Special Issue September 2019

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

ISSN Print: 2518-4261
ISSN Online: 2518-427X
Vol. 56(3), September 2019

PAKISTAN ACADEMY OF SCIENCES
ISLAMABAD, PAKISTAN

PAKISTAN ACADEMY OF SCIENCES

Founded 1953

Proceedings of the Pakistan Academy of Sciences, published since 1964, is quarterly journal of the Academy. It publishes original research papers and reviews in basic and applied sciences. All papers are peer reviewed. Authors are not required to be Fellows or Members of the Academy, or citizens of Pakistan.

Annual Subscription: **Pakistan:** Institutions, Rupees 2000/- ; Individuals, Rupees 1000/-
Other Countries: US$ 100.00 (includes air-lifted overseas delivery)

HEC Recognized, Category X; PM&DC Recognized

Published by **Pakistan Academy of Sciences**, 3 Constitution Avenue, G-5/2, Islamabad, Pakistan
Tel: 92-5 1-920 7140 & 921 5478; **Fax:** 92-51-920 6770; **Website: www.paspk.org**

Printed at **PanGraphics (Pvt) Ltd.,** No. 1, I & T Centre, G-7/l, Islamabad, Pakistan
Tel: 92-51-220 2272, 220 2449 Fax: 92-51-220 2450 E-mail: pangraph@gmail.com

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Proceedings of the Pakistan Academy of Sciences:
B. Life and Environmental Sciences 56 (3): 1–9 (Special Issue, 2019)

ISSN: 2518-4261 (print), ISSN 2518-427X (online)

Pakistan Academy of Sciences

Review Article

Therapeutic and Adverse Effects of Commonly Used Medicinal Plants: Standardization and Quality Assurance

Rizwan Faisal[1], Laiyla Shinwari[2], Iram Aziz[3], and Ali Talha Khalil[4]

[1]Rehman Medical College, Peshawar
[2]Goverment Maternity Hospital, Peshawar
[3]Cantt Military Hospital, Rawalpindi
[4]Qarshi University, Lahore

Abstract: The use of medicinal plants has witnessed an upsurge because of a general perception of being economical, effective and safe relative to allopathic medications. However, converging evidence suggests unwanted allergic reactions of herbal preparations and also toxic fatal reactions in the body signifying need for the extensive toxicity assessments. Moreover, some adverse reactions can stem from the contamination of herbal drugs which is attributed to the lack of standardization and quality control of herbal drugs. Contamination of metals, microorganisms and false identification can also end up in causing toxicity and allergic reactions which demand the dire need for pharmaco-vigilance to promote safe use of herbal preparations. In this paper, we have presented a review of literature on the toxicity profiles of most commonly used medicinal plants and presented valuable recommendations to allow safe use of the herbal medicines.

Keywords: Medicinal plants, Allopathic, Allergic Reaction, Herbal Medicines, Toxicity Profiles.

1. INTRODUCTION

Plants play an integral role in the environment and have a close association with the human civilization. Plants are used for food, clothing, shelters flavors, and fragrances and also taken in a form of medicines. Herbs or herbal preparations represents first generation of therapeutic medicines which were used to cure human ailments since time immemorial [1]. Herbs have been an integral part of traditional medicine and their use is documented from at least 5000 years [2]. Folkloric medicines are either complementary or a main source of medical treatment for about 75%–80% of the world population and used mostly in under developed and developing regions [3]. In some countries these traditional practices have been integrated through regulations into mainstream health systems. The herbal industries across the world like Traditional Chinese Medicines, Ayurvedic Medicines, Greek Medicines and Greeco-Islamic Medicines are continuously expanding [4]. In spite of their medicinal uses, several plant species contains phytochemical which are poisonous; however, they are still used for therapeutic purposes in traditional or folkloric medicines. Mostly the consumers, in general have a strict narrative about the herbal medicines (HM) for being a safer option with no side effects as being made entirely of natural ingredients.

The assumption that 'natural' can be equated with 'safe' is certainly an important factor but misleading [5]. A number of plants can be poisonous and may cause toxicity [6]. These toxicity issues remained unnoticed for thousands of years, however, with the growing scientific literature has revealed toxicity of certain herbs or their preparations manifested in different ways such as hepatotoxicity, nephrotoxicity, neurotoxicity, cardiotoxicity etc. The advantages of the herbal treatment are considerable, however, their unregulated use can have severe consequences. These herbal medicines usually contains potent

Received: Feburary 2019; Accepted: September 2019
* Corresponding Author: Rizwan Faisal; rizwan.faisal@rmi.edu.pk

and bioactive extracts of herbs containing complex phytochemistry and pharmacologically active ingredients causing varying degree of side effects [6]. Secondary metabolites of plants are not benign as these has evolved as a part of chemical defense mechanism intended to poison, repel, stun, or kill other species. Many of the herbal preparations have reported to cause hepatitis, but identification of particular component causing the disease is difficult to identify [7]. Medicinal plants have a broad action on physiology of the body which sometimes can be adverse [8]. Thus, the narrative of plant extracts being safe is misleading and signifies the need of intensive research on the safety and interaction aspects of plant based medicines.

Therefore, it is imperative to conduct the ethno-toxicological assessment of the medicinal plants, herbal medicines or their preparations in order to identify potential and possible side effects, optimize dosage, identify contamination and separate poisonous plants. The toxicity assessments are directed towards separating medicinally valuable plants from poisonous plants and develop such combinations that are effective and possess no side effects or toxicity. Furthermore, standardization and quality control is important to control the potential contamination and prepare product of highest quality and standard.

The aim of this article is to provide an overview and critical evaluation of evidence from systematic reviews (SRs) and literature of the adverse effects (AEs) associated with the use of herbal medicines [9]. It is important to remember that it does not attempt to identify or define all AEs (Adverse Effects) of HM products: in many cases, probable AEs have been implicated but were not documented in a SR. Electronic literature searches were conducted in February 2019 to identify adverse effects of HMs used in any type of clinical condition. The following electronic databases were used: Google scholar, Cochrane and Pak Medi Net. In addition, the problems associated with the standardization and quality assurance of herbal medicine manufacturing are discussed.

1.1 Ginger Rhizome (*Zingiber officinale)*

Ginger rhizomes *(Zingiber officinale*), is a well-established medicinal plant in the folkloric scriptures and used for the treatment of infectious diseases, indigestion, arthritis, rheumatism, fever, vomiting, hypertension etc. Ginger is used as an antiemetic by 80% of pregnant women to treat nausea and vomiting in the early stages of pregnancy. Mostly studies revealed that the powdered ginger does not possess any toxic repercussions and side effects [10]. In a clinical trial study conducted on concentrated extracts of ginger revealed adverse gastrointestinal effects. The 65% of the enrolled responders reported eructation, dyspepsia or nausea. Diarrhea, heartburn and irritation of mouth also reported [11]. Some of the *in vitro* studies on ginger has indicated the inhibition of thromboxane synthesis and thereby, retarding platelet aggregation and increasing bleeding time [12]. Prolong bleeding time has adverse implications in injuries and in pregnant women [13]. But certain studies have revealed that consumption of ginger in pregnancy not only increases the risk of fetal death and reabsorption but it also impairs the normal process of implantation [14].

1.2 Quina *(Cinchona pubescens)*

Cinchona is also known as quina and red cinchona. It has been used in folk medicines for many uses like increasing appetite, improving digestion, treatment of bloating and other stomach problems. It is also used as antiarrhythmic, appetite stimulant, antipyretic and hepatoprotectives [15]. The bark of the plant is a rich source for quinine a popular antimalarial drug. Therefore, *Cinchona pubescens* is cultivated in tropical regions for the isolation of quinine. In spite of being effective in malaria, Cinchona bark has an unacceptable risk of toxicity resulting from the overdoses of quinine [16]. Quinine is linked to various biological side effects like allergic reactions, cardiotoxicity, anaemia, hypoglycaemia, cinchonism (headaches, visual problems, tinnitus, dizziness decrease in hearing acuity, diarrhea, nausea). These side effects mostly stems from the lack of optimization in dose of the quinine [17-19].

1.3 Ajwain *(Trachyspermum ammi)*

Ajwain *(Trachyspermum ammi)* is among the widely used and highly valued medicinal plant. It is an annual herb and is widely distributed in Pakistan.

It has been used in the treatment of digestive disorders and possess established antioxidant and neuro protective nature [9, 20, 21]. Ajwain is also reported to have aphrodisiac and diuretic nature [22]. It also owns antispasmodic and carminative properties and is used for flatulence, diarrhea, digestive stimulant, abdominal tumors, piles, reflux, hepato protective, nausea, vomiting, abdominal cramps, lack of appetite, hypolipidemic, asthma, antitussive and amenorrhea etc. [23-26]. On the other hand, toxicity of the plant is also reported. The oil of Ajwain was found to be moderately toxic [27]. But when it is taken in larger amounts it may result in allergic reactions, stomach ulcers, and liver and heart problems. Ajwain also indicated teratogenicity in rat fetuses. Therefore, its intake during pregnancy may result in adverse effects [26]. Furthermore, there is a risk of bleeding and bruising when taken in combination with certain other drugs. *T. ammi seeds can cause hepatotoxicity when it is supplemented with other herbs like Bishop›s* weed. It can also effect the platelet aggregation when taking in combination with herbs like garlic, ginkgo, turmeric etc. Headache and nausea are also reported due to overdose [28, 29].

1.4 Kinnikinnick *(Arctostaphylos uva-ursi)*

Arctostaphylos uva-ursi (L.) has gained significant importance in traditional medicine especially for the treatment of lower urinary tract infections[30]. Reports revealed the antimicrobial and antiseptic properties which are attributed to tannins and hydroquinones in the plant [31]. It is also reported to have significant amount of antioxidants [32]. It is also recommended for the treatment of dermatitis [33]. Clinical trial studies suggested some side effects of using the *uva-ursi*. Gastrointestinal problems are reported in some studies. Some researchers have reported dermal effects to the prolonged hydroquinone exposure found in *Arctostaphylos uva-ursi*. Blurred vision due to bull's-eye maculopathy is reported due to the prolong use of *uva-ursi* [34] Studies in rats indicated nephrotoxicity. [30, 35]. Stone formation is also reported after the use of *uva-ursi* [36].

1.5 Chamomile *(Matricaria chamomilla L.)*

Chamomile (*Matricaria chamomilla* L.) belongs to the family Asteraceae and often referred to as the "star among medicinal species"[37]. Its traditional uses have been documented in the ancient folkloric scriptures. It has been recommended in hysteria, flatulence, intermittent fever and colic. It is used as antiseptic, anti-inflammatory and antispasmodic. Some of the common uses are improving digestion and treating diarrhea, hemorrhoids, urinary tract problems, oral mucositis, shingles and painful menstruation [38-40]. Chamomile tea (CT)has stress relieving and nerve relaxant properties and has been used to remove inflammations, pain and to cure rheumatic disease [2, 41]. Conjunctivitis due to the external administration of Chamomile tea is reported as pollen of *M. chamomilla* may induce allergic reactions [42]. In addition, the use of chamomile taken with warfarin caused multiple internal hemorrhages [43]. The phytochemical nature of the Chamomile can led to various drug interactions. Chamomile may increase the effects of opioid analgesics that can cause CNS depression / sedation [44]. Regular intake of Chamomile can alter absorption of other drugs.

