree to be vaccinated lies in the age group 30 to 40, and the lowest lies both in individuals aged from 15-20 as well as 30-40. The data for all three perceptions in the age group 40-50 remain the same at 33.3 % (n=4 for each perception) and people above 50 who agree to be vaccinated and those who are not sure about being vaccinated are 20 % (n=1 each) with a high percentage of 60 % (n=3) who disagree to getting shots for COVID-19 (Figure 4). The results show that most people are not sure but will most like to get vaccinated because they want to wait before others get vaccinated and not try new vaccines on themselves right away.

People that agreed to get COVID-19 shots per different professional and educational sectors among which the overall highest number is that of students (59.1 %, n=262) and people with less education were less likely to say they would get vaccinated because they were less aware of its importance. The businessmen (68.7 %, n=11) fall next in line to students followed by professional workers (3.5 %, n=20). In the group of individuals that are not willing to get vaccinated most are professional workers (35 %, n=7) and the lowest number is that of administrative professionals (12.5 %, n=1). Professional workers (40 %, n=8) also show the highest peak among individuals that are not sure if they will get vaccinated for COVID-19 with the least doubtful individuals from the business sector (12.5 %, n=2).

Figure 6 demonstrates the attitude of the public regarding COVID-19 vaccination throughout different Pakistani provinces and their capital. The most elevated peak shows that the highest number

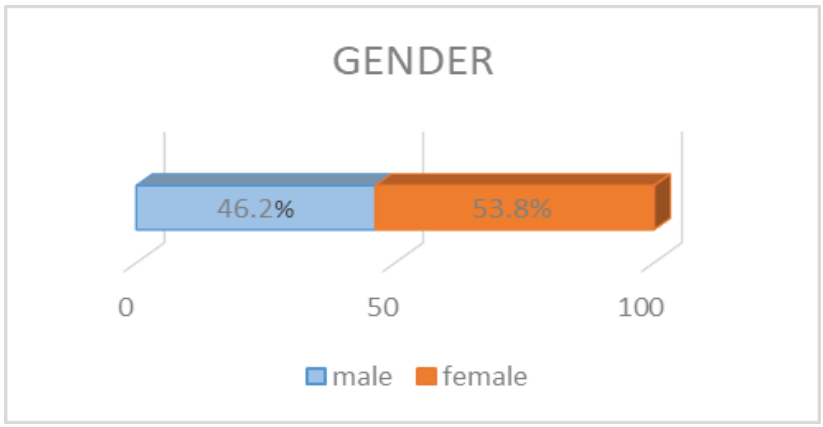

Fig. 1. No. of Male and Female individuals.

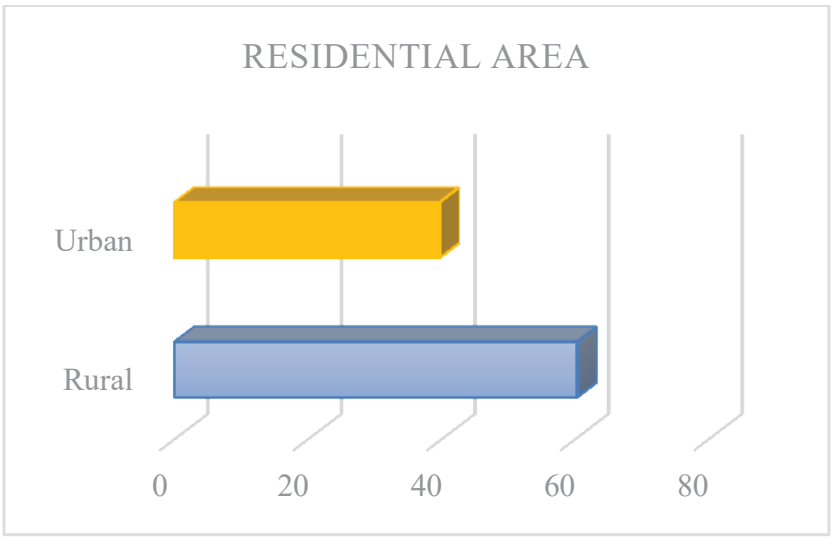

Fig. 2. Residential area wise count of participants.

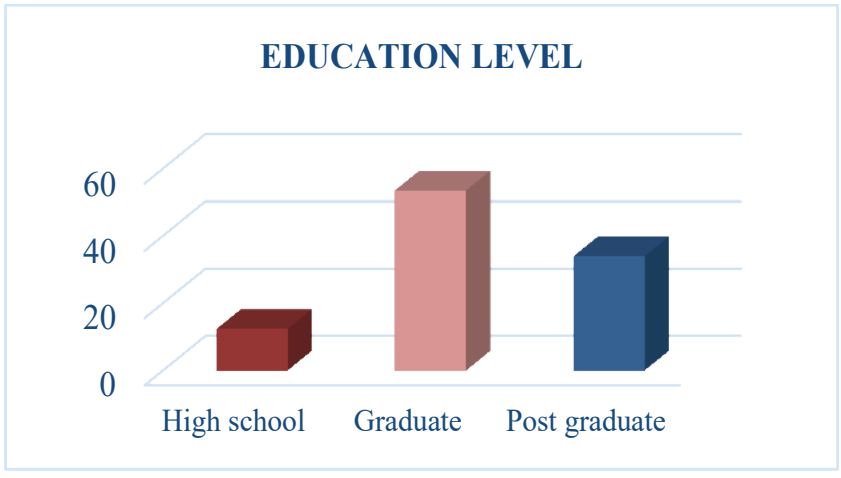

Fig. 3. Education level of the participants.

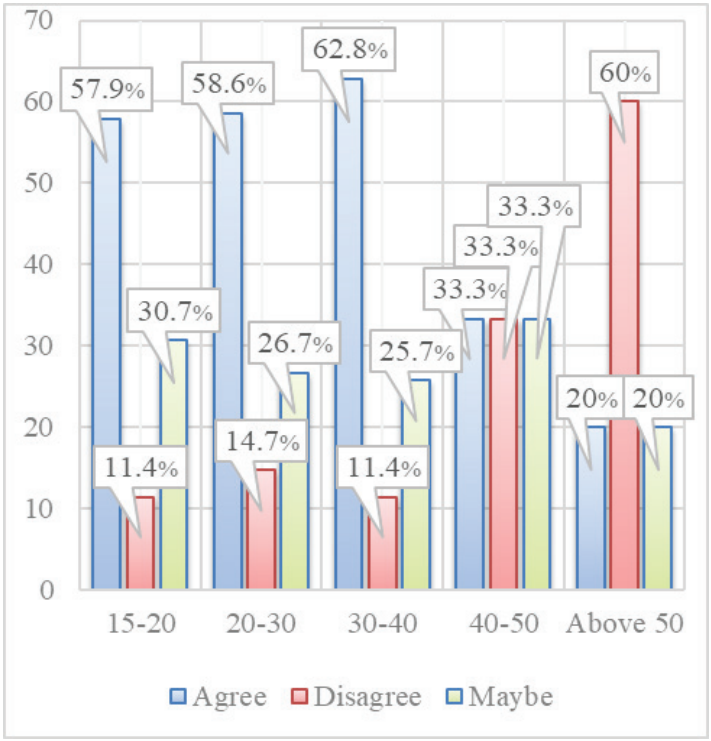

Fig. 4. Age group-wise perception about COVID-19 vaccination.

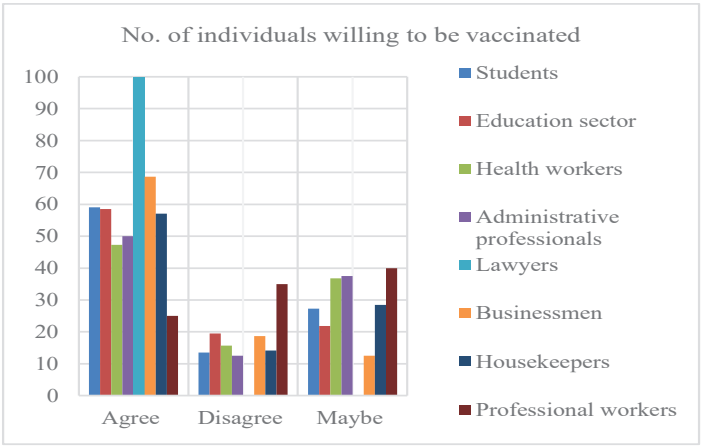

Fig. 5. No. of individuals willing to be vaccinated.

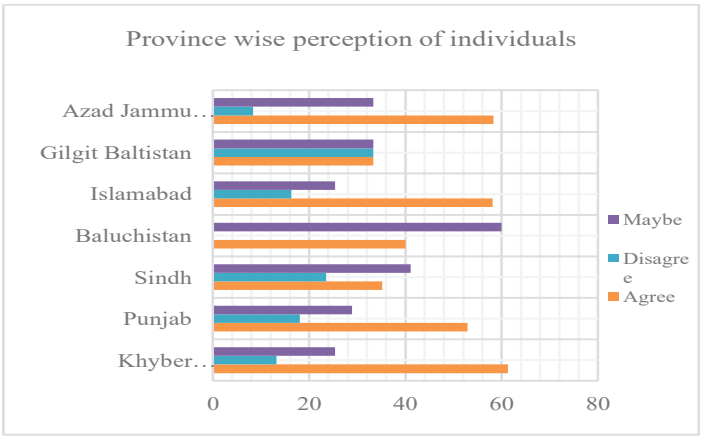

Fig. 6. Province wise perception of individuals.

of people that agree to be vaccinated belongs to KP (61.3 %, n=200) followed by the people of AJK (58.33 %, n=7) and Islamabad (58.1 %, n=32). The peak in KP can be explained as the most affected cases were from that province and the public is more aware of the severity of the disease. Most people who disagree with getting the shots are from Gilgit Baltistan with the same number of people from the area agreeing to be vaccinated and those who are not sure if they want to get vaccinated (33.33 %, n=1). The most uncertain group of people regarding the vaccines belongs to Baluchistan (60 %, n=3).

Figure 7 displays results about COVID-19 vaccine perception as per different wages of people across Pakistan. The people with income ranging between $435 - $621 (65.7 %, n=69) show the most interest in being vaccinated followed by the richest class (income more than $621). People with less income are less interested in buying expensive vaccines and choose to remain unvaccinated. Similar but the opposite trend is seen in the figure that the least people who disagree with being vaccinated for COVID-19 also belong to the group of people with stable wages of $435-$621 as they would be able to afford vaccines. The people with income between $310-$435 are most unsure if they want to spend on vaccines for COVID-19. A detailed overview of the above-said data is mentioned in (Table 1).

Unfortunately, due to the infodemics and misconceptions caused due to social media and different thoughts of people, 3.8 % of the people still think that covid-19 does not exist and there is no need of making the vaccine, while 10.4 % of people don't know whether the covid-19 a deadly virus exists or not and 85.8 % people are aware of this deadly virus and they think that a vaccine is necessary. Of the total participants 84.3 % people think that COVID-19 trials should conduct in Pakistan of which 57.7 % people are willing to participate in the trials and they will accept the vaccine shots, the remaining people are in doubt that is 6.7 % and some disagree with the trials in Pakistan 8.9 % and they would not accept the COVID-19 vaccine shots. There are 67.3 % of people who think that the COVID-19 vaccine will be effective, moreover, 4.3 % of people think that the vaccine will not be effective, 28.4 % people think that this vaccine can be effective or it can cause adverse effects and can have future complications. As initially there were myths against the COVID-19 virus that it doesn't exist and it's propaganda by the superpower countries to control people activities and to implement the new world order, same is with COVID-19 vaccine 51.8 % of people think that these vaccines can cause genetic manipulation in people while some think that these vaccines can be used to control people through 5g technology that were 26 % and 35.2 % people disagree with this myth to control people's mind with vaccines. On the other side, there are a total of 25 % of people who think that these vaccines can control the minds of people through advanced microchip technology and 20.7 % people think that this vaccine can cause a serious threat to their religious concepts and it can have the ability to control and change their religious beliefs by controlling their minds, while 51.4 % people don't accept this myth of the harm to their minds, genetics and religious concept by the COVID-19 vaccine.