1.6 Ginseng *(Panax ginseng)*

Panax ginseng has been used in folk medicines since 2000 years. Word "Panax" corresponds to "all healing", which signifies the potential diverse therapeutic uses of this wonderful plant [45]. *P. ginseng* possess diverse pharmacological activities like being rich in antioxidants, possess anti-aging, hypolipidemic, hepatoprotective, anti-fatigue, anticancer, homeostasis and antihypertensive [46-48]. It is also used to improve the cognitive functions and aphrodisiac. It is also used as a general tonic for wellbeing. Ginseng based medicines are top selling herbal medicines [47]. In one of the study conducted on consumption of ginseng in pregnant women revealed increase up to 3 folds in gestational diabetes [49]. Moreover endocrinological manifestations like mastalgia and postmenopausal bleeding are reported with ginseng use which stopped after discontinuing the intake of ginseng [50]. Tachycardia and hypertension is also reported as a potential adverse effect of ginseng use [48]. Prolong use of ginseng may cause effects like diarrhea, skin eruption, insomnia, sleeplessness, nervousness, edema and decreased appetite [51]. Drug interactions of ginseng with imatinib in a leukemia patient resulting in liver toxicity is reported [52]. Potential interaction of ginseng with

warfarin is reported [53, 54]. Ginsenosides which are the active component of ginseng has the ability to inhibited platelet aggregation and therefore not recommended for patients undergoing surgical treatments [55-57].

1.7 Garlic *(Allium sativum)*

Commonly known as "garlic", *Allium sativum* has different uses in food and medicines. Pharmacognostic studies reveals diverse properties of garlic like antiviral, antimicrobial, anti-mutagenic, antihypertensive, anti-platelet, glucose lowering, antithrombotic etc. It is also used to treat hypercholesterolemia, cardiovascular diseases, dementia, hypertension, arteriosclerosis etc [58, 59]. On the other hand, the consumption of garlic can also cause numerous adverse effects. Orthostatic circulatory problems, acute myocardial infarction, food allergy has been described in some case studies as potential adverse effects of garlic [60-62]. Small intestinal obstruction, esophageal and epi-gastric pain, hematochezia and hematemesis, nausea, blotting and other gastrointestinal problems are reported [59]. Another case study revealed allergic dermatitis in patient who used garlic powder for treating hyperlipideamia. Urticaria, Angiedema another form of skin and mouth allergies are s also reported [63-65]. For pregnant and lactating mothers, the consumption of garlic should not exceed the doses normally used as in food. doses of garlic greatly exceeding amounts used in foods should not be taken during pregnancy and lactation [59]. Herb-drug interactions are common to garlic. Ritonavir an antiviral drug can manifest gastrointestinal toxicity, when administered with garlic supplements. Other antidiabetic and analgesics drugs can also interact with the garlic supplements [66, 67]. Mouth burns, upset stomach nausea, lightheadedness are some of the other concerns after ingestion of high amount of garlic [68].

1.8 Warmwood *(Artemesia absinthium)*

In local traditions *Artemesia absinthium* is used as febrifuge, anthelmintic, stomachic antiseptic, cardiac stimulant, antispasmodic, improving nervous and liver functions. In folkloric literatures, it is also described for neurodegenerative and ulcerogenic disease, dysentery and cancer [69]. *Artemesia absinthium* is known for the preparation of Absinthe (liqueur) that which has been banned due to toxic properties, resulting in hallucinations, mental disturbances, pyschosis, digestive disorders, delirium, vertigo, thirst, dyspepsia, biliary dyskinesia and paralysis etc. Their use for lactating mothers is strictly prohibited [68].

2. STANDARDIZATION & QUALITY ASSURANCE OF HERBAL MEDICINES

2.1 Authentication of Raw Material

Authentication of herb by the collector is the foremost and important step in the development cycle of herbal medicine preparation. Mostly, herbs are collected by the local people who actually are not trained enough, neither experts in identification of plant material. Sometimes, the plant material show striking similarities that makes taxonomic verification difficult for experts. The quality of raw material plays important role in the quality of the finished product. The authentication of the particular herb should be based on different tiers of identification. Authentication cycle should be based on verification of plant material on macro morphological features, microscopic level and molecular level. Therefore, a holistic approach is required for the correct identification of medicinal plant. Microscopic level identification can be performed by studying pollen morphologies through high resolution imaging techniques. In molecular approaches, DNA barcoding is applied as an efficient technique for identification of the raw material. DNA barcoding utilizes specific gene regions and identify the biological specimen based on conserved sequence data. The commonly used DNA markers or regions that are used in DNA barcoding are *rbcL, matK, trnh-psba*, or their combination [70, 71].

DNA barcoding technique can also be used to detect any adulterant plants in the finished product. In a recent study, different herbal preparations were tested to examine if the finished product contained the same herb as prescribed on the product labels. The examination revealed that 59% of the herbal medicines did not contained the exact herb written on the product label [72]. Herbal industries are suffering adversely due to the lack of proper quality control measures compromising the quality of herbal medicines. Product substations and

adulations are frequent in herbal industries which can be controlled using DNA barcoding.

2.1.2 Metal & Metalloids Adulterants

Healing and therapeutic properties of medicinal plants and herbs are attributed to their phytochemical components and minerals, however, plants may not only contain these. Several of non-essential minerals, toxic pollutants and heavy metals may be present in the plant [44]. Plants usually growing on the road sides, and those collected from polluted areas tends to accumulate heavy metals and other toxic substances. Furthermore, in cases where plants are grown in clean environment but the water reservoir is polluted with industrial effluents, and pesticides have also revealed accumulation of such toxic materials, which after ingestion can have lethal effects [73-75]. As, Ni, Pb, Al and Cd occurring in the environment can accumulate in both plants and human organs and are categorized as the most common heavy metals causing pollution by the Environment Protection Agency [76, 77]. In one of the recent article about herbal medicine market in Jordan, authors investigated herbs species for presentence of different heavy metals. Among the assessed plants, Pb (lead) was found beyond acceptable limits in one of the herb *Satureja thymbra*, also known as Persian thyme [77]. Similarly, there are different reports on the detection of pesticides like pendimethalin, carbendazim, procymidone, phoxim along with banned pesticides herbs chlorpyrifos and aldicarb [77]. The presence of such toxic and innocuous chemicals can cause server side effects and their long term exposure can be deadly. Some of these metals like As, Cd, Pbetc can cause cancer, poisoning, dermatitis, heart shock, kidney damage, osteoporosis etc [78]. Different strategies can be applied to overcome contamination like extensive clean-up steps, dilution, more selective detection techniques.

2.1.3 Microbial Contamination

One challenging aspects for the herbal industries are to meet proper and standardized biosafety assessments to check the potential of microbial contaminants in its products. Recently, one of the herbal manufacturing company (Herbalife®) came under the critics when different from different regions, there were reports of Herbalife® associated liver injury, initially reported in Israel, and afterwards Spain, Switzerland, Iceland, Argentina, and the United States [79]. Interestingly, no significant contamination with pesticides or heavy metals were found out, however, contamination with *Bacillus subtilis* in high amount having potential to cause hepatotoxicity was identified. Other reports, later on confirmed the presence of heavy metals and pathogenic microbes like *Streptococcus, Escherichia, Acinetobacter, Klebsiella*. Both the heavy metals and pathogens were identified as a cause of liver toxicity resulting from Herbalife® products [80-82].

2.2 Optimization of Dose & Phytochemistry

The adverse reactions from herbal medicines may arise from the particular herb and dosage but sometimes the lack of proper standardization of herbal medicines. Some medicinal plants if taken in lesser amounts can manifest their medicinal potential; however, at high doses they become poisonous. *Symphytum officinale* and *Corynanthe yohimbe* which are regarded as medicinal, aphrodisiac and dietary supplements can be lethal if taken in higher amounts [83]. The optimization of the dosage of herbal medicines should be carried out through ethno medicinal, pharmacological and clinical approaches, under different conditions, and on different samples. Some of the adverse effects of herbal medicines are reported if there is already a preexisting disease in the body. With the changing physiology of the body, the response to a herbal preparation can be different. Before using a medicinal plant preparation one has investigate the effective dose, ineffective doseand overdose.

The therapeutic potential of the medicinal plant is due to its phytochemistry which signifies the need of a standardized phytochemistry of plants. The phytochemistry of a same medicinal plant can change significantly with the change in environments and in response to different stress. At present, in most of the herbal industries, in the developing regions the phytochemical standardization of a herbal preparation is negligible. Plants are collected from different phytogeographical regions having a different phytochemical profile, thus hereby, compromises the quality of the product. Similarly, the phytochemical profiles changes with the change in the growing stages of the plant. Therefore, there is increasing interest in the tissue

culture based approaches like micro propagation to cultivate medicinal plants with relatively standardized phytochemistry. This also signifies the need of understanding the rich phytochemistry through efficient analytical techniques like HPLC fingerprinting.

3. CONCLUSION

Herbal medicines play a very important part in fulfilling the health vacuum since ancient times. A massive chunk of human population relies on the folkloric traditions for treatment. The narrative of herbs being always safe is misleading that signifies the need to significant research on the safety and efficacy of herbal medicines. It's difficult to separate a medicinal plant from potentially poisonous plants. Sometimes, the adverse effects stems from the lack of quality control measures. False identification, adulteration, heavy metal contamination, herb drug interaction are some of the causes of adverse effectsand compromises the quality of herbal medicines. Research on the standardization procedures, pharmacology, drug interactions, dose optimization etc. will significantly help in reducing the risk with the use of herbal medicines. In addition, good manufacturing practices must be at the core of any herbal industry. A holistic approach is required to tackle the industry related issues. Policy measures and stringent laws for the registration of herbal medicines can be made so that herbal products with unlabeled and uncharacterized ingredients, inconsistent standard manufacturing can be dealt with.

4. Conflict of interests

Authors declare no conflict of interest.

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Proceedings of the Pakistan Academy of Sciences:
B. Life and Environmental Sciences 56 (3): 11–15 (Special Issue, 2019)

ISSN: 2518-4261 (print), ISSN 2518-427X (online)

Pakistan Academy of Sciences

Review Article

DNA Barcoding of Herbal Medicinal Products: A Challenging Task

Nadia Batool Zahra

Qarshi University, Lahore, Pakistan

Abstract: There is a global resurgence of traditional and complementary medicine, specifically the herbal products have been booming for the last few decades. However, the events of substitution and adulteration of herbal drugs/ medicinal products is an increasing concern for consumer safety. The prevailing situation of adulteration highlights the dire need of an effective scientific method for improved precision while carrying out the correct identity of the medicinal flora and their herbal products. DNA barcoding has come out as a solution for correct identification of herbs and to find the adulterants in herbal products. There are challenges involved in the barcoding method for medicinal plants in terms of developing barcodes and the analysis of data to measure the distinguishing power. Though, the solution to these problems is available and DNA barcoding can help to formulate a system to ensure the quality of herbal drugs which will help the pharma industry of herbs to regain the lost confidence of consumers.

Keywords: Herbal products, DNA barcodes, mini-barcodes, meta-barcoding, barcoding challenges.

1. INTRODUCTION

The herbal commodity market is expanding globally due to increased confidence in traditional healthcare system. The exponential rise in the business of herbals through past decade confirms the global attention towards the herbals and the associated traditional healthcare [1]. However, it has also been observed that consumer faith is damaging due to adulteration and substitution of herbal drugs. The consumer health is at risk due to the substitution events where the original herbal species is replaced by a non-medicinal plant. Similarly, the addition of fillers which are not labeled decreases the therapeutic potential of the herbal pharmaceuticals [2]. The traditional system for identification of plants is based on morphological characters which cannot typically be used for processed plant material or powdered form. The commercially available technologies employed for validation of herbal products are based on physical, chemical, biochemical analysis and recently developed molecular analysis and tools (DNA dependent) [3]. DNA is found in all tissues, less degradable and more resistant to external factors therefore, the DNA based identification is more reliable in contrast to RNA and proteins [4]. Hebert et al., [5] proposed DNA barcoding for correct identification of existing species and to unearth the new species. A standardized region of DNA (<1000 bp) called as DNA barcode is used in this method. DNA barcoding provides a simplified solution to the complex problem of correct identification of raw herbal material/medicinal products and assures a significant quality check within the market of herbal products [6]. More recently DNA barcodes have been included in pharmacopoeias, providing tools for regulatory purposes [7]. The study highlights the necessity for quality control of the marketed herbal products and shows that DNA metabarcoding is an effective analytical approach to authenticate complex multi ingredient herbal products [8]. However, there are challenges being faced in generating the barcodes and the analysis of data for estimating the distinguishing power of these barcodes [9].

2. LIMITATIONS AND CHALLENGES OF DNA BARCODING IN HERBAL PRODUCTS

2.1 DNA Extraction

There is a minimum requirement of quantity and quality of DNA to be found in herbal sample to

Received: February 2019; Accepted: September 2019
* Corresponding Author: Nadia Batool Zahra; nadia.zahra@qu.edu.pk

carry out successful DNA barcoding. Several studies showed a relatively low barcode success in their work [9-12]. The diverse manufacturing methods of herbal products and the part of plant used or type of material used in the products may be the reasons of low barcode success. Naturally there are many secondary metabolites, polysaccharides, polyphenols, glycol-proteins are found in plants. Their presence can obstruct the process of DNA isolation, gene amplification and sequencing [13]. Under good laboratory practices isolation of DNA from herbals should be carried out shortly after collection of material to stay away from such storage conditions which damages DNA and where the cross-contamination of samples can occur [14]. The widely used methods of DNA extraction are cetyl trimethyl ammonium bromide (CTAB) method [15] and commercially available DNA extraction kits [16]. However, these methods and kits are not helpful in extracting DNA from those plant tissues (roots, tubers etc.) where secondary metabolites are found in high concentration. Through early stages of DNA isolation, the high amounts of polysaccharides and polyphenols must be eliminated by utilizing methods having increased concentration of CTAB, polyvinylpyrrolidone (PVP), and β-mercaptoethanol (β-Me) [17-20].

Largely the herbal products are available in the form of tablets, capsules and liquid extracts and the DNA of plant species used is either degraded or removed during the process of manufacturing, therefore, the isolated DNA from these products is either fragmented or absent. It could also hint towards the possibility of presence of excipients (fillers, binders, lubricants, diluents, pigments, stabilizers etc.) that may affect the extracted DNA or hinder the amplification of the targeted region by the primers [13]. The manufacturing processes through which extracts and tinctures are prepared involve extensive heat treatments, filtration and distillation resulting in complete removal or degradation of DNA which make these materials unsuitable for DNA barcoding [21-22].

2.2 Selection of DNA barcoding loci

In animals, the mitochondrial cytochrome c oxidase 1 (*CO1*) is considered as a universal barcode but it cannot be employed for plants based on its slow rate of evolution and limited divergence [5, 23]. The Consortium for the Barcode of Life (CBOL) suggested the combination of two locus *mat*K-*rbc*L as the universal DNA barcode for plants in 2009 as they belong to the relatively fast- evolving plastid genome. The other commonly used regions of nuclear and plastid genome are *ITS, ITS*2*, psbA-trnH, atpF-atpH, ycf*5*, psb*K-I, *psbM, trnD, nad*1*, trnL-F, rpoB, rpoC*1*, and rps*16 [2, 24-26]. Though, none of these individual plant barcodes have both discriminating regions and the regions of attachment of universal primers simultaneously. Hence, a multi-locus barcode with two or three loci in combination was proposed [24-25]. The two locus barcode of *rbc*L-*mat*K also posed some difficulties as *mat*K is problematic in amplification in some plants because of the non-conserved primer binding site of universal primers. The other recommended two locus combination was *rbc*L + *trnHpsbA* which failed to work for some of the plants due to highly variable *trnH-psbA*. High variability of *trnH-psbA* poses difficulty in the alignment of this combination. To resolve this issue, a tiered approach was put forth by Newmaster et al. [2]. The method utilizes the easily amplifiable and alignable *rbc*L region as a scaffold on which data from highly variable non-coding regions such as *ITS*2 or the *trnH-psbA* region are employed for identification of plant species.

The short length *i.e.* 200-230 bp of *ITS*2 serves as an advantage for the identification of herbal supplements. The fragmented DNA of herbal supplements may not be able to amplify 600-800 bp long barcodes. Despite of this advantage, there are disadvantages of *ITS*2 as a plant barcode includes occurrence of multiple *ITS*2 copies in the same individual, which resulted in the inaccurate identity of species based on their resemblance to the copies of the sister species. There are also technical issues in the amplification and sequencing of *ITS*2 that can happen due to occurrence of DNA from other co-existing species of plants [27-28]. The concept of "mini-barcodes" was introduced for barcoding of herbal dietary supplements through short-barcodes (< 200 bp) of standardized *mat*K and *rbc*L regions [29-30]. Mini-barcodes provide the ease of amplification for processed dietary materials along with their ability to discriminate closely similar species.

2.3 Amplification and Sequencing

The ease of amplification and use of universal primers has been a pre-requisite of DNA barcoding

method. The inherent biases in the amplification step can result in false negative or false positive results [31]. The commercially available kits for DNA isolation utilized in the initial preparation of samples, efficacy of amplification reaction itself, differences in the melting temperatures of the primers are the factors affecting the amplification success [32-33]. The balanced melting temperature of the primer pairs and the affinity between the template DNA and universal primers are both the significant factors to carry out robust amplification [34].

The presence of inhibitory secondary compounds, inactive ingredients and excipients in the herbal supplements in the form of tablets, capsules and pills hamper the PCR reactions and may result in multiple nucleotide sequences indicating the mixed DNA sample [35].

In majority of the studies, Sanger's di-deoxy sequencing [36] is the commonly used sequencing method for DNA barcoding. It generates up to 1000 bp reads of sequences, however, the limiting factors of this method includes requirement of high conc. of DNA (100-150 ng) and its low throughput [37]. The other challenge is formation of two sequencing signal patterns (electropherograms) for each sequence generated, making it un suitable for those herbal samples that contain more than one species or excipients. Presence of multiple species in a sample results in the formation of multiple or overlapping sequence peaks causing the sequencing to be failed and making the accurate determination of barcode impossible [13]. Similarly, the fungal *ITS* barcodes in multiple copies causes problems for direct method of Sanger sequencing. Molecular cloning in an appropriate microbial/bacterial host is one of the solutions for improving the poor read quality, however, cloning introduces biases against extreme base composition *e.g.*, stretches with high guanine and cytosine contents), inverted repeats, and genes not accepted by the bacterial cloning host [38].

The recently developed high throughput sequencing technique called as the Next-Generation Sequencing (NGS) has been used as an answer to issues of the Sanger's sequencing. In this method parallel sequencing of multiple DNA fragments from various DNA templates can be performed in a single reaction [39]. NGS can generate up to one million DNA sequences, 700 bp long in a single run of sequencing. The NGS is comparatively cost effective; however, the cost of bioinformatics is additional based on the huge amount of obtained data in this technique. The next-generation sequencing "meta-barcoding" method is a combination of high throughput DNA sequencing and low-throughput DNA barcoding to conduct the analysis of DNA barcodes from environmental sediments, ancient or processed samples at a mass level [40-42].

3. CONCLUSION

DNA barcoding has both the advantages and challenges. Despite the limitations, this method has its benefits when utilized in herbal industry correctly. DNA barcoding and metabarcoding have greater prospective for quality assurance of herbal products.

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Proceedings of the Pakistan Academy of Sciences:
B. Life and Environmental Sciences 56 (3): 17–26 (Special Issue, 2019)

ISSN: 2518-4261 (print), ISSN 2518-427X (online)

Pakistan Academy of Sciences

Review Article

Traditional Chinese Medicine Going Global: Opportunities for Belt and Road Countries

Muhammad Ovais[1,2*], Ali T. Khalil[3,4], Sohail A. Jan[5], Muhammad Ayaz[6], Ikram Ullah[7], Waseem Shinwari[8], and Zabta K. Shinwari[9]

[1]CAS Key Laboratory for Biomedical Effects of Nanomaterials and Nanosafety, CAS Center for Excellence in Nanoscience, National Center for Nanoscience and Technology (NCNST), Beijing 100190, P.R. China
[2]University of Chinese Academy of Sciences, Beijing 100049, P.R. China
[3]Department of Eastern Medicine and Suregry, Qarshi University, Lahore, Pakistan
[4]UNESCO Chair in Nanosciences and Nanotechnology
[5]Department of Biotechnology, Hazara University Mansehra, KP, Pakistan
[6]Department of pharmacy, University of Malakand, KP, Pakistan
[7]Qarshi Herb Research Centre, Qarshi Industries, Hattar, KP, Pakistan
[8]Allama Iqbal Open University, Islamabad, Pakistan
[9]Pakistan Academy of Sciences, Islamabad, Pakistan

Abstract: Due to Belt and Road Initiative, Traditional Chinese Medicine (TCM) a 2,000-year-old Chinese national treasure is experiencing a fresh thrive. By 2020, China aims to issue 20 TCM international standards, register 100 TCM products and build 30 overseas TCM centres in BRI (Belt and Road Initiative) countries. BRI initiative is intended to expand TCM understanding and increase exchanges between researchers and healthcare professionals, for instance, through a new hospital alliance and a health policy research network. Worldwide trade in TCM services, including clinic treatment, education and training, and health tourism is estimated to be at about $50 billion. TCM has become a vital area of health and trade cooperation between China and the ASEAN, EU, Africa, and Central and Eastern Europe. In the present scenario, BRI countries (~70) also have the opportunity to promote their own traditional and complementary medicine systems globally such as Ayurveda, homeopathy, and unani medicines. A flagship project of BRI, China–Pakistan Economic Corridor (CPEC) Pakistan is intended to rapidly modernize infrastructure of Pakistan and strengthen its economy through diverse projects with the value of ~ $62 billion as of 2017. In this scenario, it's vital for the herbal practitioner's, scientists, industry and policy makers in Pakistan to explore the opportunity given by BRI and CPEC to promote the herbal industry by forging the practices of TCM. This will result in a massive move towards the achievement of SDGs (Sustainable Development Goals) globally and will nurture herbal industries to develop on solid scientific and legal grounds.

Keywords: Traditional Chinese medicine (TCM), Belt and Road Initiative, Ayurveda, Homeopathy, Unani medicine, SDGs, China–Pakistan Economic Corridor (CPEC).

1. TRADITIONAL CHINESE MEDICINE (TCM): MYTHS AND REALITIES

Traditional Chinese medicine (TCM) is practiced in China for over thousand years. It is believed that Chinese medicines started from the time of Shen Nong, who was the ruler in ancient China and was known for his knowledge of plants. He identified hundreds of plants and also found some poisonous plants at that time [1]. Huangdi Neijing is the first record of Chinese medicines written in 3rd century BCE, which are used to day to provide the basic concept and usage of Chinese medicines. Traditional Chinese medicine is not only confined

Received: February 2019; Accepted: September 2019
* Corresponding Author: Muhammad Ovais; movais@nanoctr.cn

to medicinal plans but it is also associated with the beliefs, philosophical theories and therapies such as Chinese acupuncture exercise and diet. These methods of practices are still in use in many countries of Asia as well as in Western world.