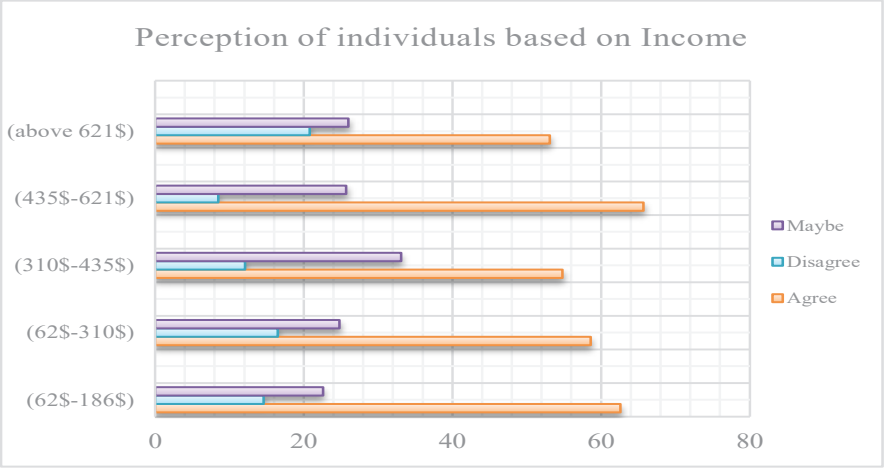

Fig. 7. Perception of individuals based on income.

Table 1. A detailed overview of the opinions of individuals from multiple localities about multiple aspects including vaccination

Age groups	**Total no, % value**	**No. of individuals willing to be vaccinated**		
		Agree	Disagree	Maybe
15-20	88 (15.8 %)	51 (57.9 %)	10 (11.4 %)	27 (30.7 %)
20-30	416 (74.8 %)	244 (58.6 %)	61 (14.7 %)	111 (26.7 %)
30-40	35 (6.3 %)	22 (62.8 %)	4 (11.4 %)	9 (25.7 %)
40-50	12 (2.2 %)	4 (33.33 %)	4 (33.33 %)	4 (33.33 %)
Above 50	5 (0.9 %)	1 (20 %)	3 (60 %)	1 (20 %)
Profession	**Total no, % value**	**Agree**	**Disagree**	**Maybe**
Students	443 (79.7 %)	262 (59.1 %)	60 (13.5 %)	121 (27.3 %)
Education sector	41(7.3 %)	24 (58.5 %)	8 (19.5 %)	9 (21.9 %)
Health professionals	19 (3.4 %)	9 (47.3 %)	3 (15.7 %)	7 (36.8 %)
Administrative professionals	8 (1.4 %)	4 (50 %)	1 (12.5 %)	3 (37.5 %)
Lawyers	2 (0.3 %)	2 (100 %)	-	-
Businessmen	16 (2.9 %)	11 (68.7 %)	3 (18.7 %)	2 (12.5 %)
Housewives	7 (1.2 %)	4 (57.1 %)	1 (14.2 %)	2 (28.5 %)
Professional jobs	20 (3.5 %)	5 (25 %)	7 (35 %)	8 (40 %)
Province	**Total no, % value**	**Agree**	**Disagree**	**Maybe**
KP	326 (58.6 %)	200 (61.3 %)	43 (13.2 %)	83 (25.4 %)
Punjab	138(24.8 %)	73 (52.9 %)	25 (18.1 %)	40 (28.9 %)
Sindh	17 (3.1 %)	6 (35.2 %)	4 (23.5 %)	7 (41.1 %)
Baluchistan	5 (0.9 %)	2 (40 %)	-	3 (60 %)
Islamabad (Capital territory)	55 (9.9 %)	32 (58.1 %)	9 (16.3 %)	14 (25.4 %)
Gilgit Baltistan	3 (0.5 %)	1 (33.33 %)	1 (33.33 %)	1 (33.33 %)
AJK	12 (2.2 %)	7 (58.33 %)	1 (8.33 %)	4 (33.33 %)
Income Groups (Monthly)	**Total no, % value**	**Agree**	**Disagree**	**Maybe**
10k-30k ($62-$186)	75 (14.1 %)	47 (62.6 %)	11 (14.6 %)	17 (22.6 %)
30k-50k ($62-$310)	133 (25 %)	78 (58.6 %)	22 (16.5 %)	33 (24.8 %)
50k-70k ($310-$435)	124 (23.3 %)	68 (54.8 %)	15 (12.1 %)	41 (33.1 %)
70k-100k ($435-$621)	105 (19.7 %)	69 (65.7 %)	9 (8.5 %)	27 (25.7 %)
Above 100k (above $621)	96 (18 %)	51 (53.1 %)	20 (20.8 %)	25 (26 %)

The majority of the people think that govt. and media are playing a sufficient role in awareness against this virus and they are trying their best for the acceptance of the COVID-19 vaccine among people.

To the best of our knowledge, this is one of the first kinds of study conducted in Pakistan. Moreover, in terms of age group and profession, similar findings have been reported in several international studies [4, 23-27].

The study has a few limitations that need to be considered, the samples count is more enough to represent the surveyed areas but the results could not be generalized over areas where the number

of participants was very low. In addition, due to lockdown and pandemic situations, the study was conducted online through a questionnaire and no personal interviews were conducted. This is the first survey conducted in Pakistan that highlights the knowledge and behavior of the individuals regarding COVID-19 vaccination.

4. CONCLUSION

This survey assessed the perceptions of the individuals of different ages, regions, and income groups of Pakistan regarding the acceptance of COVID-19 vaccination. It has been concluded that the major number of individuals who were willing to get vaccinated were teenagers and students. Senior citizens were found more hesitant of vaccination due to negative perception of side effects or mythical effects. Based on geographic distribution, the highest number of individuals willing for vaccination were from Khyber Pakhtunkhwa. Moreover, based on income, the highest willingness for COVID-19 vaccination was found in middle-income group individuals. To recapitulate, we suggest that only authentic sources such as WHO, CDC, and NIH website should be followed for seeking information regarding COVID-19. Exposure to malicious and disinformation could lead to infodemics that negatively influence people's perceptions regarding COVID-19 vaccination.

5. ACKNOWLEDGMENTS

We would like to thank all the participants who voluntarily took part in this survey.

6. CONFLICT OF INTEREST

The authors declared that they do not have any conflict of interest.

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Proceedings of the Pakistan Academy of Sciences: B. Life and Environmental Sciences 58(3): 75-82 (2021)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(58-3)667

Pakistan Academy of Sciences

Research Article

Prevalence and Association of Different Levels of Intellectual Disability with Prenatal, Perinatal, Neonatal and Postnatal Factors

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Abstract: Intellectual disability (ID), also called mental retardation, is defined by below-average intelligence or mental aptitude as well as a lack of life skills. It has a significant association with residency, family history, and chromosomal disorder. An analytical cross-sectional study was performed over a period from December 2019 to January 2021 in special educations centers and hospitals of Lahore, Faisalabad, Shahkot, Sialkot, Gujranwala and Sangala, Punjab, Pakistan. This study was aimed to access the prevalence and risk factors of Intellectual disability (ID). Questionnaires were designed and filled with the help of general doctors, pediatricians, and psychiatrists who diagnosed both intellectual and adaptive functioning of individuals Association between these parameters was analyzed by using SPSS software (Chi-square test) between ID and risk factors and the level of significance was considered as $P<0.05$. The frequency of mild, moderate, severe, and profound ID was 46.7 %, 32.1 %, 14.6 %, and 6.7 % respectively. More males (56.82 %) as compared to females 43.17 % were observed.

Keywords: Neonatal; Perinatal; Postnatal; Prenatal and Prevalence.

1. INTRODUCTION

Intellectual disability (ID), characterized by embryonic development impairments in intellectual function and adaptive behavior which are determined by the low-level intelligence quotient (< 70) [1,2]. Intellectual disability (ID) can be divided into a mild group (IQ: 50 to 70), moderate group (IQ: 35 to 49), severe group (IQ: 20 to 34), and profound group (IQ below 20) which depends on IQ level [3]. Approximately 1-3 % of the world population is getting affected by this syndrome [4]. Accordingly, the occurrence of Intellectual disability (ID) is about 2-3 % but some areas only have 1 % of occurrence. In developed and undeveloped countries, risk factors of ID vary from region to region. According to the latest survey conducted, for mental retardation among 8-year children about 13.6/1000.

The prevalence rate is recorded for the surveillance year 2010. 2.5 % of the Pakistani population suffers from ID and out of this about 55% of the affected population belongs to the province Punjab and 28.4 % in Sind according to the National Census of Pakistan 1998. However; the incidence rate may vary from mild ID (65/1000) to serious (19.1/1000) [5].

Children having ID show low expressing level with having speaking and language development difficulties. There are various etiological and heterogeneous forms of ID. Some of these are deprived mental abilities that may cause birth-related injuries or infections [6]. Genetics, as well as environmental factors, are involved in ID.

Received: July 2021; Accepted: September 2021
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Malnutrition repeated prenatal period without break, prenatal/ perinatal brain ischemia, infections after birth, emotional, social deprivation experienced, exposure to chemicals during pregnancy, and inadequate medical services are non-genetic causes of ID [7]. Fifty percent of intellectual disabilities have a genetic basis out of which 25-50 % can be divided into chromosomal aberrations or transformations in most gene and metabolic disorders [8].

In Pakistan, one of the main reasons behind the higher incidence rate of genetic diseases (ARID with 6.2/100 live births and 1.1/100 cases of severing ID) is due to a high level of endogamy. The present study was aimed at assessing the prevalence rate, the association between ID, demographic variables, and etiology factors such as prenatal, perinatal, and postnatal. Behaviors such as sensory impairments symptoms as well as signs of disorders were also included in this study. This study was performed over a period from December 2019 to January 2021 in special educations centers and hospitals of Lahore, Faisalabad, Shahkot, Sialkot, Gujranwala and SangalaPunjab, Pakistan. The primary goal of this research is to provide information to individuals and groups who are recognized to be at risk for ID, as well as their family members and to those who deal with them.

2. MATERIALS AND METHODS

2.1 Nature of Study

Hospitals and special education centers based analytical coss-sectional study was performed over a period from December 2019 to January 2021 in special educations centers and different hospitals of Lahore, Faisalabad, Shahkot, Sialkot, Gujranwala and Sangala, Punjab, Pakistan.

2.2 Questionnaire Design and Data Collection

Questionnaires were designed to collect information about variables such as age, area, gender, level of ID (mild, moderate, severe, and profound), family history, and genetic factors (Monogenic and chromosomal disorders). Non-genetic factors such as prenatal (before birth), perinatal (around birth) and postnatal factors (in infancy or childhood) include behavior, sensory impairments symptoms as well as signs of disorders. Questionnaires were filled with the help of general doctors, pediatricians, and psychiatrists who diagnosed both intellectual and adaptive functioning of individuals. They categorized the level of ID with different IQ test measurements and adaptive functioning is assessed through standardized measures with the individual and interviews with others, such as family members, teachers, and caregivers.

Physical examination by mental health clinicians and pediatricians was also done to categorize disorders with the help of symptoms associated with ID e.g. Down syndrome. Parents' meetings were also arranged for the completion of these questionnaires. Ethical clearance was taken on 10th December 2019 from the institutional Ethical committee of the Zoology department of Lahore College for Women University of Lahore (Report number: RERC/LCWU/Zoo 681). Consent forms of sampled ID individuals were signed from respective families. Permission from the respective institutions and hospitals was also taken.

2.3 Statistical Analysis

Statistical packages for social sciences (SPSS), version 22 were used to analyze the recorded data. Chi-Square ($\chi 2$) technique was used to test the association between two qualitative parameters. P-value at <0.05 was considered as significant, P-value at <0.01 was considered as highly significant and P-value at >0.05 was considered as insignificant.

3. RESULTS

3.1 Distribution of variables

About 315 patients of ID were collected in which 56.2 % (n=177) were male and 43.8 % (n=138) were female. 36.50 % (n=114) cases come from rural area while 63.50 % (n=200) cases come from urban areas. The frequency of mild, moderate, severe and profound ID were 46.7 % (n=147), 32.0 % (n=101), 14.6 % (n=46) and 6.7 % (n=21) respectively. Frequency of parent's consanguinity was 72.1 % (n=227) and non-cousin marriage was 27.9 % (n=88) (Table 1).

In this study risk factors for ID at prenatal,

perinatal, neonatal and postnatal stages were also studied. At the prenatal stage, genetic and non-genetic factors were recorded in this study. In genetic factors, two types of disorders were including one is chromosomal disorders and the other one is related to the monogenic disorder.