In the era of Shang (1600 BC) the Chinese philosopher started collecting information about plants regarding the practices of TCM. At that time alcohol was introduced in the medical field as great discovery which was used as solvent for enhancing the curative effect of medication. The process of decoction was also introduced in the same era of Shang, by making liquid medicines. The earliest books in which different kind of plants were recorded with respects to its habitat, localities, medicinal and edible properties were published as "The Book of Songs" and "The Book of Mountains and rivers". However, the Chinese were greatly influence with the idea of the great philosophers Confucius and Laozi and other leaders who knew and understood the function of the body and the surrounding environment [2]. The Chinese tradition is mostly based on stories about the cultural heroes who taught the useful things to their offspring and the wider community and it passes from generation to generation orally [3]. A book "Yellow Emperor's Classic of Medicines" written by Huang Di (206 BC-220AD) is considered as the symbol of medical theories in China. Patricia B. Ebreyin her Book "Chinese Civilization (1993) quotes about the medical theory of "Yin" and "Yang". Similarly, the Chinese culture is rich of many mythical stories and belief about the TCM, its usage, identification of poisonous and non-poisonous plants. However, some healing techniques in Chinese medicines are very significant with respect to disease identification. For example, the determination of pulse rate was a significant factor for disease determination and this technique adopted by Korean, Japanese, lately spread to the Arab and Persian world and now it is considered as very important factor in the modern-day Western world [4]. Some authors have made great comparison in some techniques between China and Europe and they found that Chinese were much earlier aware of some techniques that are known to European today. For instance, Chinese acupuncture, which was developed almost 2500 years ago, attracted the attention of Western patients and medical professionals in the 1960s. Similarly, antiseptics and vaccination were first developed in China centuries ago. Moreover, "The Compendium of Materia Medica" which describes the classification of botanical was written by a Chinese Li Shizhen in 1596 about 200 years earlier than that written by Carolus Linnaeus (1707-1778) in Europe [5].

The healing of the body in TCM is based on the body's vital forces known as "Qi". According to "Qi" concept the body has two types of forces, the "active" and the "passive" which flow through channels in the body and connect different organs, tissues, cells, nerves and veins etc. When these two forces are balance the body is healthy. The disproportion in these forces causes unhealthy conditions. A TCM practitioner diagnoses the source of unbalanced condition and the specific channel involved in that particular condition. He then treats the unhealthy condition by re-activating and balancing among different organs, cell, tissue, nerves and veins etc., by stimulating these channels. The TCM uses different methods for stimulating these channels, either by physical therapy and or herbal medicines. In Chinese herbal formulation, combination of different herbs is used to alleviate the function and or vibration of the nearby organs [6]. Today the image of Traditional Chinese Medicine (TCM) is quite controversial. While some people choose to trust the practitioners of TCM and claim to be healed by it, others see it as a traditional or cultural practice based on pseudoscience, consider its practices as a sham, or even magic [7]. Myriads of tests and experiments have been carried out in an effort to find out how such practices such as acupuncture works, or how herbal tea eliminates various illness symptoms. However, scientists struggled to find a right way to test TCM's efficacy [8]. The demand of Chinese medicines is increasing in west day by day. However, acceptance, scepticism and debate in the use of Chinese medicines have been observed. It can be said that TCM is a globalized body of knowledge and practices that is practice in many countries of the world including Germany, France, Italy and the United States etc. Candelise in her research article "Chinese Medicine Outside of China" describes the complete encounter between Chinese Medical Practices and Conventional Medicine in France and Italy. In last decade the complementary medicines industry was worth 1.5-2.5 billion US dollars [9].

2. TRADITIONAL CHINESE MEDICINE (TCM): CLINICAL TREATMENT

Traditional Chinese Medicine (TCM) is commonly used in more than 140 countries for the control and treatment of many lethal diseases [10]. Various TCM approaches are used to solve many health related problems. The conventional use of TCM have very poor efficacy for many diseases. Hence, proper clinical trials are vital to improve its efficacy and biosafety against specific diseases [11]. Many trials have been conducted to check scientific evidence of various vital TCM [12, 13].

In early clinical trials, maximum enzyme-reducing and hepatoprotective activity of an important TCM herb *i.e. Schisandra chinensis* (Turcz.) Baill was reported [14]. After this discovery, many important secondary metabolites were isolated and were used against Hepatitis B (HB)[14]. Since the end of 1980 many animal liver injury model experiments were conducted by the Chinese Academy of Medical Sciences and recorded positive hepatoprotective and other biological activities of key compound (Bicyclol) [15]. The bicyclol gave strong antifibrotic and hepatoprotective effects in liver injury rats and mice model experiments. It also demonstrated strong positive affect against hepatitis in 2.2.15 cell line and duck model experiments [16]. In chronic HB patient the Bicyclol decrease HB virus replication by increasing the concentration of serum alanine aminotransferase and by minimizing the level of aspartate aminotransferase [17]. This potent drug was approved in China since 2004 for the control and treatment of chronic HB disease [17]. Similarly, another important drug *i.e.* Taxol was approved for the treatment of cancer [18]. The TCM showed positive correlation with many disease with no or minimal adverse effect [19]. Only minimal adverse effects were reported for TCM against type II diabetes indicating certain advantages in the prevention of type II diabetes [20]. The TCM can be used with other western medicine. For example TCM showed synergistic affect with insulin and used for the treatment of gestational diabetes [21].

To improve the precision and ease to access the information of clinical trials, trial registration is being required by the International Committee of Medical Journal Editor [22]. Many institutes have developed trial registries, and ClinicalTrials.gov which is freely available to anyone. It is one of the biggest registries containing approximately 200,000 clinical trials from 174 different countries including many trials on TCM [23]. Chen et al., [24] examined 1,270 TCM trials in ClinicalTrials.gov from October 2000 to September 2015. Maximum trials 970 (1270) were found for treatment purposes followed by 168 and 96 trails for prevention and other purposes. Maximum 691 (54.4 %) trials were acupuncture; while rest 454 (35.8 %) were herbal medicines. They also recorded 55.7 % small studies enrolled < 100 subjects. USA was found the second (28.3%) after China (41.5 %), which conducted more trials on TCM. Only 50 trial (8.7 %) results were reported on ClinicalTrials.gov. In addition the disease specific TCM trials were almost similar from year 2010-15. For acupuncture trials maximum trials (18.1 %) were studied for musculoskeletal system and connective tissue. While for herbal medicine trials, maximum trials (16.6 % and 15.7 %) were tested for neoplasms and other circulatory system diseases. For cite wise information, maximum trials (52.8 %) were conducted in Asia-Pacific region followed by North America (28 %) and Europe (15.4 %). A similar study was conducted by Zhang et al. [25] to check the clinical trials of TCM against Chronic hepatitis B (CHB). They summarized the results of randomized controlled clinical trials (RCTs) of TCM during 1998-2008. Their findings envisaged the effectiveness of TCM in a separate dose or in combination with other interferon or lamivudine (IFN/LAM). Their findings showed that TCM gave positive effect as that of IFN or LAM. In addition, it increase liver function and increase antiviral activity of IFN and LAM. It also helps to maintain normal serum ALT level. They also noted that many clinical trial based study published didn't include data of adverse effect. The outcome measurement is important to check the efficacy of TCM. A total of 22 outcomes have been reported in previous study. For example, a systematic review of 35 trials investigated the efficacy of TCM on chronic obstructive pulmonary diseases [12]. One other comprehensive study reported 11 key outcomes for the improvement of the post-stroke function [26]. The correct outcomes measure is important for any clinical trial [27]. Some of the common diseases treated through the TCM are indicated in Table 1.

Table 1. TCM herbs for common diseases

S. No.	Condition / Disease /	TCM Treatment	Reference
1	Atopic dermatitis	Scutellaria baicalensis, Glycyrrhiza uralensis,, Corydalis yanhusuo, Platycodon grandiflorum	48
2	Allergic rhinitis	Platycodon grandiflorum, Angelica dahurica, Fritillaria chrrhosa, Xanthium sibiricum	48
3	Virus infection	Forsythia, Folium isatidis, Scutellaria baicalensis	49
4	Parasitic infection	Betel nut, Omphalia, Artemisia annua	50
5	Cervical spondylosis	Pueraria thunbergiana, Paeonia lactiflora, Zingiber officinale, Cinnamomum cassi, Ephedra equisetina, Glycyrrhiza gkabr	51
6	Alzheimer Disease	Panax ginseng	52
7	Parkinson disease, Cardiovascular and Coronary Heart diseases	Ginko biloba	53, 54
8	Rheumatoid arthritis	Codonopsis Radix, Atractylodis macrocephalae, Salviae miltiorrhizae	55

3. TRADITIONAL CHINESE MEDICINE (TCM): EDUCATION AND HEALTH TOURISM

The Chinese medical education system has been undergoing state-mandated reform since the turn of the millennium, and with greater momentum since 2008 [28]. The health care system and medical education has been affected by these developments in which both biomedicine and traditional medicine are together delivered and administered at each level. The vital place of plurality in the health care system of Chinese is specified by the State Council's 2015 instructions on health care development, which specifies that the Chinese state will support 15 % public hospitals will subsidize by 2020 are reserved for traditional medicines. The distinctiveness of this dual-track system has been reported by many studies [29]. However, few studies reported that in clinical practice doctors that are trained in biomedicine used regularly traditional medical.

Over the past 60 years the use of TCM has increased under state administration, with medical universities, professional status and hospital systems along with the "Western medicine." Comparison of teaching TCM in Western medical schools (or biomedical) in China with the Complementary and Alternative Medicine (CAM) treatment in medical schools in U.S. shows more dissimilarities. The American Medical Association suggested integrating elective CAM curriculum in medical schools in 1997, while individual schools are free to choose the level and makeup of requirements for course [30]. The Ministry of Education in China specifies that as part of mandatory coursework medical programs of 5 years includes training in traditional medicine. Traditional Chinese medicine is considered an important aspect of a biomedical education than CAM in the U.S. Over ten years ago, a survey done in China indicates that students in biomedicine universities obtain training in traditional Chinese medicine in two semesters, accounting to over 200 hours of informative experience [31]. Across institutions how the distribution of these hours differ and how such coursework has been gained is still need to be investigated, this study tries to fill this part of the gap.

A Survey was done by Fan et al., [32] on 60 post-graduate students, 33 undergraduate's and18 clinicians. The survey consists of open-ended and forced-choice questions and evaluating professional and personal experiences regarding TCM. Mixed quantitative and qualitative measures were utilized to study trends in open-ended survey reactions. Their findings showed that undergraduate students (67 %), post-graduate students (60 %) and clinicians (89 %) have great experienced of TCM treatments personally. Traditional Chinese medicine is recommended by the majority of all three groups to patients. In case of extra professional experience,

Fig. 1. A pharmacy in a traditional-medicine hospital in Shijiazhuang dispenses medications. Photo credits: Muhammad Ovais (Chinese Academy of Sciences) (2019).

respondents showed an overall positive attitude with TCM; however, their professional experience was mixed with TCM. The various types of TCM for a diverse range of indications the professional and extraprofessional experiences of students and clinicians show the constant clinical presence of TCM. They also envisaged the significance of more training in TCM applying, particularly on clinical level, and imminent difficulties that must be overwhelmed in implementing clinical training developments [33]. Some of the countries which had already established strict regulations regarding the TCM are indicated in Table 2.

4. BELT AND ROAD INITIATIVE BOOSTS TCM

The initiative of BRI was presented in 2013 by Chinese president Xi Jinping. The initiative has brought an economic revolution in the BRI countries by investing in the joint trading to develop infrastructure and further stimulate investment in trade, health and medicine. Almost 90 countries which add one third of global GDP have committed to join the BRI programme [34, 35]. Under this programme, China has signed deals of worth 5 trillion USD to augment trade volume in the BRI countries. Least developed countries

Table 2. Regulations in different countries about TCM

S. No.	Condition / Disease /	TCM Treatment	Reference
1	United States	Exists	56
2	Russia	Exists	56
3	Vietnam	Exists	56
4	Australia	Exists	56
5	Europe • Portugal • Austria, Bulgaria, Estonia, Hungary, Romania, Serbia, Slovenia, Switzerland, UK (Regulated treatment but profession is not regulated) • Other European countries	• Exists • Regulation for treatment exists but not for profession • No regulations	http://cam-regulation.org/en/maps

got the opportunity to develop their infrastructure to improve connectivity with neighbour countries and actively participate in the global trade [36]. In addition to infrastructure development, these countries are improving in the development of science-technology, education standard, innovation transfer and development of their own resources. Though BRI is basically an economic project, yet it has considerably contributed to the global heath development in collaboration with World Health Organization. In a BRI high level meeting, director general of WHO commented that the BRI project is a dire need for the united efforts to ensure fundamental needs including infrastructure, human resources, access to medicine throughout the world [37]. Thus the Health Silk Road, part of the BRI project is a significant effort in promoting the combined effort of BRI countries for the prevention of communicable diseases, improvement in health polices, medical training, TCM, health education and disaster management system [38].