Out of 315 cases of ID, the prevalence of chromosomal disorders is 15.87 % (n= 50) which includes 94 % (n=47) were Down syndrome, 2 % (n=1) with Pradarwilli syndrome and 4 % (n=2) with Klinefelters syndrome while the other patients show no disorders. 42.53 % (n=134) patients shows single gene disorders in which 73.88 % (n=99) microcephaly, 3.73 % (n=5) were phenylketonuria, 2.24 % (n=3) hypothyroidism, and 20.15 % (n=27) were other diseases. Environmental influences include deficiency of Iodine, malnutrition, exposure to chemicals, maternal influences, Rh incompatibility were studied with incidence of 4.4 % (n=14), 22.2 % (n=70), 5.4 % (n=17), 7.6 % (n=24), 9.5 % (n=30) respectively. 50.9 % (n=160) have unexplained etiology (Table 2).

At the time of birth (perinatal) prematurity, birth trauma, complication in delivery causes were observed with 9.8 % (n=31), 1.3 % (n=4), and 18.1 % (n=57) incidence rate while 70.80 % (n=223) not show any cause. During neonatal stage .6 % (n=2) cases of septicemia and 22.6 % (n=71) cases of jaundice were observed in ID patients. 20.6 % (n=65) have Unexplained etiology and 56.2 % (n=177) have no causes.

Brain infection 22.5 % (n=71), head injury 8.6 % (n=27), lead exposure 0.3 % (n=1) and 13.3 % (n=42) malnutrition causes were calculated at postnatal stage while 55.24 % (n=174) have no cause at this stage. Distribution of these non-genetic risk factors with respect to ID levels is explained in Table 3.

3.1.1 Association of Intellectual disability with d emographic variables

Table 1 is describing that gender, parent consanguinity has some non-significant effect with ID, while residency and family history has significant result with ID.

3.1.2 Association of Intellectual disability with prenatal risk factors (Genetic variables)

Chromosomal disorder and ID level have significant relations. It shows that ID has significantly associated with different syndromes like Down's syndrome, Fragile X syndrome, Prader-Willi syndrome and Klinefelter's syndrome. Gene mutation diseases (Microcephaly, Phenylketonuria, Hypothyroidism and other disorders) have insignificant effects with ID (Table 2).

3.1.3 Association between ID with other variables (non-Genetic variables)

Family history is significantly associated with ID. As shows that prenatal risk factors have some non-significant effects on IQ level. While perinatal factors (Placental dysfunction, severe prematurity, birth trauma, and complicated delivery) have a significance association Neonatal factors (Septicemia Jaundice) and postnatal are also significantly associated with the ID (Table 3). Some variables are associated with signs and symptoms

Table 1. Distribution and association of demographic variables with intellectual disability patients

		Frequency of mild ID	Frequency of moderate ID	Frequency of severe ID	Frequency of profound ID	Total Percentage	P-Values
Residency	Rural	36 (11.4 %)	29 (9.2 %)	35 (11.1 %)	15 (4.8 %)	115 (36.5 %)	**.000**
	Urban	111 (35.2 %)	72 (22.9 %)	11 (3.5 %)	6 (1.9 %)	200 (63.5 %)	(53.942,3)
Parent consanguinity	Yes	101 (32.1 %)	76 (24.1 %)	34 (10.8 %)	16 (5.1 %)	227 (72.1 %)	**.662**
	No	46 (14.6 %)	25 (7.9 %)	12 (3.8 %)	5 (1.6 %)	88 (27.9 %)	(1.587)

Table 2. Distribution and association of risk factors of intellectual disability

Characteristics	Intellectual Disability				Total Percentage	P-Values (χ2,df)
	Mild	Moderate	Severe	Profound		
Prenatal Factors						
1. Genetic Factors Chromosomal disorder						
Down's syndrome	12 (3.8 %)	25 (7.9 %)	8 (2.5 %)	2 (0.6 %)	47 (14.9 %)	
Pradarwilli syndrome	0 (0.0 %)	0 (0.0 %)	1 (0.3 %)	0 (0.0 %)	1 (0.3 %)	**0.004** (24.355,9)
Klinefelter's syndrome	0 (0.0 %)	2 (0.6 %)	0 (0.0 %)	0 (0.0 %)	2 (0.6 %)	
NIL	35(42.9 %)	74(23.5 %)	37(11.7 %)	19(6.0 %)	265 (84.1 %)	
Single Gene Disorder						
Microcephaly	56 (17.8 %)	24 (7.6 %)	13 (4.1 %)	6 (1.9 %)	99 (31.4 %)	
Phenyl	1 (0.3 %)	1 (0.3 %)	3 (1.0 %)	0 (0.0 %)	5 (1.6 %)	**0.052** (20.915,12)
Hypothyroidism	2(0.6 %)	0 (0.0 %)	1 (0.3 %)	0 (0.0 %)	3 (1.0 %)	
Unexplained disorder	8 (2.5 %)	14 (4.4 %)	4 (1.3 %)	1 (0.3 %)	27 (8.6 %)	
NIL	74 (23.5 %)	62(19.7 %)	23 (7.3 %)	13(4.1 %)	172 (54.6 %)	
Missing	6 (1.9 %)	0 (0.0 %)	2 (0.6 %)	1 (0.3 %)	9 (2.9 %)	
2. Environmental factors						
Iodine	3 (1.0 %)	7 (2.2 %)	3 (1.0 %)	1 (0.3 %)	14 (4.4 %)	
Malnutrition	35 (11.1 %)	22 (7.0 %)	6 (1.9 %)	7 (2.2 %)	70(22.2 %)	
Exposure to chemicals	13 (4.1 %)	3 (1.0 %)	1(0.3 %)	0 (0.0 %)	17 (5.4 %)	
Maternal infection	14 (4.4 %)	5 (1.6 %)	5 (1.6 %)	0 (0.0 %)	24(7.6 %)	
Rh income	16 (5.1 %)	5 (1.6 %)	7(2.2 %)	2(0.6 %)	30(9.5 %)	**0.077** (23.350,15)
Others	66 (21.0 %)	59 (18.7 %)	24 (7.6 %)	11(3.5 %)	160(50.8 %)	

Table 3. Distribution and Association of Non-Genetic Risk Factors of Intellectual Disability

Prenatal factors						
	Intellectual disability				**Total Percentage**	**P-Values**
Characteristics	**Mild**	**Moderate**	**Severe**	**Profound**		**(χ2,df)**
Birth time prematurity	19 (6.0 %)	7(2.2 %)	1(0.3 %)	4 (1.3 %)	31(9.8 %)	
Birth trauma	2 (0.6 %)	0(0.0 %)	0(0.0 %)	2 (0.6 %)	4(1.3 %)	**0.004** (23.966, 9)
comp delivery	28 (8.9 %)	17(5.4 %)	11(3.5 %)	1(0.3 %)	57 (18.1 %)	
Others	98(31.1 %)	77(24.4 %)	34(10.8 %)	1 (4.4 %)	23 (70.8 %)	
Neonatal Factors						
Septicemia	1 (0.3 %)	0 (0.0 %)	0 (0.0 %)	1 (0.3 %)	2 (0.6 %)	
Jaundice	33 (10.5 %)	22 (7.0 %)	13(4.1 %)	3 (1.0 %)	71 (22.5 %)	
Unexplained Etiology	41 (13.0 %)	14 (4.4 %)	10(3.2 %)	0 (0.0 %)	65 (20.6 %)	**0.005** (23.422,9)
NIL	70 (22.2 %)	64 (20.3 %)	23(7.3 %)	16(5.1 %)	173(54.9 %)	
Missing	2 (0.6 %)	1 (0.3 %)	0 (0.0 %)	1 (0.3 %)	4 (1.3 %)	
Postnatal Factors						
Brain infection	42(13.3 %)	21 (6.7 %)	9 (2.9 %)	0 (0.0 %)	72 (22.9 %)	
Headinjury	14 (4.4 %)	2 (0.6 %)	8 (2.5 %)	3 (1.0 %)	27 (8.6 %)	
Lead exposure	0 (0.0 %)	0 (0.0 %)	0 (0.0 %)	1 (0.3 %)	1 (0.3 %)	
Malnutrition	22 (7.0 %)	7 (2.2 %)	9 (2.9 %)	4 (1.3 %)	42 (13.3 %)	**.000** (44.146,1)
NIL	69 (21.9 %)	71 (22.5 %)	20 (6.3 %)	13 (4.1 %)	173 (54.9 %)	

that are highly linked with ID like delay in sitting, delay in standing, delay in walking, difficulty in seeing at daytime, difficulty in seeing at night, difficulty in hearing, difficulty in understanding, difficulty in moving arm, loss of consciousness, lack of learning, not able to speak at all, not name object and mentally backward. (Table 4).

3.1.4 Association between Physical disorder and Intellectual disability

The results are describing here that physical disorder somehow, is no more associated with the ID, as an only floppy limb, problem in feeding and lump a navel has a significant association with IDs (Table 5).

Table 4. Association of ID with Signs and Symptom

Variable * IQ	Chi-Square value	P-Value
Delay in sitting	11.728	.008
Delay in standing	11.374	.010
Delay in walking	9.267	.026
Difficulty in seeing at daytime	7.514	.057**
Difficulty in seeing at night	7.811	.050**
Difficulty in hearing	16.096	.001
Difficulty in understanding	11.508	.009
Difficulty in moving arm	20.055	.000
Loss of consciousness	1.262	.738**
Lack of learning	6.900	.075**
Can’t speak at all	21.991	.000
Can’t name object	54.668	.000
Mentally backward	5.162	.000

Table 5. Showing the Association between Physical Disorder and Intellectual Disability

Variable * IQ	Chi-Square value	P-Value
Floppy Limb	21.7	0
Problem in Feeding	26.216	0
Cleft Lip	2.494	.476**
Large Head	6.415	.093**
Weak limbs	7.62	.055**
Club feet	4.542	.209**
Lump on Back	1.979	.577**
Lump at navel	15.275	0.002

4. DISCUSSION

In this study 315 patients of ID were collected in which 56.2 % (n=177) were males and 43.8 % (n=138) were females. 36.50 % (n=114) cases were come from rural area while 63.50 % (n=200) cases from urban areas. These results can be compared with one study conducted in Assiut, Egypt in which the total number of cases was 90, 63 males and 27 females, 76 % of cases were coming from rural areas while 24 % of cases were coming from urban areas [10]. The frequency of mild, moderate, severe and profound ID were 46.7 % (n=147), 32.0 % (n=101), 14.6 % (n=46) and 6.7 % (n=21) respectively, which is relatable with a study in which non-genetic ID patients were 97 in number which includes 24 % mild ID, 40 % moderate, 23 % severe-profound and 10 % unspecified ID [11].

Frequency of parent's consanguinity was 72.1 % (n=227) and non-cousin marriage was 27.9 % (n=88). In contrast, a study conducted to investigate the parental consanguinity among mentally retarded children found that (63 %) were born to non-consanguineous marriages [12]. In the present study, male gender remains dominant in all types of ID. This is in agreement with one study in which the gender ratios for severe/profound ranges of cognitive impairment (i.e., male-to-female ratio, 1.2:1in the severe range of mental retardation and 1.4:1 in mild mental retardation.

In this study, 315 cases of ID the prevalence of chromosomal disorders is 15.87 % (n= 50) which includes 94 % (n=47) were Down syndrome, 2 % (n=1) Pradarwilli syndrome and 4 % (n=2) with Klinefelter syndrome while the other patients show no disorders which is relatable with a study done by Harbour and Maulik [14]. Most cases were of unknown etiology (30-50 %). Down syndrome is the most common known cause and accounts for about 5-20 % of all cases. Congenital hypothyroidism accounts for 1-2 % of cases 42.53 % (n=134) patients shows single gene disorders in which 73.88 % (n=99) were microcephaly, 3.73 % (n=5) were phenylketonuria, 2.24 % (n=3) hypothyroidism and 20.15 % (n=27) were other diseases. Environmental influences include deficiency of Iodine, malnutrition, exposure to chemicals, maternal influences, Rh incompatibility were studied with incidence of 4.4 % (n=14), 22.2 % (n=70), 5.4 % (n=17), 7.6 % (n=24), 9.5 % (n=30) respectively. 50.9 % (n=160) have unexplained etiology.

In a study, the causes and associated disorders among children with mental retardation, cerebral palsy represent 7.8 % of cases with mental retardation, unexplained cause represented 70 %, Down syndrome represented 6.7 %, hypothyroidism represent 2.2 %, phenylketonuria represent 3.3 %, brain trauma represent 3.3 %, post-CNS infection cases represent (3.3 %) and MR associated with epilepsy (3.3 %).10 In this study, at the time of birth (perinatal) prematurity, birth trauma, complication in delivery causes were observed with 9.8 % (n=31), 1.3 % (n=4) and 18.1 % (n=57) incidence rate while 70.80 % (n=223) not showed any cause. During neonatal stage 0.6 % (n=2) cases of septicemia and 22.6 % (n=71) cases of jaundice were observed in ID patients. 20.6 % (n=65) have unexplained etiology and 56.2 % (n=177) have no causes. 22.5 % (n=71) with brain infection, 8.6 % (n=27) with head injury, lead exposure with 0.3 % (n=1) and 13.3 % (n=42) with malnutrition causes were calculated at postnatal stage while 55.24 % (n=174) have no cause at this stage. Intellectual disability has a significant association with residency, family history, chromosomal disorder, perinatal, neonatal and postnatal factors while gender, parent consanguinity, gene mutation diseases, and prenatal show non-significance levels. The significant statistical attachment between intellectual disabilities and family's socioeconomic profile in terms of residence, maternal, paternal education, father's job, and parent's consanguinity [10] is relatable with our study. The goal of this study is to give parents of ID patients advice on how to help their children survive in the environment. This research has immediate and long-term consequences for the diagnosis and treatment of ID patients and also advances our scientific understanding of this complicated disorder. Furthermore, it improves our knowledge of the prevalence of certain ID factors.

5. CONCLUSION

Analysis of recent study indicates significant association of intellectual disability with residency, family history, and chromosomal disorder, perinatal, neonatal and postnatal factors while gender, parent consanguinity, gene mutation diseases, and prenatal

shows non-significance level. With the discovery of incidence of predominance and risk factors of ID, it will help to diagnose the disease accurately and this discovery can also help in the couple plan in future.

6. ACKNOWLEDGMENTS

We are thankful to patients and their families for giving data and also thankful to the staff of Institutions and hospitals for their cooperation.

7. CONFLICT OF INTEREST

There is no conflict of interest among coauthors.

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Proceedings of the Pakistan Academy of Sciences: B. Life and Environmental Sciences 58(3): 83-94 (2021)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(58-3)676

Pakistan Academy of Sciences

Research Article

Traditional Process of Dates, COVID-19 Pandemic Observation, and Challenges in Sindh

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Abstract: This study brings to the front the reports on the importance of dates, its local processing, and associated all the challenges in the Thari Mirwah sub-district, Khairpur. A survey was planned to collect data based upon the interviews of local farmers. A questionnaire was structured with a total of 20 respondents (each of 4 per village). The demographic results show that the major proportions of the population under study were male (100 %). A 50 % of them were of an age ranging between 30-50 years. Almost, all the respondents were married, with a literacy rate of 20 %. The analysis showed that the dwellers make use of date fruit at variable stages of maturity and prepare different traditional dishes from the flesh of the fruit. Also, they responded that because of the COVID-19 pandemic, there were observable losses that occurred without any proper attention and influence on a timely harvest and supply chain of date fruit. It is concluded that a strong ethnobotanical relation exists between fruit trees and local dwellers of studied Taluka. Limited studies were conducted about the importance and challenges of date fruit in this area. It is recommended that the consumption of natural fruit with enormous antioxidants may help to trigger effective control over challenging issues of food insecurity or malnutrition, being an indigenous food source for the local population of the studied region.

Keywords: Date Fruit, Health Benefits, Challenges, Processing.

1. INTRODUCTION

Date palm (*Phoenix dactylifera* L.); belongs to the Kingdom Plantae, Palmae (*Arecaceae*) family, phylum Spermatophyta, sub-phylum Angiospermae, and class Monocotyledonae. It is an outstanding drought-tolerant, a lifesaver (for a livelihood in desert situations), healthy fruit. It is well-known nourishment as well as having a financial side [1-6]. Besides, the rest of the parts are also useful, such as leaves that can be used as raw substantial for making the roof of the house. It is an amazing fruit crop significantly cultured in the arid regions of the domain comprising the Arabian Peninsula, the Middle East, and North Africa since pre-historic eras and amongst the eldest plants cultivated on the ground for its eatable fruit [7], broadly planted in hot and dry weather. It is an imperative food means for the people of areas and shows a significant character in daily survival [5,8]. As per the report of the Food and Agriculture Organization 2014, Pakistan was the 6th largest producer of dates. In addition, over 300 varieties have been identified in the country in 2011. The study found that socioeconomic as well as food security situations of areas where date palm grow are poor in Pakistan. Universally, dates are eaten in current cultures for the pleasing flavor, odor, and biting texture, their use for flavoring foods, beverages, and medicine [9] These are a vital part of the diet in the Middle East. Dates play a prominent role in inhibiting human illness promoting well-being advancement as they are the basis of various dietary fibers and other bioactive mixes i.e.

Received: April 2021; Accepted: September 2021
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vitamins, carotenoids, polyphenols, glucosinolates, and minerals. Date palm [9] measured a well-known versatile, with multi-purposes like feed, food, and shelter. It had an extremely maintained national legacy in various sections globally. The key fate of Date palm cultivators is their growth, export, and selling capability [10]. The fruit is classified into basic parts: flesh, skin, pit [8]. The flesh consists of carbohydrates (73–79), total dietary fibers [14-18], ash (2.5), protein, (2.1–3.0) [11], and fat (2.0–3.2) in percent [5]. Dates too possess numerous medicinal abilities such as anti-bacterial, anti-fungal, anti-tumor, anti-ulcer, and immuno-modulatory possessions. An action of antioxidant about Date palm cultivars has been credited to phenolic mixes [5,12, 13, 14]. Traditional harvesting techniques and processing of dates were observed in the studied area. Farmers are not given advancement, awareness, and key attention from the stakeholders, policymakers, and non-governmental as well as governmental bodies. They are facing many obstacles during harvesting, processing, and marketing. During the study period, a major threat named coronavirus was observed with strict restrictions of human mobility, farmers and laborers were at home that results in a big delay in harvesting and processing. To know the socioeconomic importance, traditional processing, and challenges of date palm for a rural population, a study was conducted on dates in the Khairpur district of Pakistan.

2. MATERIAL AND METHODS

2.1 Study Area and Population

A total of five villages were selected for study from Taluka Thari Mir Wah, district Khairpur Mirs, Sindh provinces as depicted in maps below (Fig. 1). Thari Mirwah (a capital city of Mirwah division) is present 45 Km distant from the Khairpur Mirs district located on the Southern side (coordinates 27.068310 ^{o}N and 68.602252 ^{o}E) shown in Fig. 2. The selection of locations was based on the date palm plantation around the area. A compiled survey was taken up, to collect the data regarding date palm fruit, various techniques used in processing, and losses observed during harvesting in association with the human mobility due to COVID-19 pandemic total lockdown on production and supply chain. The current production of date has remained 33000 ha acreage per year. The survey was conducted during the year 2020, from June to July. The selection of 20 random respondents was done from selected villages (4 from each village). The general population i.e., farmers interviewed is for the study. The method of collecting information regarding the fruit-based interviews from local farmers of various age groups residing in rural, semi-urban, and urban areas is followed by Fatima *et al.* [19]. A well-structural questionnaire was developed for the proper study comprising 2-distinctive sections, namely.

1. Demographics of the people working at date palm orchards to monitor the trees and fruits.
2. Information regarding Date Palm fruit harvesting and challenges that farmers were facing and the influence of the current pandemic on a field operation and production.

2.2. Statistical analysis

The data collected from the survey was analyzed according to Snedecor and Cochran [20]. The Statistical Package for Social Sciences (IBM SPSS Statistics 20) was used while percentages and frequencies were used to interpret the findings from the recovered data.

3. RESULTS AND DISCUSSION

3.1 Demographics

Table 1 depicts the demographics of all the respondents. It was observed that all respondents were male (100 %) in taluka Thari Mir Wah which all were married. Out of a total of 20 respondents, 50 % were of the age between 30-50 years. Illiteracy was dominant with 80 % illiterate, only 02 attained educations (Primary and Secondary).

3.2 Ripening, Harvesting, and Traditional Processing

The Ripening steps of dates are starting from Hababauk, a creamy white color appearance that after 1-week changes into another growing form called Kimri, it takes 5 weeks to produce green color and after 17 weeks' yields turning yellow color or red color Kimri. Later, it goes to form Khalal, a yellow or red-colored. Rutab, another phase after Khalal that takes 26-28 weeks to get brown color and finally results in Tamer as depicted in Fig 3.

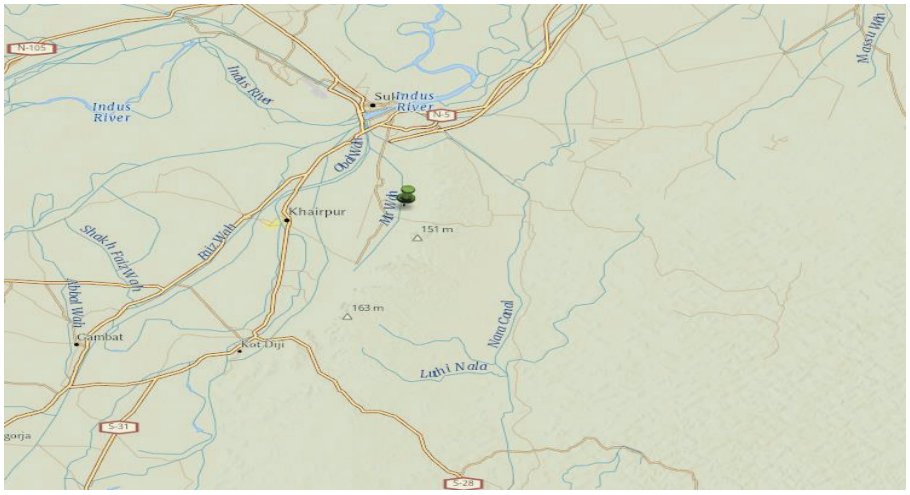

Fig. 1. Map showing Mirwah Sub-district, Khairpur Mirs, Sindh, Pakistan

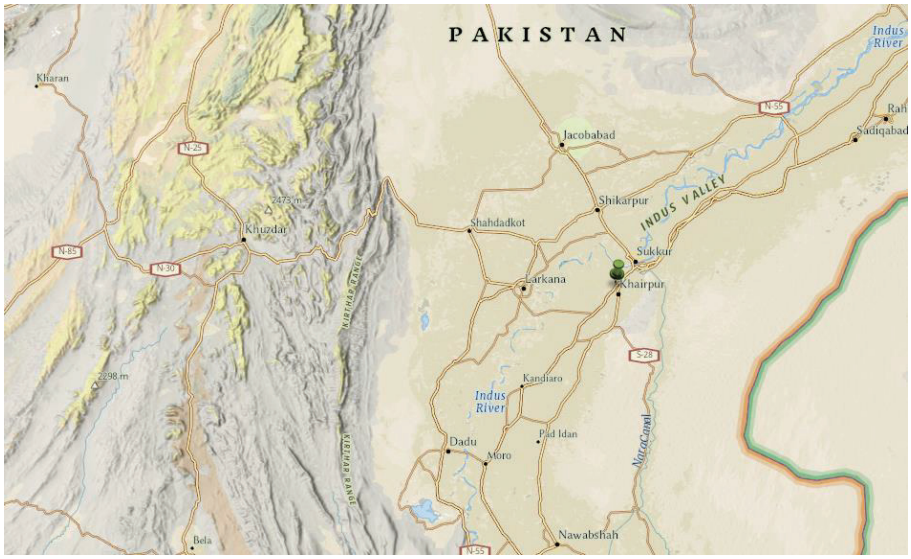

Fig. 2. Map showing Khairpur Mirs district of Sindh, Pakistan.

Table 1. Information regarding demographics of respondents from Taluka Thari Mir Wah

Demographic variables	%	Frequency
Gender		
Male	100	20
Female	00	00
Age (years)		
30-50	50	10
51 above	50	10
Marital status		
Married	100	20.
Unmarried	00	00
Education level		
Illiterate	80	18
Primary	10	01
Secondary	10	01
Matric	00	00
Intermediate	00	00

After maturation or ripening, harvesting was carried out through the traditional way using a sickle, etc., to cut dates bunches manually. Bunches of dates dropped down from the date palm that resulting in dispersion of dates, breakdown, and damage. In this way, a big loss of dates was observed during the study period that is shown in Fig 4. The harvested dates were stored on the farm sometimes covering it or not. The Dates were washed properly and boiled for about half an hour and dried under sunlight for about 12-24 hours to get products more effective and attractive. Sun-drying is the oldest method of drying those results in a long time for dates drying, environmental contamination, and several other factors that can influence the quality of dates.