The BRI initiative has boosted the international recognition of Chinese traditional treasure 'Traditional Chinese Medicine" (TCM) [39]. In May 2018, the state administration for TCM reported that 57 TCM projects under the BRI programme have been initiated in various countries including United Arab Emirates, France, Germany and Poland. In Spain, under the umbrella of European TCM development and promotion centre, a signature project will be initiated including TCM training-education centre, health clinic and trade centre. Moreover, a master degree programme will be initiated with the support of Beijing University of Chinese Medicine, University of Barcelona and Universitat of Pompeu Fabra. Up till now, about twenty nine countries have established laws and regulations for the use of TCM and eighteen countries had given a legal status to acupuncture in their health care system. For instance, Portugal as a first European country has approved laws to open 4 years bachelor's degree programme in acupuncture in 2017 in Polytechnic Institute of Setubal (IPS). Further, IPS has signed an MOU with Tianjin University of TCM to allow students to study for one extra year in Tianjin University of TCM for their bachelor degree [40, 41]. The Portuguese government has also approved laws about offering TCM bachelor's degrees in other institutions.

5. TCM VITAL AREA OF HEALTH AND TRADE COOPERATION BETWEEN CHINA AND WORLD

Traditional Chinese medicine (TCM) is among the best preserved influential forms of traditional medical system [43] which symbolizes the Chinese culture and heritage. TCM is getting popular with passing days as the world faces numerous issues with conventional medicines like antibiotic resistance. Thus, traditional medicines are considered as a window of opportunity to cope with deadly diseases. Noble prize to Tu Youyou, for discovery of Artemisinin from Artemisia [44] is an excellent example to demonstrate the power of TCM and other forms of traditional practices. TCM has much strength as compared to western system. The impact of the TCM is now valued domestically but also internationally. Reports suggest that there are 12,807 TCM resources in China distributed as medicinal plants (11,146), medicinal animals (1581), and medicinal minerals (80). About 600 Chinese medicinal materials are common in use, in which near 400 plant and animal species are artificially cultured. In addition, a statistical analysis of 320 commonly used plant medicinal materials indicates that the total resources storage has reached approximately 8.5 million ton [45]. BRI possess a great potential towards the internationalization of the TCM system. In a holistic view, traditional Chinese medicines has many advantages as compared to the conventional medicines. The focus of the TCM is holistic that is curing both the root cause and symptoms of the disease. Synthesis of chemical entities is a hard and time consuming process often takes decades in development. The developmental cost of synthetic drugs is very high, and moreover, side effects are one of the major concerns. The development of new synthetic drugs is slow paced and very few satisfy the commercial and economical aspects. On the other hand, traditional systems are in place from thousands of years, whether it be TCM, Greek Medicines or Ayurveda Medicines, using natural resources for healing purposes with minimal or no side effects. Different pharma products finds its bases in plant medicines. Some classic examples are anticancer drugs from *Catharanthus roseus* [42], antiviral drug from *Silybum marianum* [46] and antimalarial drug from *Artemisia annua* [47]. Therefore, TCM should be among the priority areas

regarding health corridor among the BRI countries. It will provide extensive opportunities to develop health sector among the partnering countries and execute potential research and development projects regarding natural medicines.

5.1 A Flagship Project of BRI, China–Pakistan Economic Corridor (CPEC) Pakistan is Intended to Rapidly Modernize Pakistan.

CPEC is a multibillion dollar project not only intended to improve the connectivity among different regions but also holds the potential of massive socio-economical uplift, well fare, technological cooperation and mobilization in different fields. CPEC can have significant impact on the aggravating economy of Pakistan. With reference to traditional medicines, Pakistan has a medicinal flora which can be easily exported for revenue generation. CPEC will significantly increase the market outreach for Pakistani made herbal products across different regions of the world. Through scientific and technological cooperation, Pakistani herbal industries can transformed into modern research based traditional medicine industries. Chinese experience in TCM and their internationalisation can be a motivation for the local herbal manufacturing companies in Pakistan. Chinese herbal plants, R & D, pharmaceutical equipment, management and marketing, export strategies and transportation of herbals have continued intermittent development and are rapidly expanding. Pakistan can seize on CPEC opportunity to develop its local herbal industry and devise strategies for effective utilisation of the local manufacturing of herbal medicine. Convergence of TCM with Pakistani folkloric practices will indeed result in fascinating advancement of the health care sector in the region.

Provision of a holistic quality care is considered an important area of cooperation under CPEC, which will be incomplete without considering TCM. TCM is one of the greatest gifts of Chinese culture to the mankind in which natural resources and products are used to cure various diseases and infections. With the health corridor now materializing in the form of CPEC, TCM creates massive opportunities for the extensive cooperation between Pakistan-China and other countries part of the initiative. Traditional Chinese Medicine is one of the areas that can be used in creating opportunities for improving the health care in general and creating evidence based natural solutions to major health issues. Herbal medicine industry is growing in an expeditious manner and still 80 % of the world population relies on herbal medicine [42]. Folkloric and natural medicines have deep foundations in Pakistan, in the form of "Greek Medicines"/ "Tibb-e-Unani", and "Prophetic Medicines" / "Tibb-e-Nabwi" [42]. The amalgamation of TCM practices with traditional medicine practices in the region and rich biodiversity of Pakistan can led to massive leaps in evidence based natural medicines Moreover, it would further create opportunities for entrepreneurial setups dealing ultimately transforming in the big herbal industries. This will further enable this region to capture a handsome portion in the gigantic international market for herbal medicines. For the local manufacturers it will provide easy trade routes to different regions to export their indigenously developed herbal medicine around the world. Pakistan and China have already embarked into joint ventures in different sectors however; the operation in TCM and herbal sector is scarce. CPEC intend to create a link between herb research centres, academics, teaching institutes, universities, researchers and experts for the capacity-building Pakistan in the herbal sector. The exchange of knowledge and information would further pave ways for setting-up of a joint department of herbal and traditional medicine, traditional medicine hospitals through which the public will have access advanced herbal treatment, and additional employment opportunities will be created.

6. RECOMMENDATIONS BY AUTHORS

- Consumer confidence on alternative systems can be a driving force for fostering the future of these traditional systems. Increase in the consumer confidence is subject to the scientific approaches and evidence based research. Therefore, it's imperative to undertake measures which entice traditional medicine manufacturers for adopting scientific techniques, processes and procedures. The umbrella of CPEC and BRI can have significant impact in bringing latest technologies for standardization and quality assurance of traditional medicines. Practitioners of alternative medicines need to adapt a

scientific approach for conducting herb-drug interaction studies. It will further provide new avenues for conducting research, innovation and development, ultimately transforming the alternative medicine industries leading to acceptability and consumer confidence.

- Recognition of traditional medical systems and bringing them to the mainstream necessitates the need of mass awareness. A strong advocacy for promoting the use of traditional medicines is required to stimulate the policy makers, stake holders and other relevant parties for accepting traditional medicines in mainstream.
- Proper use of herbal medicines in the general public and consumers may be promoted. The narrative of traditional medicines, always being safe is misleading. Recognition of Drug interactions. The widespread belief that whole herbs formulations are harmless is not correct. Concurrent use of herbs with modern medicine may mimic, magnify, or oppose the effect of drugs. The apparently harmless garlic can interact with some modern drugs and cause serious interaction like bleeding when taken with low dose aspirin and Warfarin etc.
- Herbal practitioners, scientists and industrialists in Pakistan should take benefit from the opportunity given by BRI and CPEC. Under the umbrella of CPEC, entrepreneurial setups in traditional systems and partnerships can be nurtured which will have a long lasting positive impact.
- It is recommended to have a joint centre of excellence dedicated to preserving the fascinating heritage of alternative medical systems like Traditional Chinese Medicine, Eastern Medicine and others. Building shared and common resources can be pivotal to harvest the benefits of such traditional medicinal systems. Shared repositories and databases can be created for documentation of medicinal systems, herbals, their uses with open access to all. Like convergence of economic interests through CPEC among BRI countries, the traditional medical systems needs to converge together to fulfil the health vacuum in this region. In this regard, joint hospitals offering treatments through TCM and Eastern Medicine can play significant role.
- With the expected rapid industrial activities under CPEC and BRI, one can assume a rapid rise for the demand and supply of crude materials used in the manufacturing of traditional medicines. Most of these medicines are made from the unique and rare herbs and plant material. Strategies and policies are required to ensure the growth of herbal industries but not at the cost of disturbing the natural balance. Therefore, all policies must be based on the No Net Loss (NNL) for preserving the natural habitat as well as preventing significant loss in the number of species for preventing their extinction.
- Policies with reference to traditional drugs regulations and licencing are very crucial. A vibrant regulatory body with holistic capacity for 360 degrees evaluation of the products is required.
- Incentivising the traditional medicine industry, promoting educational programs related to traditional medicines can have a positive impact on the future of TCM, and other forms of traditional systems in BRI regions. There is also need to educate common people on the subject matter as the TCM spreads in BRI countries.

7. ACKNOWLEDGMENTS

Muhammad Ovais acknowledges Peking University Guanghua School of Management for arranging series of lectures on Belt & Road Initiative. He is also grateful to "The First China International Biomedical Technology Development Forum" 2018, Shijiazhuang, China, for guest invitation and field visit of a TCM hospital.

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Proceedings of the Pakistan Academy of Sciences:
B. Life and Environmental Sciences 56 (3): 27–36 (Special Issue, 2019)

ISSN: 2518-4261 (print), ISSN 2518-427X (online)

Pakistan Academy of Sciences

Review Article

Current Trends in the Popular Sector Traditional Medicine in Sri Lanka

Rathnayake M. Abeyrathne

Department of Sociology
University of Peradeniya, Kandy, Sri Lanka

Abstract: It is well known fact that Sri Lanka was able to produce a unique tradition of knowledge in the field of traditional medicine over the last two thousand five hundred years of its history. The country is placed where its inhabitants were able to develop a traditional medicine-based medical pluralism, comprising Desheeya Chikithsa, Ayurveda, Unani, Siddha, Homeopathy and various other informal traditions to as remedies cure various illnesses. In this paper, I focus on discussing major developments and trends in the popular sector traditional medicine in Sri Lanka at present. They include new trends in the use of traditional medicines as reliefs for minor illnesses, personal health and beautifying items, supplementary food substances and positive health and wellness practices. At present, traditional medicine-related items have become common consumable items among Sri Lanka public as a deterrent to control non-communicable diseases and traditional herbal products are more popular as beatification therapies. In order to minimize the problems that this sector faces, the state can take numerous measures such as strengthening the law to crack down illegal practitioners, register all service providers under the local government's health departments and conduct periodic health campaigns to educate people about the importance of this sector as a way to prevent the etiologies of illnesses and diseases to promote health behavior in the long-run to combat the spared of all types of diseases and to cut down staggering cost of health expenses that people have to face in-day-to-day life.

Keywords: Traditional medicine, popular sector, herbal medicine, non-communicable diseases.

1. INTRODUCTION

Sri Lanka is an island nation, located on the Southern tip off India, with a history of more than two thousand five years of indigenous rule (6th Century B.C. 1505 A.D) and nearly four hundred fifty years of colonial rule by three western colonial rulers, first by the Portuguese, then by the Dutch and finally by the British (1505-1948 A.D.). Sri Lanka population comprises three main ethnic groups, the Sinhalese represent seventy per cent and they are mainly Buddhist and Christian followers; the Tamils consist of fifteen per cent of the population while they practice Hinduism and Christianity; the Muslims comprise ten percent of the population and they are mainly the followers of the Islamic religion and the rest belongs to various small ethnic groups. Sri Lanka as a welfare-based society, the state provides universal health care and free education facilities to all its citizens irrespective of their socio-economic backgrounds. In addition, the private sector also plays a pivotal role in providing all types of health services to the public to make Sri Lanka a healthy nation.