3.3 Some Health Benefits

It is to believe that the antioxidants in fruit are very significant [14]. Fruits, as well as vegetables, are the main source of antioxidants. Epidemiology depicts that frequent intake might cause the hazard, leading to numerous long-lasting ailments such as cancer, cardiovascular diseases, and diabetes, [16, 17]. Various antioxidants include polyamines, phenolics, and glutathione. Phenolics are hydroxyl-cinnamates, flavonoids, and phenolic acids. The pointed antioxidant activity of dates is related to phenolics.

We can broadly characterize the biological properties produced by phenolics into two core sets. The first set includes anticipation of nucleic acids, lipids, and proteins of oxidative harm [18, 22]. The date fruit has been commercially added in numerous homeopathy drugs to cure various ailments including hypertension, diabetes, cancer [23], atherosclerosis as well as an anti-bacterial, anti-fungal, and immunity modulator [24]. The dietary antioxidants of dates fight against various degenerative problems of our body such as CVDs, neurological, ulcers [25, 26], gastric ulcers by depressing oxidative anxiety [3].

Dates are extremely nutritious food with innumerable health benefits. The fate of Dates as an emergent "healthy" food has been studied by several authors [12, 18]. Seeds are the main waste of marketable handling. Fruits and seeds are of high research focus being high in nutritious aspects [27]. Actuality dioicous plant, both flowers (M/F) are innate on two dissimilar palms.

3.3.1 Antimicrobial properties

Dates have anti-bacterial, antifungal, and antiviral characteristics due to very high phenolic insides. The fruit is very rich in carotenoids, ascorbate, selenium, and many antioxidants. These antioxidants support our body against oxidative harm caused by the

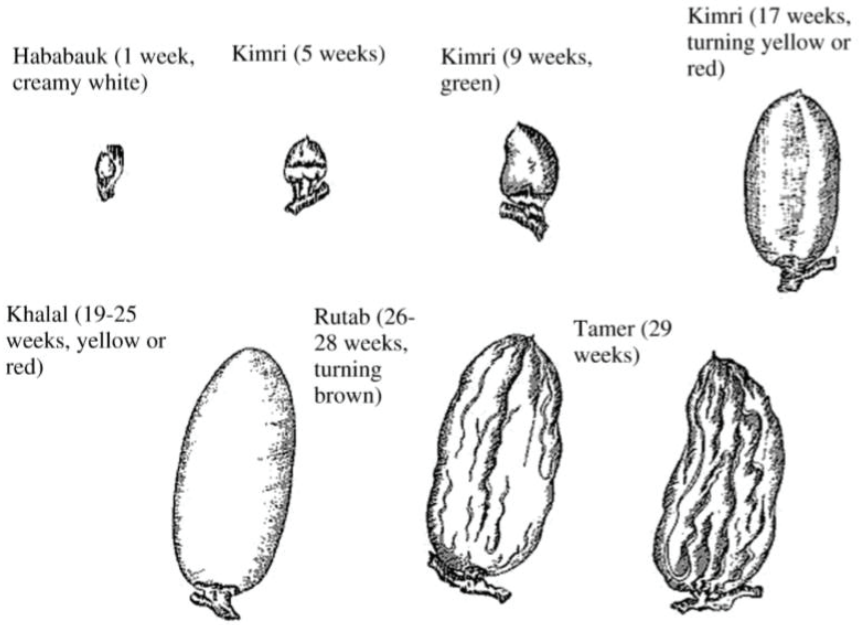

Fig. 3. Different stages of forming and ripening of dates [21]

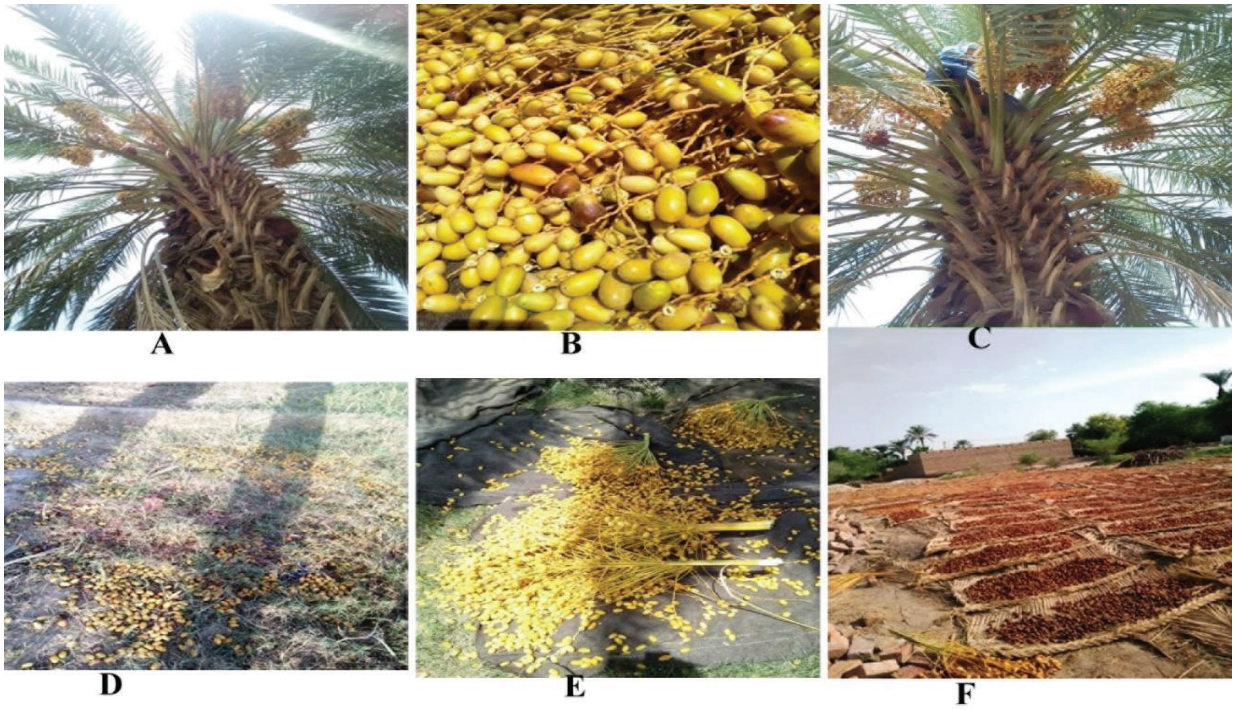

Fig. 4. Different steps of date processing from ripening, harvesting, sorting, grading to traditional drying are shown in A, B, C, D, E and F. A shows a date palm tree in scorching sunlight having ripe mature as well immature dates. In picture B, a bunch of dates is shown. C shows harvesting by hand, D shows a waste of date during the season, E shows a collection of harvested bunches and F shows sun-drying of dates at the local level.

activity of lymphocyte phagocytosis of pests and pathogens.

3.3.2 Anti-tumor and anti-ulcer properties

Almost all phenolics were verified as anticarcinogenic and found rich in dates [28, 29]. These phenolics depress the development of malignant tumors at various steps [30]. The anticancer activity of phenolics may be helpful to stop the enzymes catalyzing the development of pro-carcinogens [5, 31]. Caffeic and ferulic acids are major acids in dates that hinder the growth of skin tumors [32].

3.3.3 Immuno-modulatory properties

Dates have elevated fiber and phenolic contents that have a role in the inhibition of cardiovascular ailments and variation of an immune system. Stoppage ailments are resulted inhibited platelet accumulation and low-density lipoprotein oxidation. Phenolics with anti-inflammatory and anti-thrombotic results may be effective in minimizing the pressure of blood [33]. Additionally, phenolics also inhibit the activity of α-glucosidase and α-amylase to increase blood glucose levels [34, 35]. The immuno-modulation of phenolics involves anti-inflammatory responses which are triggered by the suppression of pro-inflammatory pathways.

3.3.4 Bioactive compounds in date fruit

The composition and nutritional value proposed that it has a good blend of fiber, invert sugars, and antioxidants. Date seeds with high-value anti-oxidants might be due to the good defense of seeds, the most fundamental constituent for plant beings. It states that the supreme total phenolic content is associated with dehydrated fruits i.e. apricots, plums, raisins, figs, and cranberries [36]. The antioxidant and phenolic content capability of Dates of Middle Eastern countries calculated broadly directing on dates grown in Oman [37, 38], Algeria [39], Saudi Arabia [4], and Iran [40]. It is ironic with bioactive mixes as shown in Table 2, studied by different researchers.

3.4 Dates Processing and Value-adding

Ordinary treatments involve the elementary stages

of fumigation, physical categorization, washing, desiccation, classification, and packing as well. More progressive methods for 1st mark dates include value-adding phases namely, removal of seeds also called pitting, earlier packing, refilling of dates without seed in eatable nutty products, packaging separate in good resources. 2nd and 3rd degree dates include, agglomeration then sorting that yields bits/pulp used (breakfast cereal or confectionary products, Dates paste, syrup concentrate, and wine, etc.) Pastes have a high market due to having uses i.e. fillers, binding components, and sweeteners. Dates bars comprising almonds, cereals, and sesame seeds were established. An assessment of juice handling data, interim goods, and end-products e.g., chaotic, or dim date juice and unblemished date sugar resolution brought is done. Complex Dates structures along with business techniques of numerous value-added products are defined [42].

Dates are particularly delightful fresh fruit by straight eating complete Dates are usually made variety diverse goods like date juice concentrates i.e., syrup, spread, and liquid sugar, agitated goods i.e. alcohol, wine, organic acids, vinegar and date pastes for varied uses (e.g., confectionery, bakery). Similarly, end-products of data processing are used for diverse energies [42]. Provide excellent taste to the final product and integral in food measures i.e. snacks, sweets, baking products, confectionery, health foods, and official feeding [7].

3.5 Varieties

In our country Pakistan, many varieties of dates have been observed, Begam Jangi, Aseel, Dhakki, Fasli, Karabalian, Halawi, and Muzawati, etc. [43]. Pakistan was graded 7th in all rest countries producing data during 2011. The country produces above 150 varieties of dates generally in Baluchistan, districts of Punjab and Sindh. Aseel and Dhakki are leading types. Dates are ordered semi-soft, soft, and dry dates [44]. In Pakistan, varieties come in a group of semi-dehydrated dates, highly rich in sugars (~81-88 %, mostly glucose, fructose, sucrose), dietary fiber (~5-8.5 %) and insufficient in additional

Table 2. Different properties of Dates and bioactive compounds

Compounds/ plant parts	Characteristics	Sources
Date fruit	Antimutagenic	[46]
	Neuroprotective	[47]
	Anti-allergic	[48]
	Cellular resistant motivation	[48]
	Anti-diabetic result	[49]
	Defense to stress in a liver of rat due to oxidation	[50]
	As a medicinal food	[12]
Flavonoid sulfates	During maturing and large volume of procyanidin/tannin at khalal stage	[51]
43 volatile compounds	Noticed in Tunisian Dates at various maturity phases	[52]
Phenolic compounds (anti-oxidants)	A defensive role for humans and plants in contradiction of antagonistic ecological surroundings, offering confrontation against microbial and parasitic contaminations	[53, 54, 55]

Table 3. The Nutritive contents of Dates with its properties

Parameters/elements	Quantity and properties	Source
Protein, moisture, fat, carbohydrates, and ash of Dates /100 g (wet)	42.4%, 1.5%, 0.14%, 1.16%, and 54.9%, separately. 16 varieties including Deglet Nour and Barhee.	[12,18,57,58]
potassium content in Deglet Nour and Medjool types.	96, 6.56 mg/g (wet weight), individually.	[18]
Carbohydrates in palatable flesh	70-80% (glucose, fructose)	[5,18,58]
Sugar content	Above 50%, declared a tree of life	[34]
consumable sugars generally glucose, sucrose, and fructose dietary fibers and enfold fewer proteins and fats	(70%), pulp hold it and bounded vitamins like riboflavin, biotin, thiamine, ascorbic, and folic acid that are vital for the body	[18]
Phosphorus, calcium, copper, iron, cobalt, fluorine, magnesium, potassium, manganese, sodium, boron, copper, zinc, sulfur, and selenium.	Good amount.	[18,59]
Oil	0.2-0.5% fleshy material of dates and seed holds 7.7-9.7%	[57]
Numerous nutrients	Dates embraced	[60]
Sugar, dietary fiber, minerals	Mineral and vitamins turn dates nutritionally further vital for mankind.	[61,62]
Rich in fat-soluble and water-soluble vitamins	Due to functional and nutritional ingredients, dates share an important part of human health and diet	[60]
15 different minerals	rich source of minerals	[63]
Vitamins	wanted by the human body for growth, waste removal, and digestion	[64]
Minerals	Involvement with organic doings, behave as catalysts in metabolic feedbacks of the body.	[65, 66, 67, 68, 69]
	Crucial for digestion, creation of nerve compulsion, engagement of food nutrients.	[62,66,67,70,71]
polyphenols and tannins	valuable to cure intestinal hitches	[72]

Table 4. Harvesting the season of dates, challenges, and an impact of COVID-19

Region	Harvesting time	Challenges	Investment	Effect of COVID-19
Khairpur Mirs is the hub of Date palm trees. The study was conducted in Thari Mir Wah villages	July-Aug each year	Rain Whitefly attack Low market value Diseases Water shortage Loss during harvest i.e., 40% The negligible focus of government on date palm trees and fruits	A lot of investment is needed. Approximately values are, Labor cost- 400 Cost of 1 sack of fertilizer per acre 200 per tree for pollination 50 per tree chemical cost	Not timely harvest Labor shortage due to lockdown, human mobility. No proper look after A whitefly attack was not measured early. No Marketing No Export to other countries.

nutrients [45].

3.6 Nutritional Value of Dates

It is rich in nutritive properties with enormous macro-nutrients as well as micro-nutrients shown in Table 3. It also contains phytochemicals that improve the nutritious and organoleptic possessions of the fruits [1, 2, 56].

Due to Vit-C and E, dates have antioxidants properties [18]. 100g of dried dates contains 193.7-239.4 mg of phenolic mixes, Some varieties have improved compounds after drying issue of these compounds from tannins afterward ruin through temperature and enzyme growth [37].

3.7 Challenges

One of the biggest challenges of the date palm is R. ferrugineus (an unseen and deadly tissue borer) with verminous palms in the initial phase of the outbreak. These palms respond to different chemical actions and can be saved unlike palms in the progressive stage of occurrence, where larvae cause wide tissue losses. The infestation mostly occurs at the base of the tree trunk near soil [73], whereas in P. canariensis the crown is typically damaged [74].

The tree can rise in a terrestrial array, specified circumstances are obligatory in blooming of flowers as well as the growth of fruit. The lengthy heat of summer is very good for the maturation of the fruit, while rainy and highly moisture conditions resulted in cracking, darkening, agitating, and mold growth of fruit [75]. While fruits can survive in a harmed situation like elevated (45-50 °C) and low temp. (–7 °C). Further, results show that it is a delicious fruit, consist of carbohydrates, protein, minerals, and vitamins. A study reported that one kg contains 2500-3000 calories [76].

Currently, the COVID-19 pandemic has left footprints on agriculture and food security as well; therefore, people interviewed deny sharing the information about different risks for the production of both quality and quantity of fruit due to various factors. These factors include harvesting, rainfall during fruit ripening, whitefly attack, a high cost of investment, lockdown, and lack of restricted human mobility presented in Table 4.

4. CONCLUSION

It is concluded from the present survey study that Dates are very ideal dietary intake for human health because of the enormous antioxidants, phenolic compounds and bioactive elements contained. If consumed properly the fruit can prevent numerous chronic ailments to ensure public health. Therefore, keen attention is required by various governmental and non-governmental organizations to empower our farmers with trending technologies to decline losses and mitigate other biotic and abiotic impacts on the quality and quantity of fruit.

5. RECOMMENDATIONS

There is a further need for research studies to be carried out to educate people regarding its dietary importance and get rid of ailments by evaluating nutritional characteristics and other quality parameters.

6. ACKNOWLEDGMENTS

The main author is grateful to the Institute of Food Sciences and Technology, Sindh Agriculture University, Tandojam to facilitate research work.

7. AUTHOR CONTRIBUTIONS

All authors have contributed equally to perform this study.

8. 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 58(3): 95-108 (2021)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(58-3)635

Pakistan Academy of Sciences

Research Article

Comparative Evaluation of Compost, Wheat Straw and Sawdust on Soil Structural Stability, Plant Available Water and Sorghum (*Sorghum biocolor* L.) Yield

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Abstract: The decreased soil structural stability with a resultant reduction in soil porosity, availability of water and nutrients has declined the crop yield. Compost, wheat straw and sawdust, the organic wastes can be effectively used as soil amendments to improve soil structure, porosity and water holding capacity and the crop yield. The study was conducted in randomized complete block design (RCBD) and three types of amendments i.e. compost (CM), wheat straw (WS) and sawdust (SD) were applied at three rates of 0, 4 and 8 t ha^{-1}. The higher sorghum growth was at 8 t ha^{-1}, however, 4 t ha^{-1} also indicated statistically comparable trend. The highest grain yield of 1357 kg ha^{-1} and 1374 kg ha^{-1} was observed in amended soils and type of amendments showed statistically ($P \leq 0.05$) similar yield. The water contents at field capacity (35.4 %), wilting point (22.5 %) and plant available water (13.25 %) were higher in WS 8 t ha^{-1} amendment. The higher soil structure stability of 90.78 % was observed in WS with rates of 8 t ha^{-1}. The crop residue WS 8 t ha^{-1} have the potential to increase soil water retion and soil structure stability as well. The CM, WS and SD amendments significantly improved organic matter (OM), porosity, nitrogen (N) and phosphorus (P) of soil in 4 and 8 t ha^{-1} than control. The low bulk density (BD) of 1.07 g cm^{-3} was in WS 8 t ha^{-1} and the lower pH was recorded in CM 8 t ha^{-1}. The study showed that all three types of amendments (CM, WS and SD) at 4 and 8 t ha^{-1} rates have potential to improve the soil structural stability, plant available water and yield of grain sorghum.

Keywords: Compost, Wheat straw, Sawdust, Water retention, Sorghum growth, Yield

1. INTRODUCTION

Land degradation lowers the soil quality and reduce the potential productivity of the soil [1] Aggregate stability is considered to be an indicator of soil structure. Organic matter serves as a binding material that lead to the formation of water stable aggregates through the formation of clay, humus complex [2]. Different organic matter inputs have a potential to improve soil water at field capacity and wilting point [3]. Organically amended soils increased soil organic carbon (SOC) by 49 % and 29% than an unfertilized and fertilized control, respectively [4]. Compost additions have increased SOC significantly [5]. The slow release of nutrients, especially N [6] from organic amendments builds up mineralizable N and increase the crop yield. The existence of a large quantity of water stable macroaggregates controls the degradation of soil to a great extent [7].

Sorghum (*Sorghum bicolor* L.) is one of an important kharif (summer) season crop of Pakistan that known as jowar. It is grown both in rain-fed and in irrigated areas of Pakistan for fodder and grain purpose [8]. It belongs to Poacea family. With regard to economic importance, sorghum attains fifth position worldwide among different cereal crops that give production of about 60 million tons annually. In Pakistan, it is cultivated in an

Received: December 2020; Accepted: September 2021
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area of 214 000 hectares that give approximate 137 000, tones sorghum grain production [9]. Sorghum has a wide adaptability to different types of soil however, subsoil compaction due to poor structural condition of soil usually decreases root growth of the crop. This limits the availabilityy and uptake of nutrients and water by plants which results in reduction in grain yield of the crop [10]. Grain yield of sorghum is also severely diminishes if water is scarce at the critical stages of crop [11]. Water stress at the vegetative stage alone can reduce yield more than 36 %, and water stress at the reproductive stage can reduce yield more than 55 % [12]. For that reason, this crop needs better management measures such as moisture conservation, enhanced nutrients availability, soil temperature maintenance and low soil compaction.

Organic matter of soil usually decreases due to exhaustive cropping of soils [13]. Physical deterioration of soil due to extensive agriculture activities without proper management of soil is a serious and alarming issue in Pakistan. It causes crop yield reduction and this problem can be overcomed by applying organic inputs in soil [14]. Organic amendments play a major role in the improvement of physical, chemical as well as biological properties of soils [15, 16]. Many studies [17, 18] proved that organic amendments improve the quality of degraded soils. These materials supply nutrients and organic matter to the soils. Amongst the most important organic amendment compost application trials resulted in increased SOM concentrations [19]. Compost has a potential to sustain good soil tilth, better aeration and nutrients supply, thus makes its greatest contribution to soil productivity [20]. It has been studied that application of composted organic wastes in a silt loam soil result in better aggregate stability [21].

Sawdust is also an additional plant residue or organic waste that is a rich carbonaceous substance. Once applied in soil, sawdust has a promising role in supplying humus that lead better soil hydro-physical properties [22]. Similarly, crop residues, e.g. straw also improve and increase soil properties and crop yield. Compared with control, the average soil available potassium (K), available P and available N and SOC levels were higher in the 0–40 cm soil layers after straw incorporation treatments [23].

Most of the soils of Rawalakot (latitude 33º51′32.18″N and longitude 73º45′34.93″E) Azad Jammu and Kashmir are usually low in fertility, compacted, eroded and prone to crusting. These soils have degraded structure, higher runoff and low infiltration. The study was conducted with the hypothesis to improve the soil physico-chemical properties and subsequent increase in sorghum yield by organic inputs i.e. compost, wheat straw and sawdust. These soil amendments are low in cost, easily available in this area and have potential to improve physical properties of soil, restoring soil fertility and enhancing crop yield.

2. MATERIAL AND METHODS

The field experiment was performed to assess the effect of amendments (compost, wheat straw and sawdust) on soil structural stability, plant available water and sorghum (*Sorghum bicolor* L.) yield at the Research Farm of the University of the Poonch Rawalakot, Azad Jammu & Kashmir during the year 2016. The study was conducted in randomized complete block design (RCBD) having two factors; (i) types of organic amendments (compost CM; wheat straw WS and sawdust SD) and (ii) rates of amendments. i.e. 0, 4 and 8 t ha^{-1}. There were a total of nine treatment combinations with three replications. Net plot size was $4m^2$. The variety of grain sorghum (*Sorghum bicolor* L.) "Johar" was grown. The distance maintained from plant to plant was 15 cm and the distance from row to row was 60 cm after germination of the crop. The recommended rates of NPK fertilizers (100 kg ha^{-1} nitrogen, 50 kg ha^{-1} P_2O_5 and 40 kg ha^{-1} K_2O) were also applied to sorghum crop as basal dose to all treatments.

Presowing composite and post-harvest soil samples from each replication were collected from a depth of 0-15 cm and analyzed for physico-chemical properties of soil.

2.1 Soil Analyses

To determine the texture of the soil the Boyoucos hydrometer method was used [24]. The suspension ratio of soil and water was 1:2 to measure the pH [25]. The 1:2 soil and water suspension were used to measure the soil electrical conductivity of extract ECe. The ECe reading was noted on EC meter by

inserting an electrode into suspension [26]. The Nelson and Sommers, [27] method were followed in the determination of soil organic matter (OM). The OM as percent oxidizable organic carbon (OC) was then determined. By multiplying % organic carbon with factor 1.724, the percentage of OM was computed. To determine the bulk density (BD), core method as proposed by Blake and Hartage, [28] was used. For that an intact soil sample was taken with the help of core sampler. The intact sample was placed in moisture can. Then moisture can weight was measured prior to and after drying in oven at temperature of 105 ºC.

"BD (g cm^{-3}) = Oven dry weight of soil (g)/volume of core sampler"

Determination of particle density (PD) was done by method of Bray [29].

Percent pore space (PS) was computed as:

% PS = 1-Db/Dp x100

Where % PS = percent pore space; Db = bulk density; Dp = particle density

The Kjeldahl method [30] was applied to determine the total nitrogen in soil. The available P was measured by the AB-DTPA method. The absorbance of the blue colour of the solution at 880 nm was measured on a spectrophotometer [31]. Soil structural stability was measured by wet sieving method [32, 33]. The plant available water (PAW) was determined by the pressure plate apparatus. The method determines soil water content at the Permanent wilting point (PWP at 15 bars) and field capacity (FC at 1 bar) thus calculates PAW as difference between PWP and FC [34].