Since independence, Sri Lanka has been able to maintain a remarkable progress in improving the health status of its population. The recent human development indexes reflect that Sri Lanka has achieved the highest statuses among South Asian countries. The life expectancy of the population of Sri Lanka has been increased from 43 years in 1946 to 80 years in 2018 (81 years for females and 76 for males), maternal mortality in 2018 (6.2 per 1000 live births), infant mortality in 2018 (8.4 per 1000 live births) and literacy rate in 2018 was 92 per cent (Department of Censes, Sri Lanka). Sri Lanka's achievement is even more remarkable when considering its level of income and its low expenditure on health. It spends a total (public and

Received: February 2019; Accepted: September 2019
* Corresponding Author: Dr. Rathnayake M. Abeyrathne; abeyrathnayake87@gmail.com

private) of approximately 4.2 per cent of GDP or US$57 per capita on health. However, many of its health indicators are comparable to those found in South East Asian countries with income levels two to six times higher, adjusting for purchasing power parity, which spend 1.5 to 10.0 times more on health per capita "Sri Lanka Health Accounts [SLHA] 2017]".

Many argue that Sri Lanka has been able to maintain satisfactory heath indicators when compared to other South and South East Asian countries because of various welfare programmes that the Sri Lankan government has introduced over the years to develop health facilities in the country. At the same time, many people are of the view that it is because of western medicine that Sri Lanka has been able to increase health statuses of its people. However, this view is partially correct because it is not only western medicine but also health and medical pluralism that the country has been practicing over millenniums has played a significant role in promoting health of its people. However, this has not been given due recognition by the state and previous researchers and scholars in the field of Sri Lankan health. Sri Lankan medical pluralism comprises multiple therapeutic traditions ranging from Western Medicine, Ayurveda, Siddhayurveda, Unani, Deseeyachikithsha, Acupuncture, Homeopathy, Religion-based medicine, Vedda's indigenous medicine (Sri Lanka's indigenous people rea recognized as the Vedda) occult practices and to numerous home remedies "Abeyrathne [1]".

Even at present, the majority people in Sri Lanka seek more traditional medicine at time of illness but information or statistics is meager to substantiate this argument because there is no formal practice to maintain records of patients in traditional medicine unlike in western medicine (it should be highlighted that now only in the formalized Ayurveda sector patients' details are kept at hospitals). The purpose of this paper is to discuss new trends in the popular sector-based traditional medicine in Sri Lanka. In order to contextualize the present analysis, this paper uses the framework that the American Anthropologist Arthur Klienman put forward on the provision of health care services to explain the current trends in the field.

In this framework, he divides health care services in a modern society into three main parts, namely, the popular sector, the folk sector and the professional sector health services. The popular sector deals with the use of informal sector-based health practices that include western and traditional medicine practices to maintain health. The folk sector focuses on traditional medicine services that people use as health care provision to enhance their health. Finally, the professional sector relates to western medicine that people practice at time of illness "Helman [2]". However, the present discussion on the new tends in the popular sector traditional medicine begins with a very brief introduction about traditional medicine in Sri Lanka and then focuses on the new trends in the popular sector-based traditional in Sri Lanka. They include new trends in the use of traditional medicine as reliefs for minor illnesses, personal health and beautifying items, supplementary food substances and positive health and wellness practices.

2. TRADITIONAL MEDICINE IN SRI LANKA

According to the World Health Organization (WHO), traditional medicine refers to health practices, approaches, knowledge and beliefs incorporating plant, animal and mineral based medicines, spiritual therapies, manual techniques and exercises, applied singularly or in combination to treat, diagnose and prevent illnesses or maintain well-being. Further the term complementary and alternative medicine (and sometimes also non-conventional or parallel) are used to refer to a broad set of healthcare practices that are not part of country's own tradition, or not integrated into the dominant healthcare system.

As mentioned earlier, Sri Lanka provides free and universal health care coverage for all its citizens irrespective of their all socio-economic differences. Sri Lanka's health care service consists of five main sectors: the government funded western medical sector; the government supported Ayurveda sector (traditional medicine); the privately run western medicine sector; the privately financed Ayurveda (traditional medicine sector; and what is often referred to as the privately run 'other sector' "Simeonov [3]". There are number of synonyms that scholars and researchers in this field use to refer to traditional medicine and they range from native medicine, indigenous medicine, complementary

medicine, supplementary medicine, aboriginal medicine, local medicine and traditional medicine. However, the term 'indigenous' to describe all varieties of traditional medicine in Sri Lanka- they do not differentiate local health traditions, known as the Deseeya Chikithsa (indigenous medicine), from other practices in the island.

Therefore, this study uses the term traditional medicine, which includes traditions ranging from Deseeya Chikithsa, Ayurveda, Siddhayurveda, Unani, Acupuncture, Homeopathy, and to various other local practices (this study reveals that the Sri Lankan government uses TM, Indigenous Medicine, and Ayurveda interchangeably. Such terms are embedded in the names of institutions and roles, such as, the Department of Ayurveda, Ayurveda Hospital, Ayurveda College, Commissioner of Ayurveda, Ayurveda Pharmaceutical Corporation, Ministry of Indigenous Medicine, and the National Institute of Traditional Medicine).

However, it should be mentioned that the Sri Lanka government uses the term Ayurveda to refer to all types of traditional medicine practiced in all parts of the country. Based on my own study findings of the subject, it is more rational, logical and scientific to use the term traditional medicine because Ayurveda is one type of traditional medicine used in the northern part of India. Nevertheless, the term, traditional medicine includes all types of local medical traditions irrespective of any cultural difference exists among them.

The early medical history of Sri Lanka is abound in legends, according to which the demon king Ravana, the prehistoric ruler of the island, was said to have been a versatile medical practitioner (he is believed to have hailed from a family specializing in folk medicine)"Kumarasignhe [4]". It was said that his grandfather, Pulasthi Rishi, represented Sri Lanka at a Bharatha Irshi (Bharatha sage) medical conference, held in the Himalayas. According to the Lanka version of Ramayana, the Indian epic, Ravana himself allegedly participated at the conference. In addition, Ravana is believed to be the author of medical manuscripts, namely, *Arkaprakasaya, Nagavignanaya, Kumarathatraya*, and *Udishasasthraya* "Uragada [5]".

The written history of TM in Sri Lanka goes back to the sixth century B.C. The strategic location of the country, at the nexus of crucial maritime routes in the middle of the Indian Ocean connecting Europe, the Middle East and China to the rest of Asia, made it a focal point of population migration and trade. Diverse ethnic, linguistic, and religious groups arrived in the island and contributed to the development of TM pluralism "Hettige [6]". Infused with knowledge from India, Arabia, and China, Sri Lanka's diverse traditional medicine comprises the following formally and informally recognized and informal traditions: Vedda medicine, *Deseeya chikithsa*, Buddhist practices, Ayurveda, Siddha, Unani, Acupuncture, Homeopathy, and various other local practices.

In pre-colonial Sri Lanka, starting 6 B.C and ending 1815 A.D. all monarchs considered that it was obligatory for them to care for the sick and encouraged the study of medicine as well as the building of hospitals. Medicine was an esteemed profession practiced under royal patronage and some of the ancient kings were well known physicians. There was a well-known saying among the Sinhalese that "if a person were not able to become a king he should become a physician". All available ancient medicine- related literary sources, inscriptional and other archaeological information indicate that there was a well-developed and sophisticated traditional medical system providing health care services to its citizens in ancient Sri Lanka society. For instance, the concept of hospital where a number of patients could be collectively housed in special centers with the attendant advantages to the sick was recognized as early as the fourth century BC during the reign of King Pandukabhaya (294 - 307 AD). The existence of the first hospital in the city of Mihintale on a ninth century BC site, described as being perhaps the oldest hospital in the world as shown in the Figure 1 & 2, has been verified archeologically "Uragaoda [7]".

However, in his book, *A History of Medicine in Sri Lanka,* U. C. Uragoda argues that with the arrival of western colonial rulers, traditional medicine lost the state patronage that it received under the ancient Sinhalese kings. Anyway, as a result of the ongoing nationalist activities, the British later decided to provide state financial support and legitimacy to traditional medicine. Since independence in 1948, all successive governments that came to power

Fig. 1. The ruins of ancient hospital at Mihinthale, Anuradhapura

Fig. 2. The medical trough at the ancient Mihinthale hospital

took important initiatives to promote traditional medicine as one of the main streams of medicine to provide free and quality health care service to all its citizens. In the 1990s, Sri Lanka established a separate cabinet level ministry for the development of traditional medicine and it was the first time in the world that a separate ministry was formed to enhance traditional medicine in a nation state.

At present, government and private sectors involve in providing traditional medicine services at small, medium and large scales to enhance people's curative, preventive, rehabilitative and promotive aspects of health to build a healthy nation. There are nine integrated health ministries, including the national and provincial council government ministries to promote traditional medicine along with western medicine to enhance people's health. Sri Lanka has three state funded university level traditional medicine colleges to provide education both at undergraduate and graduate levels and annually state run traditional hospitals absorbed them into state run hospitals located in all parts of the country. In addition, there are some formally and informally run state and privately funded small traditional medical colleges located in many parts of the country to provide education and to train paramedical professionals to work in traditional medicine-related allied health centers. Now traditional medicine and western medicine works side-by-side in tandem in all parts of the country even while facing a fierce competition and integration between the two systems of medicine.

3. TRENDS IN THE POPULAR SECTOR TRADITIONAL MEDICINE

When observing Sri Lankan people's health seeking behavior, it indicates that there is a remarkable growth in the use of traditional medicine in the popular sector-related health care service when compared to the past. According to the American anthropologist, Arthur Klienman the popular sector medicine involves all therapeutic options that people use without any payment and without consulting medical practitioners. Among these options include self-treatment or self-medication, advice or treatment given by parents, a relative, a family member, a friend, para-medics and neighbour or worker. In addition, the popular sector medicine involves in providing health maintenance practices to prevent ill health in families and communities. They include the beliefs about dress, food, drink and religious prayers. This sector depends on informally trained individuals as service providers and sometimes today's patient becoming tomorrow's healer. However, the growing new trends in this sector witnesses that the use of modern technologies have transformed some of its traditional practices to be more efficient, efficacious and professional to cater to modern demand.

In the past, it was a custom for Sri Lankan people to grow day-to-day needed medicinal items in their homesteads so that it would help them use when they needed to use them because they considered frequently used medicinal material food items and food items medicine. However, with the advancement of technology, both state and private sector health care producers in Sri Lanka produce traditional medicine items in multiple forms for easy use for all age groups. They range from alepa (external applications), arista (fermented preparations made out decoctions), asava (fermented preparations made out of material), guli (pills), kalka (paste), modaka (sweet-based large bolus), nethra bindi (eye drops), thel (oil), swarasa (juice), curna, (powder), gruta, (oily paste), mallum (heated mixture of medicine material), basma, (ashes), basna, (keeping in medicinal water) and kenda (medicinal gruel). These varieties are easily accessible to people at traditional medicine pharmacies, super markets and sometimes at treatment centers throughout the country as over the counter medicinal items.

As mentioned earlier, the following discussion is focused on the new trends in the use of the popular sector-based traditional medicine by people from all walks of Sri Lanka society for enhancing various aspects of their health. Thus, the most commonly used practices include using traditional medicine as reliefs for minor illnesses, improving personal health and beautifying purposes, taking as supplementary food substances and practicing them as positive health and wellness practices.

4. POPULAR SECTOR MEDICINE FOR MINOR ILLNESSES

It is a usual practice in Sri Lank that people maintain a mini-pharmacy with both western and traditional medicine drugs in their homes to use them as basic precautions for minor illnesses until they consult western or traditional medicine experts if required irrespective of their socio-economic backgrounds. The range of minor illnesses comprises cold, early childhood diseases, early pregnancy related ailments, fever, stomachache, tonsil, headache, toothache, diarrhea and joint pains, pimples, mensuration pains, arthritis, gastritis and various other minor diseases. Sometimes, people use traditional medicine simultaneously with western medicine as well.

As in other societies, women, mothers and grandparents in Sri Lanka also play a significant role in recognizing diseases and illnesses and providing health care services to affected members of the family and community. Sometimes, those who have suffered previously from similar symptoms, those with extensive life experience like giving birth to children, paramedical professionals and husbands and wives of traditional medical practitioners also function as resource persons to provide basic health advice to overcome minor illnesses in this sector. For instance, young pregnant women with less experience in delivering babies seek advice from their mothers and other senior women the types of symptoms that they should expect and how to deal with them.

The most commonly used such brands are produced by well-known companies, such as, Siddhalepa, Link Natural, State Ayurveda Pharmaceutical Corporation, Vendol, Nature Secret

and many other state and private companies. In addition, people in Sri Lanka still use local home remedies to cure minor illnesses and they collect required material from nearby surroundings, from home gardens or from traditional medicine pharmacies located in many parts of the country. At present, Sri Lankan people use more Indian Ayurveda products as basic remedies to cure minor illnesses and annually both local and Indian pharmaceutical companies organize promotional trade exhibitions, workshop and conferences to increase public awareness of traditional medicine. The promotors of traditional medicinal items use both electronic and print media facilities belonging to state and private sectors to popularize their products through regular advertisements, discussion, interviews and documentary programmes. Some companies have wide local and foreign clientele to purchase their products from Sri Lanka and sometime they have trade outlets in some foreign countries as well. For example, privately owned Sri Lankan traditional medicine pharmaceutical companies annually export large amount of health items to North American and European countries.

5. POPULAR SECTOR MEDICNE AND PERSONAL BEAUTY PRODUCTS

Unlike in the past, at present, both Sri Lankan men and women use more traditional medicine products for beautification and personal health and hygiene purposes. Especially, traditional medicine is more popular than western items because the former is regarded as more natural while the latter is considered artificial and causes more side-effects. There is a growing market for beautifying products in the country and people use both locally and internationally produced traditional medicine items for daily and special social occasions-related events. Thus, various local and foreign produced medicinal items are readily available at super markets, regular saloons, both traditional and western pharmacies and at beauty parlors located island-wide. Traditional medicine beauty products are used as baby care, oral care, skin care, body care and skin care. Both men and women use them on daily basis and at special occasions like weddings, graduation ceremonies, parties and other social events.

All beauty products range from powder, cream, oil, soap, die, paste, tablet, drinks, tablets, pills and to face wash liquid. Similar to popular medicine products, large number of state and private sector companies currently engage in producing beauty products for both local and international markets and they use electronic and print media facilities to promote their products in competitive ways. At present, a significant number of women maintain traditional medicine-based beauty salons or parlors as self-employed entrepreneur business ventures in many parts of the country.

As far as traditional medicine-based personal and beauty products are concerned, the private sector plays a pivotal role when compared to the state sector. The main reasons are that the former invests funds heavily in research, technology, training, both print and electronic media, organizing local and international conferences to update knowledge and modifying traditional medicine products to match modern day needs. Many companies employ well trained scientists with foreign and local university qualifications to improve the quality of health products and maintain international standards to face any completion from other countries. Some of them even conduct join business ventures with other countries like Germany, Japan and India to absorb updated technology and other innovative good practices in the field to popularize their health producers.

It is a usual practice for companies that produce traditional medicine items to grow and maintain herbal gardens on their own to supply raw material to minimize the cost of productions and improve the quality of their productions. However, some companies provide subsidies to farmers to grow some rare herbal plants as a livelihood and sometimes they buy some products from private farmers to use them in their own factories. On the other hand, this way they help farmers earn extra income in addition to their main other earnings. Such initiatives help companies also save money from importing material mainly from India.

The commonly grown species range from inguru (*Zingiber officinale*), katuwelbatu (*Solanum verginianum*), komarika (*Aloe barbedensis*) and to pawatta (*Justicia adhatoda*). For example, the Forever personal and Ayurveda beauty producing company organizes such famer community-related herbal gardening programme at the Kandy export

zone and the same company has established a branch in Australia to promote traditional medicine herbal products. Similarly, the Ministry of Indigenous Medicine of Sri Lanka introduced a very similar programme in 2005, it is known as the Herbal Farmer Village (Osu Govi Gammana). Under this project, the farmers sell their products either to the State Ayurveda Pharmaceutical Manufacturing Corporation or provincial council governments to run such facilities to earn their living. At present, there is a growing trend for the state and the private sectors to organize traditional medicine trade fairs, conferences and health promotional campaigns jointly to popularize traditional medicine in the country.

6. POPULAR SECTOR MEDICNE AND FOOD BEHAVIOUR

If diet is wrong, then medicine is of no use. If diet is correct then medicine is of no need. Let the correct food to be your medicine. This is the traditional belief in food behavior in Sri Lanka. When observing people's day-to-day food behavioral practices, one can observe that there has been a growing trend at present among all ethnic groups that they are more conscious about their food consumption patterns than in the past. It has been a long tradition in Sri Lanka that people used traditional medicine-based food items as supplementary dietary items to maintain healthy lifestyle for millenniums. It is well known that people in ancient Sri Lankan society considered some traditional medicinal items food and food items medicine for a healthy living. This belief became more popular in the wake of the unprecedented speared of non-communicable diseases, such as, diabetes, cholesterol, cancer and high blood pressure among a significant percentage of its population. Especially, people who live in urban areas tend to give up unhealthy modern dietary practices and embrace traditional food items to stay healthy.

The people in Sri Lanka grow traditional medicine items as organic items at state, private sector's and privately owned lands and distribute them to island-wide established food processing companies to prepare them. These food items are manufactured by using modern technology and they are sold as drink, tea, bread, rice, curry, soup, biscuits, medicinal gruel at traditional and modern medicine pharmacies, state and private food outlets, health food stores, restaurants, super markets, tourist hotels and community markets throughout the country. However, some people prepare them at home by using ingredients collecting from their homesteads and buying from traditional medicine outlets. In order to promote these items people widely use social media, other types of print and electronic media and periodic health promotional campaigns, such as, health campaign on the World Diabetes Day in many parts of the country. In addition to local markets, there are wider trade networks to promote these food items especially among Sri Lankan clientele living in many other countries in the world.

Recently, the state agriculture department took an initiative to promote traditional medicine food outlets in many parts of the country as a measure to control the spread of non-communicable diseases among a significant percentage of population in the country. As a result, now there is a shift among people to move away from unhealthy modern dietary practices and return to thousands of years old indigenous food practices to lower the spread of threatening non-communicable diseases. Similarly, the chains of these food places have directly and indirectly benefited women, farmers and other service providers to secure employment opportunities in running these places.

In addition, food outlets located at public places like schools, hospitals and other places, service providers are discouraged from selling unhealthy food items and artificial drinks. But, instead, they are recommended to provide healthier natural food items. For example, parents are required to provide more grain items, vegetables, green leaves, fruits and other fiber substances for their children's daily school food packages as a way to combat the spread of non-communicable diseases among school children. It should be highlighted that those who suffer from non-communicable diseases consume these dietary items as supplementary medicine as a way to control them even while taking western medicine simultaneously. For instance, the Sambodhi Buddhist temple in Colombo conducts weekly traditional medicine- based food programme known as, the Hela Suwaya medicinal gruel programme and it tests whether traditional medicine practices could be applicable to lower diabetes

patients' insulin dependency. It was conducted together with western medical practitioners to test the efficacy of such practices and the results of the test indicated that some patients with diabetes have become totally free from insulin dependency.

7. POPULAR SECTOR MEDICNE AND HEALTH AND WELLNESS PRATICES

In addition to the above-mentioned health practices, Sri Lankans use popular sector medical practices as allied forms of health knowledge as wellness techniques to improve personal, family and community health. Wellness means a way of life and living in which one is always exploring, searching, finding ways to lead healthy living to keep the balance among three primary dimensions of health. They include the physical, the mental and the social dimensions related to health to achieve holistic fulfillment in human life. Some of the commonly used wellness practices consist of yoga, tai-chi, qigong, karate, judo, tykondo and various forms of meditations, breathing techniques, massage techniques at traditional clinics, traditional medicine resorts and traditional medicine spas. In addition, religion-based healing rituals, religious retreats, religious prayers other forms of practices include in the popular sector medicine as well. Although, these are not purely medical systems but they have been adapted as health applications and contribute to health sector immensely in many countries.

Among the above-mentioned long list of wellness- related popular sector- based traditional medicine practices, the most frequently used two techniques in Sri Lanka, namely, religious retreats and traditional medicine-related therapeutic techniques will be discussed at length in the following section, considering the uniquenesses of the two practices. As far as religion is concerned, Sri Lanka is a unique place because it is a meeting place for four main world religions, Buddhism (main religion), Hinduism, Islam, and Christianity. People practice them harmoniously side-by-side within a very limited geographical proximity. Sri Lanka is the only one country in the world where all regions' holy days are recognized as public holidays for people to engage in spiritual activities and to promote psychological and social aspects of health. All full moon days are holydays for Buddhists, three for Hindus, three for Muslims, and two for Christians. Nevertheless, all holydays are holidays for all religious practitioners.

All religious followers have daily religious ritual that they practice either at home or designated religious places to maintain balanced health among physical, social, biological and spiritual aspects of health. Except Buddhists, all other religious groups have short retreat sessions during religious holy periods. However, Buddhists practice extensive retreats through organized and institutional manner at various Buddhist temples, hermitages and public places on short-term and long-term basis for both local and foreign Buddhist and non-Buddhist followers. As an island-nation, Sri Lanka is a well-known destination with exotic beaches as well as a rich cultural heritage, mainly Buddhist. The traditional tourism offer multiple opportunities from beach tourism to eco-tourism, adventure/ wild-life and to religious experiences.

Thus, large numbers of foreign tourists, especially, from western countries visit Sri Lanka throughout the year to participate in meditation or retreat programmes at various places in the country. Some forest hermitages and meditation centers offer well organized residential retreat programmes to foreigners and local Buddhists in many parts of the country. These religious groups believe that Buddhist meditation practices help them find solutions for various etiologies of illnesses and diseases, such as, stress, anxiety, suicide, mental disorders and non-communicable diseases created by modern artificial and hectic lifestyles. For instance, research findings on high blood pressure-related studies conducted in many countries prove that Buddhist meditation positively affects to reduce drug dependency among such patients.

In addition, business executives in many successful companies worldwide highly praise that Buddhist medication has been the driving force behind their success because it has helped them cultivate right concentration for attending to all matters pertaining to running their business organizations. Even the British Royal family has professionally employed a Sri Lanka Buddhist meditation teacher to advise them on enhancing mental health of its members irrespective of them

being the leading custodians of the Anglican Church in the Great Britain. Not only the Great Britain, but many western countries use Buddhist mediation practices as one of the most common wellness programmes to restore mental health.

Moreover, Sri Lankan people use traditional medicine practices to enhance their personal, family and community health. At present, such places function in multiple forms, namely, Ayurveda clinics, Ayurveda resorts and Ayurveda spa. These places are run by professional trained traditional medicine expert with university qualifications, those who engage in this profession from family inheritance and those who have training in traditional medicine therapeutic practices. Mostly, the private sector provides wellness services to both local and foreign clients who seek help from these places for various ailments, such as, arthritis, back pain, paralysis condition, joint pains, weight loss and stress. Some places specialize in providing services to women. Women practitioners, therapeutic assistants, or male of female masseuses run these places. Treatment methods include alepa (external applications), arista (fermented preparations made out decoctions), asava (fermented preparations made out of material), guli (pills), kalka (paste), modaka (sweet-based large bolus), nethra bindi (eye drops), thel (oil), swarasa (juice), curna, (powder), gruta, (oily paste), mallum (heated mixture of medicine material), basma, (ashes), basna, (keeping in medicinal water), kenda (medicinal gruel) sambahana (massage), Vaspha snana (steam bath).