PAW (%) = FC-PWP

FC = Field capacity (%); PWP = Permanent wilting point (%)

2.2 Crop Growth Parameters

The crop growth parameters including plant height, leaf surface area, number of leaves per plant chlorophyll content of leaf, biological yield, grain yield, dry matter yield, harvest index were also determined. To measure chlorophyll contens a piece of leaf (1 cm^2) was taken and put into a test tube and 5 ml acetone was also decanted in a tube. It was placed in dark for overnight [35]. The spectrophotometer was then used to read the absorbance at 663 nm and 645 nm for chlorophyll a and b. Then total chlorophyll content was computed as described:

Total Chlorophyll = 8.02 x (A 663 nm) + 20.2 x (A 645 nm)

2.3 Plant Analyses

Kjeldahl's method as prescribed by Bremmer and Mulvaney, [30] was used to calculate the total nitrogen in plant. Instead of soil, plant material was used in this case. However, all other steps were almost same in this method as adopted for total nitrogen analysis in soil. To measure P in plant Olsen and Sommers, [36] method was used. Spectrophotometer was used to measure the color intensity at wavelength 410 nm.

2.4 Total N and P Uptake

Uptake was calculated as total N and P uptake (kg ha^{-1}) = " [percent N and P content of yield x yield (kg ha^{-1})]/100"

2.5 Statistical analysis

The Statistica 8.1 software was applied to analyze the data. The variations between the means of different treatments were observed using LSD test at the 5 percent level of probability [37].

3. RESULTS AND DISCUSSION

3.1 Pre-Sowing Properties of Soil

The pre-sowing properties of soil are given in Table 1. shows neutral pH with no salinity or alkalinity problem. The bulk density and particle density were within the range of mineral soil. The soil porosity 43.34 %. The total N was 0.55 g Kg^{-1}, available P 7.27 mg Kg^{-1} and OM was 1.37 %. The soil texture was sandy loamy.

3.2 Growth and Yield of Sorghum

Plant height, number of leaves per plant, leaf surface area and chlorophyll content of sorghum plants as

Table 1. Presowing properties of experimental field

Soil properties	Values
Soil pH	7.21
ECe (dSm^{-1})	0. 34
BD ($g\ cm^{-3}$)	1.49
PD ($g\ cm^{-3}$)	2.63
Soil porosity (%)	43.34
N ($g\ kg^{-1}$)	0.55
Available P ($mg\ kg^{-1}$)	7.27
OM (%)	1.37
Sand (%)	52.4
Silt (%)	31.6
Clay	16
Texture	Sandy loam

affected by the interactive effect of amendments types x rates of amendments are given in Table (2). The results showed that all the growth characters had positive response when all three types of amendments were applied.

The maximum plant height of 175.93 cm in CM 8 t ha^{-1} was recorded and it was 12 % higher than no amended soils. Whereas the leaf surface area was maximum (74.25 cm^2) in case of SD 8 t ha^{-1} that was 25 % higher than no amended soils.

The high chlorophyll contents of 11.5 mg cm^{-2} were observed in CM 8 t ha^{-1} compared to rest of the treatments. This high rate was 59 % than its no amended soils. All the growth characters of sorghum were minimum at zero rates of all three amendments.

The yield data (Table 3) showed a higher grain yield (1454.2 kg ha^{-1}) at treatment CM 8 t ha^{-1} and it was statistically comparable with WS 4 t ha^{-1}. The means of amendment types showed 25 % higher grain yield in CM compare to WS and SD, however, rates of amendments showed higher yields at 4 and 8 t ha^{-1} compared to no amendments. The percent increase was 25 and 27 at rates of 4 and 8 t ha^{-1}, respectively compare to zero amendments.

The highest dry matter yield (7469 kg ha^{-1}) was obtained in WS 8 t ha^{-1} that was at par with WS 4 t ha^{-1} (7453 kg ha^{-1}) and SD 4 t ha^{-1} (7456.8 kg ha^{-1}). The lowest dry matter yield (5098.5 kg ha^{-1}) was recorded in WS0. The means of amendment type had higher yield with WS and SD compare to CM. The percent increase compare to respective control was 31, 24 and 9 by WS, SD and CM, respectively. The zero rate of amendment showed a minimum yield.

The higher harvest index (19.89 %) was found in CM 4 t ha^{-1} followed by HI (18.2 %) recorded in CM 8 t ha^{-1}. Among the amendment type, CM 8 t ha^{-1} had higher HI (18.12 %) while the rates of amendments showed statistically similar HI.

3.3 Concentration and Uptake of Nutrients

The results in Table (4) illustrating the response of total nitrogen to the treatments and data showed the highest soil total N (0.720 g kg^{-1}) in SD 4 t ha^{-1} and it was similar with CM 8 t ha^{-1} (0.677 g kg^{-1}) and SD 8 t ha^{-1} (0.680 g kg^{-1}). Whereas WS 8 t ha^{-1} (0.537 g kg^{-1}) and CM 8 t ha^{-1} (0.577 g kg^{-1}) did not show a marked increase in total soil nitrogen. However, the lowest total soil nitrogen 0.33 g kg^{-1} was observed in SD0. The means of amendment types had higher total N with SD and it was 24 % than its control and means of rates had higher N at 4 t ha^{-1} and it was 55 % higher than no amendment.

Table 2. Effect of compost, wheat straw and sawdust on growth parameters of sorghum

Rates of organic amendments (t ha^{-1})	Types of organic amendments			Means
	Compost	Wheat straw	Sawdust	
Plant Height (cm)				
0	156.43^{d}	156.17^{d}	163.80^{bcd}	158.80^{b}
4	171.80^{ab}	166.27^{abc}	159.73^{cd}	165.93^{a}
8	175.93^{a}	167.57^{abc}	166.53^{abc}	170.01^{a}
Means	168.06	163.33	163.36	
Leaf surface area (cm^2)				
0	56.43	59.03	59.56	58.34^{b}
4	71.75	73.63	63.88	69.76^{a}
8	69.84	67.27	74.25	70.45^{a}
Means	66.00	66.64	65.90	
Chlorophyll content				
0	7.29	7.26	7.29	7.28
4	8.70	9.16	8.03	8.63
8	11.57	10.11	8.49	10.06^{a}
Means	9.19^{a}	8.84^{ab}	7.94^{b}	

The different letters in columns are statistically significant at $P \leq 0.05$

Table 3. Effect of compost, wheat straw and sawdust on yield of sorghum

Rates of organic amendments (t ha^{-1})	Types of organic amendments			Means
	Compost	Wheat straw	Sawdust	
Grain yield (kg ha^{-1})				
0	1024.0^{d}	1098.2^{c}	1135.8^{c}	1086.0^{b}
4	1346.7^{b}	1413.3^{a}	1311.3^{b}	1357.1^{a}
8	1454.2^{a}	1335.0^{b}	1334.2^{b}	1374.4^{a}
Means	1274.9	1282.2	1260.4	
Dry matter yield(kg ha^{-1})				
0	5274.7^{c}	5098.5^{c}	5218.2^{c}	5197.1^{b}
4	5422.7^{c}	7453.0^{a}	7456.8^{a}	6777.5^{a}
8	6544.5^{b}	7469.0^{a}	6677.5^{b}	6897.0^{a}
Means	5747.3^{b}	6673.5^{a}	6450.8^{a}	
Harvest index (%)				
0	16.25^{cde}	17.81^{bc}	17.93^{bc}	17.33
4	19.89^{a}	15.79^{de}	14.67^{e}	16.78
8	18.21^{ab}	15.19^{de}	16.70^{bcd}	16.70
Means	18.12^{a}	16.26^{b}	16.43^{b}	

The different letters in columns are statistically significant at $P \leq 0.05$

The lowest available phosphorus (7.24 mg kg^{-1}) was found in CM0. The highest available phosphorus (10.077 mg kg^{-1}) was found in CM 8 t ha^{-1} followed by WS 8 t ha^{-1} (8.813 mg kg^{-1}). The means had higher P with CM and it was 18 % higher than its control and among rates higher P was at 8 t ha^{-1} and this was 23 % higher than no amendments.

The higher total N uptake was at WS 8 t ha^{-1} and higher P uptake was also in the same treatment. The means of amendment types showed higher total N uptake of 149.83 kg ha^{-1} by WS compare to CM and SD. The higher total N uptake of 172. 62 kg ha^{-1} was at rate of 8 t ha^{-1}. Similar trend was shown by total P uptake, the type of amendments had higher p uptake of 18.16 kg ha^{-1} by WS and at rate of 8 t ha^{-1} the higher total p uptake was 21. 61 kg ha^{-1}.

3.4 Soil Water Content at Field Capacity, Wilting Point and Plant Available Water

Data as depicted in Figure 1 showed that at field capacity the higher water content of 35.4 % in WS 8 t ha^{-1} was found and each control had minimum water contents. The means of amendments showed higher water contents at field capacity (32.1 %; SD ± 3.9) in WS and means of rates had higher water content of 33.1%; SD± 2.0 at 8 t ha^{-1}. The higher water contents at wilting point were 22.5 % in WS 8 t ha^{-1}. The amendments mean had higher water contents (22.5 %; SD ± 0.91) at WS and means of rates had higher water content of 21.7 % ; SD ± 0.7 at 8 t ha^{-1}.

The higher PAW of 13.25 % was observed in WS 8 t ha^{-1}. The means of amendments showed higher water contents (10.5 %; SD ± 0.91) at WS followed by SD. Among rates 8 t ha^{-1} showed higher PAW of 11. 6 %; SD ± 0.7.

3.5 Soil structure Stability

Data (Fig. 2) revealed that the interaction among rates x types of amendments significantly increased soil structural stability (%). The WS 8 t ha^{-1} had resulted in the highest soil structural stability (90.78%) followed by CM 8 t ha^{-1}. i.e. 88.5 %. The

Table 4. Effect of compost, wheat straw and sawdust on concentration and uptake of nutrients

Rates of organic amendments (t ha^{-1})	Types of organic amendments			Means
	Compost	Wheat straw	Sawdust	
Tota N (g kg^{-1})				
0	0.55^{e}	0.54^{e}	0.66^{de}	0.58^{c}
4	0.89^{bc}	0.74^{cd}	1.07^{a}	0.90^{a}
8	0.92^{ab}	0.79^{bcd}	0.73^{d}	0.81^{b}
Means	0.79^{b}	0.68^{b}	0.82^{a}	
P (mg kg^{-1})				
0	7.24^{d}	7.30^{d}	7.34^{d}	7.29^{c}
4	8.33^{bc}	8.09^{c}	8.37^{bc}	8.26^{b}
8	10.08^{a}	8.81^{b}	8.06^{c}	8.98^{a}
Means	8.548^{a}	8.07^{b}	7.92^{b}	
Total N Uptake (kg ha^{-1})				
0	97.80^{e}	92.00^{ef}	91.31^{f}	93.70^{c}
4	143.29^{d}	177.66^{a}	171.30^{b}	164.08^{b}
8	177.09^{ab}	179.82^{a}	160.95^{c}	172.62^{a}
Means	139.39^{b}	149.83^{a}	141.19^{b}	
P uptake (kg ha^{-1})				
0	11.47^{e}	10.75^{e}	10.28^{e}	10.83^{c}
4	15.33^{d}	21.12^{b}	18.12^{c}	18.19^{b}
8	23.38^{a}	22.62^{a}	18.84^{c}	21.61^{a}
Means	16.73^{b}	18.16^{a}	15.75^{c}	

The different letters in columns are statistically significant at $P \leq 0.05$

amendment types had soil structure stability of 75.49 % ($P \leq 0.05$; SD ± 17) with WS and rates of amendments showed higher soil structure stability of 87.27 % (SD ± 4.3) at 8 t ha^{-1}. The statistically lower soil structure stability was found in non amended soils.

3.6 Physico-chemical Properties

The response of soil physico-chemical properties of different amendments applications is shown in Table (5). At 0 rate, all plots (without amendments) had higher pH compared to amended ones (Table 5). The interaction of rates x amendments had a lower soil pH (6.41) with CM 8 t ha^{-1} while type of amendments had pH 6.78 with SD and rates of amendments showed lower pH compare to no amended soil.

The highest percentage of OM 2.462 % was recorded for WS 8 Mg ha^{-1}. Meanwhile CM 8 t ha^{-1} and SD 8 Mg ha^{-1} had also resulted in a slight increase in OM i.e. 2.387 % and 2.364 %, respectively. The lowest OM was recorded for WS0 that was 1.423 %. The means of amendment types showed higher OM of WS followed by CM and SD. However, the rate 8 t ha^{-1} showed higher OM (2.41) compared to 4 t ha^{-1} and no amended soils.

The lower BD of 1.073 Mg m^{-3} was observed in WS. The amendment types showed lower BD with CM and it was statistically similar to WS. The no amended soils had a higher BD (1.23 Mg m^{-3}) than amended ones. The lower PD (2.20 Mg m^{-3}) was

Table 5. Effect of compost, wheat straw and sawdust on post-harvest soil physico-chemical properties

Rates of organic amendments (t ha^{-1})	Types of organic amendments			Means
	Compost	Wheat straw	Sawdust	
pH				
0	7.10	7.13	7.18	7.14^{a}
4	6.51	6.68	6.63	6.61^{b}
8	6.42	6.58	6.52	6.51^{c}
Means	6.68^{b}	6.80^{a}	6.78^{a}	
Organic matter (%)				
0	1.43^{g}	1.42^{h}	1.43^{h}	1.43^{c}
4	2.25^{e}	2.27^{d}	2.22^{f}	2.25^{b}
8	2.39^{b}	2.46^{a}	2.36^{c}	2.40^{a}
Means	2.02^{b}	2.05^{a}	2.00^{c}	
Bulk density (g cm^{-3})				
0	1.21^{ab}	1.24^{a}	1.22^{ab}	1.22^{a}
4	1.09^{ef}	1.12^{de}	1.18^{bc}	1.13^{b}
8	1.12^{def}	1.07^{f}	1.15^{cd}	1.11^{b}
Means	1.14^{b}	1.15^{b}	1.18^{a}	
Particle density (g cm^{-3})				
0	2.40^{a}	2.39^{a}	2.38^{a}	2.39^{a}
4	2.36^{ab}	2.367^{ab}	2.26^{c}	2.32^{b}
8	2.27^{c}	2.32^{b}	2.20^{d}	2.26^{c}
Means	2.34^{a}	2.36^{a}	2.28^{b}	
Pore space (%)				
0	48.18^{f}	49.16^{def}	49.10^{ef}	48.82^{b}
4	52.33^{ab}	50.04^{cde}	51.917^{ab}	51.43^{a}
8	53.05^{a}	50.77^{bcd}	51.35^{bc}	51.72^{a}
Means	51.19^{a}	49.99^{b}	50.79^{ab}	

The different letters in columns are statistically significant at $P \leq 0.05$

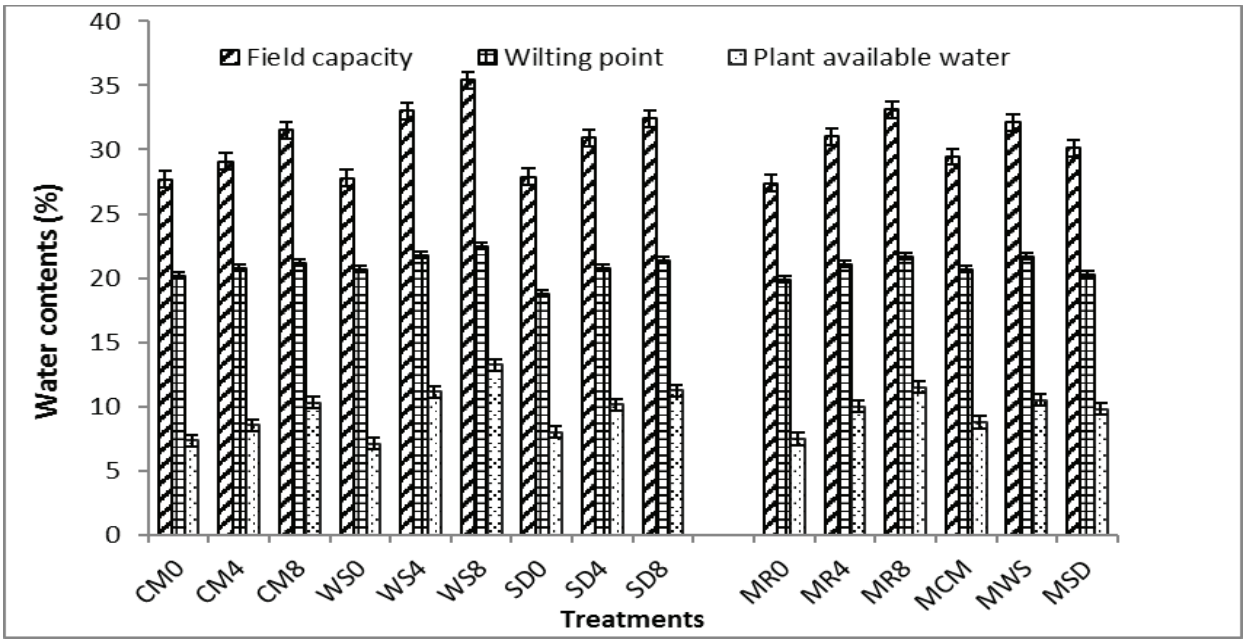

Fig. 1. Effect of compost, wheat straw and sawdust on soil water contents at field capacity, permanent wilting point and plant available water.
CM=Compost; WS= wheat straw and SD= sawdust. 0,4,8 t ha^{-1} are rates (R) of CM, WS and SD; M= means

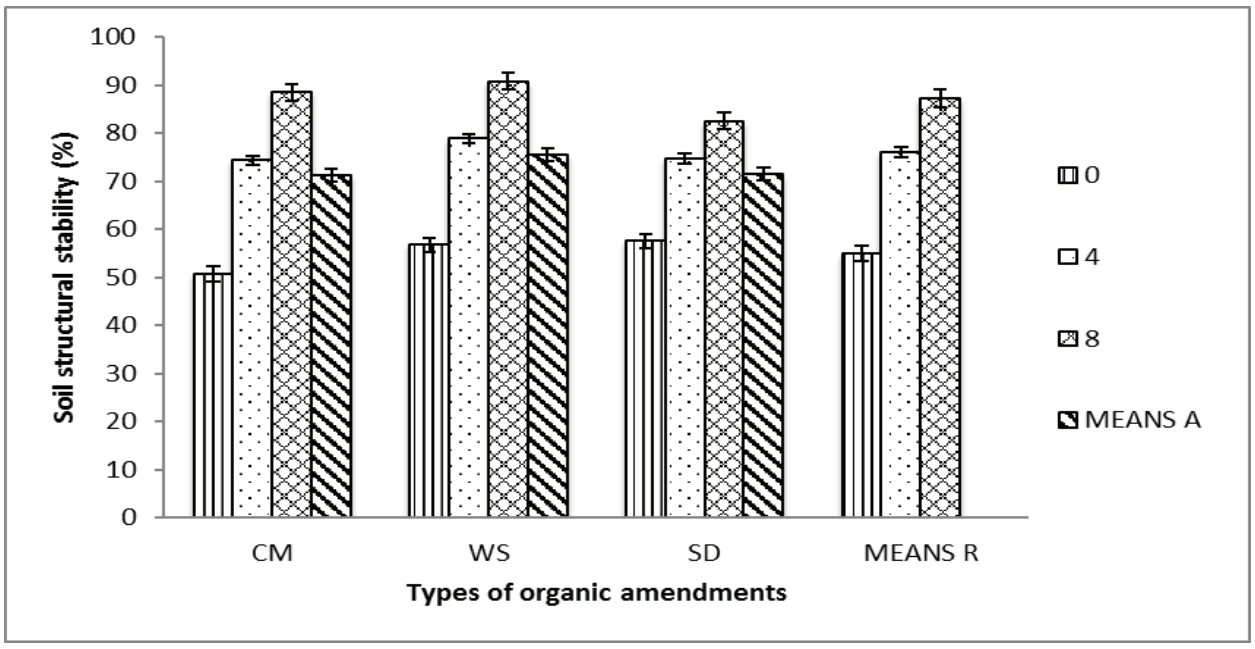

Fig. 2. Effect of compost, wheat straw and sawdust on soil structure stability.
CM=Compost; WS= wheat straw and SD= sawdust. 0,4,8 t ha^{-1} are rates of CM, WS and SD; Means R= means of rates of amendments; Means A= means of types of amendments

observed in SD 8 t ha^{-1}. The means of amendment types showed lower PD of 2.28 Mg m^{-3} followed by CM and WS. However, the means of rates showed PD of 2.26 Mg m^{-3} with a rate of 8 t ha^{-1} followed by 4 t ha^{-1-1}.

Soil porosity also positively affected in response of treatments. The lowest soil porosity (48.18 %) was recorded in CM_0 at par with soil porosity in WS0 (49.10 % and SD_0 (49.16 %). The highest soil porosity (53.04 %) was found in CM 8 t ha^{-1} statistically similar with CM 4 t ha^{-1} (52.33 %) and SD 4 t ha^{-1} (51.91 %). The means of amendment types showed lower porosity of 49.99 % with WS. However, the means of rates showed lower porosity of 48.8 % with no amended soils compare to amended soils.

4. DISCUSSION

The organic amendments stimulate plant growth and yield by providing essential plant nutrients and improved soil properties [15, 16]. The three organic amendments increased crop growth while rates of the amendment also showed higher growth (Table 2) compared to no amended soils. The performance of co-applied fertilizers might have increased with organic amendments. Therefore, organic amendments could be a good strategy to sustain and improve yield in the long term [38]. The highest yield (Table 3) in organically amended soil could be due to supplementation of essential nutrients and improvement of soil physical properties. Previous studies showed that organic inputs had recovered degraded soils by sustaining soil properties [39, 40]. Studies showed that organic amendments lower the soil compaction by improving water penetration and aeration [41]. The higher content of total N and P in amended soils (Table 4) shows the decomposition of organic material during growing season of crops. The organic wastes were found to increase the availability of NO_3-N in soil [15, 42]. The positive response of waste on plant growth was also reported [43]. Wei *et al*. [23] reported that soil with straw incorporation had higher available K, available P, available N, SOC compared to no amended soils. Our results also showed higher nutrients and organic matter in amended soils. Similarly, the amendment compost was found to improve not only soil properties but also has improved crop production and quality [19]. Compost has significant amounts of macro nutrients, and have beneficial effects on the plant-soil syetem [44, 45]. Therefore, compost can be termed as multi nutrient organic fertilizer [46].

Total N and P uptake (Table 4) were higher in wheat straw amended plots and 8 t ha^{-1} showed higher uptake than 4 t ha^{-1} and no amended soils. It might be attributed to higher yield in wheat straw amended plots. Straw incorporation into the soil has improved the soil physical and chemical properties [47]. It improves soil structure [48-50] and also increased use efficiency of nutrient [51-53]. The post-harvest soil properties (Table 5) showed lower pH with amended soils and it is attributed to release of organic acids as organic material decomposed and this release was higher in 8 t ha^{-1}. The higher organic matter content with wheat straw showed higher decomposition rate compare to other amendments. Straw enhances soil organic matter [54] and also provide soil nutrients, thus improve the soil quality and increase the soil productivity.

The organic amendments by improving the status of soil OM had also decreased soil BD and improved soil pore space. The particle density was also found lower due to impact of organic matter accumulation in amended soils (Table 5). Eldridge *et al*. [5] showed increase in SOC following compost additions. The organic amendments had increased SOC by 49% compared to non amended and 29% compared to an inorganic fertilized control [4]. Blanchet *et al*. [55] reported higher SOC with organic inputs compared to inorganic inputs. Comparable results were reported by Nest *et al*. [56]. Most of the studies [57-59] revealed same results as present study showed that the addition of manure has reduced BD, increased porosity and SOC and consequently overall improved soil mechanical properties.

Among the soil amendments, the crop residue retention and organic manure are important for improving soil quality. In eroded soils of Rawalakot Azad Jammu and Kashmir the indigenous organic amendments have the potential to improve soil structure stability, water retention and OM. Environmental changes that result in increases in soil OM will increase available water contents [60]. The higher plant available water in amended soils with higher rates of residues (Figure 1) could be

due to improvement of soil physical conditions. The organic amendments had improved soil physical fertility by decreasing soil BD and by enhancing soil aggregate stability [61, 62]. The organic amendments have also improved water contents at field capacity and wilting point [63]. Liang *et al*. [64] reported that in long term study corn straw in combination with inorganic fertilizer had significantly increased soil water content in the plow layer, reduced soil bulk density, and increased plant available water contents in the top soil. Higher soil structure stability was observed in amended soils and with 8 t ha^{-1} (Figure 2) that could be due to higher OM. Soil OM enhances soil aggregation and the development of soil porosity. It further improves the rate of soil infiltration and retention of water that is ultimately available to plants.

5. CONCLUSION

Organic amendments improved soil OM status, which is contributing factor for improving physico-chemical properties of soil and associated soil productivity. The indigenous organic amendments have the potential to improve the poor physical condition, water retention and yield of degraded soils. The three types of amendments increased yield compared to no amendments. There is the need to apply organic amendments in degrades soils to enhance both sustainability and productivity.

6. CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Volume 58, No. 3, September 2021 **Page**

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