It should be highlighted that popular sector-based wellness programmes are very much associated with respect to medical/wellness tourism, as in other countries, there is no official statistic on how many of the tourist arrivals are in Sri Lanka for the purpose of health related tourism, but anecdotal evidence points to an increasing number of foreign patients coming to Sri Lanka for treatment. The cost of medical treatment, even including travel and accommodation, is on average, 50% cheaper than developed countries. Large numbers of holidaymakers come to Sri Lanka for the purpose of staying in resorts that offer ayurvedic spa and treatment. Ayurveda is already advertised in the Sri Lankan official tourism website.

Almost all tourist hotels located in all parts of the country, whether they be large or medium level business ventures, maintain Ayurveda resorts and spas as an integrated part of their business to run them as profitable ventures. However, some small tourist hotels specialize in providing medical tourism-related services only to selected foreign clientele. The most well-known Ayurveda resorts and spas are located along the southern coastal belt. At these places, the most popular wellness service is the provision of Ayurveda panchakarma treatments to both local and foreign tourist groups. Panchakarma is the purification therapy used in Ayurvedic medicine. The word panchakarma means five actions and refers to five procedures intended to intensively cleanse and restore balance to the body, mind, and emotions. The five procedures include vamana (therapeutic vomiting or emesis), virechana (purgation), vasti (therapeutic enema), nasna (elimination of toxins through the nose and raktamokshana (bloodletting). The panchakarma process reflects the influence of Indian Ayurveda on Sri Lanka traditional medicine.

8. DISCUSSION

The details in the above discussion witness that the popular sector traditional medicine in Sri Lanka has undergone significant transformations in the recent time and in general they have impacted positively on the enhancement of people's health in the country. However, everyday newspapers and other electronic media provide information about the ignorance of the use of some medicinal items, abuses of the system by various groups merely for making more financial gains. Those who use traditional medicine as reliefs for minor illnesses, sometimes over use them until patient develop into critical conditions rather than taking them to more professional sectors to save their lives. Especially, mothers often depend on home remedies for treating their children's minor diseases and illnesses and face negative consequences because they postpone taking them to more qualified health care providers to save their lives from danger.

The other challenge that this sector faces is that some individuals and companies produce inferior products by using low quality material merely for making financial gains and they negatively affect people's health. Especially, such products are common in medicine, food products, personal

wellness-related products, beauty related heath goods and tourism-related health products. There is a reckless competition among various companies that manufacture various traditional medicine-related products for making more profits by using all modern market strategies. Some companies use western medicinal substances with traditional medicine to improve the efficacy of their products.

Moreover, some service providers in this sector do not fulfill all required qualifications to practice the popular sector-based traditional medicine because daily all types of media report cases reported about illegal practitioners providing low quality services and putting people's lives in hazardous situations. In Sri Lanka, there is abundance of distorted and misleading information relating to traditional medicine in daily and weekend newspapers, electronic media and social media programs to promote it. However, some of them tend to misinform the public and eventually they exploit those who seek their help find solutions to various problems that they face. Such illegal and disqualified practitioners are common in beauty and wellness-related field and those who provide service using various religio-magical teachings in many parts of the country. For instance, there were numerous incidents in the recent past that some men and women became disfigured and they developed side-effects due to severe chemical reactions developed from the substances that they used for beatifying purposes. Sometimes, few men and women lost their lives at illegally established places run by disqualified people.

9. CONCLUSION

The popular sector-based traditional medicine contributes to improve all aspects of health of the people of Sri Lanka and minimize health expenditures in the wake of skyrocketing cost of health expenses in all modern societies. The uses of such practices have become more popular as basic remedies to control the spread of non-communicable diseases in the country. Traditionally these informally used medicinal items were looked down upon as rural poor and less-prestigious people's health habits. However, at present, people from walks of society in Sri Lanka use them as a part of their primary health care practice irrespective of the fact that they come from estate, rural and urban social backgrounds.

In addition, this sector directly and indirectly contributes considerably to provide employment opportunities to millions of people in the county irrespective of abuses by some segments of the service providers as highlighted earlier in the current discussion. Similarly, it helps people reduce their health expenditures significantly because some of the medicinal items are cheaper when compared to western medicine-related items. Not only those benefits but it assists Sri Lanka to get easy flow of new technology into the country to promote the quality of its products to compete with other drugs producing countries.

Finally, in order to minimize the problems that this sector faces, the state can take numerous measures such as strengthening the law to crack down illegal practitioners, register all service providers under local governments' health departments and conduct periodic health campaigns to educate people about the importance of this sector as a way to prevent the etiologies of illnesses and diseases to promote health behavior in the long-run to combat the spared of all types of diseases and to cut down staggering cost of health expenses that people have to face in-day-to-day life.

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Proceedings of the Pakistan Academy of Sciences:
B. Life and Environmental Sciences 56 (3): 37–47 (Special Issue, 2019)

ISSN: 2518-4261 (print), ISSN 2518-427X (online)

Pakistan Academy of Sciences

Review Article

Bioinspired Synthesis of Nanoparticles and their Biomedical Potential: the Pakistan Experience

Sana Ullah[1], Muhammad Ali[1*], Misbahud Din[1], Shakeeb Afridi[1], Samina Bashir[1], and Zabta Khan Shinwari[1,2,3]

[1]Department of Biotechnology, Quaid-i-Azam University Islamabad
[2]National Council for Tibb, Islamabad, Pakistan
[3]Pakistan Academy of Sciences, Islamabad, Pakistan

Abstract: Nanotechnology is an emerging field that play pivotal role in a wide range of scientific fields. Applications of nanotechnology have been successfully applied in healthcare, drug delivery, gene delivery, diagnostics, and energy sciences. Nanoparticle synthesis involves different methods like physical, chemical approaches and biological methods. The physical and chemical methods are associated with pitfalls as they pose potential threat to the environment; and hence ecofriendly routes of bioinspired nanoparticle synthesis are preferred. The biogenic synthesis of nanoparticles has attracted numerous researchers because of their potential advantages such as simplicity, safety, easy production, biocompatibility, and low production costs. Green synthesis of nanoparticles involves the mixing and processing of metal salts with plant/bacterial/fungal extracts. Secondary metabolites from biological sources have potentials for reducing the metal salt(s); herby synthesize respective nanoparticles. The current review is aimed to discuss reports and studies conducted in Pakistan that have used biological approach for nanoparticle synthesis, as well as their potential biological and pharmacological application/s. Future directions should involve market oriented approaches for the commercialization of nanoparticles-based products that can help in up-left of national economy.

Keywords: Nanoparticles; Green synthesis; secondary metabolites, Pakistan

1 INTRODUCTION

Nanotechnology is an important field of modern research that deals with the synthesis, designing and manipulating particle structures having size from 1-100 nm [1, 2]. The synthesized nanoparticles (NPs) are used in various fields such as health-care, biomedical science, drug and gene delivery, food industry, energy science, light emitters and catalysis [3-6]. The hybrid field of Bionanotechnology, that uses biological starting materials, biological design principles or has biological or medical applications, has found the vast variety of application due to the manipulation of living matter at the nanoscale [7, 8]. The synthesis of nanoparticles with different size, morphology and chemical composition is important area of nanobiotechnology research. This multidisciplinary approach results from the tentative use of nanoparticles in various disciplines such as biology, chemistry, biochemistry, medicine, physics and engineering. Furthermore, this hybrid fields also serves as imperious practice in developing clean, safe and environment friendly process for synthesizing metallic nanoparticles that reduce metals in a specific metabolic pathway [5, 6].

For nanoparticles synthesis, various approaches are applied comprising chemical, physical and biological methods (Figure 1). Traditionally applied methods (i.e. physical and chemical synthesis) have potential harmful effects such as environmental damage, cost and prolong time consumption; researchers are looking for other possible approaches for the synthesis of NPs [9]. Biological production of NPs has preference over other mentioned protocols as they are having more dynamic nature, comparatively safer and efficient

Received: February 2019; Accepted: September 2019
* Corresponding Author: Muhammad Ali; alibiotech01@gmail.com

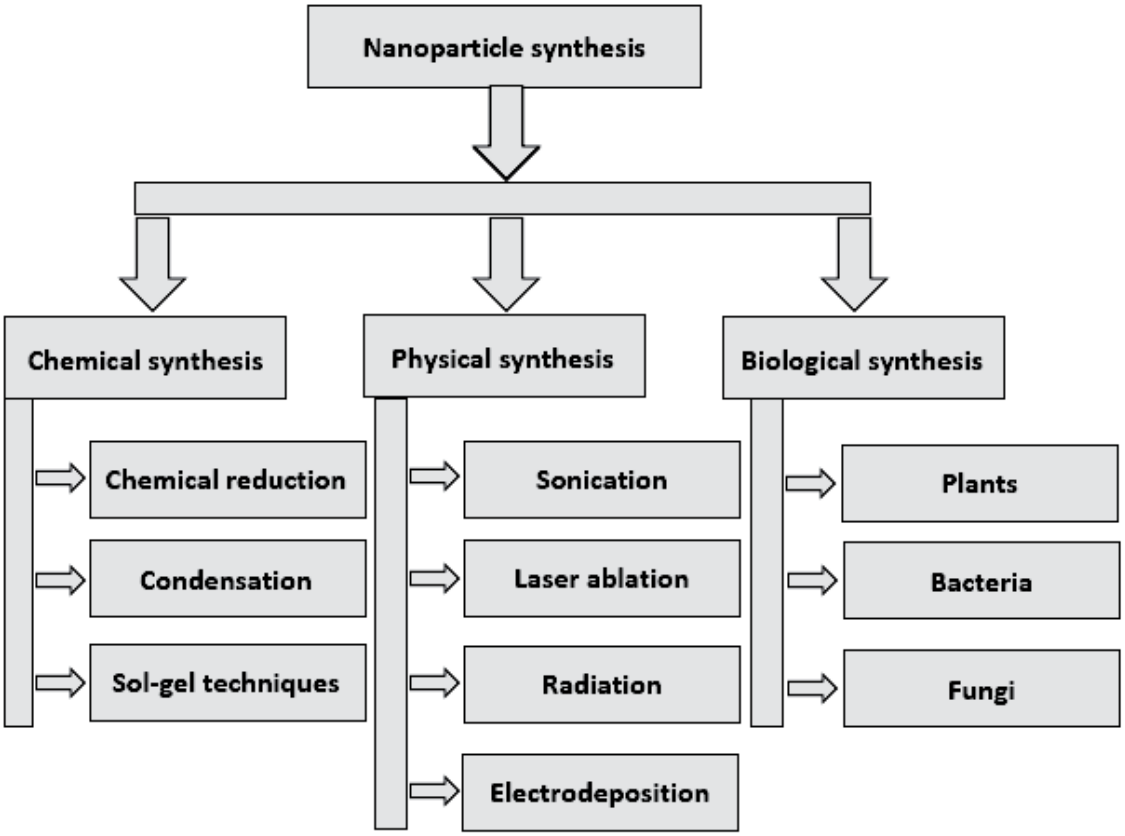

Fig. 1. Methods for NPs synthesis

in terms of energy input [10, 11]. NPs are produced in *in-vivo* biological systems including eukaryotes and prokaryotes [12].

The development of ecofriendly process that avoids the use of toxic chemicals is the need of hour. Different techniques such as chemical, physical, biochemical and green synthesis are used for synthesis of nanoparticles. Green synthesis in particular, has been used on a large scale in Pakistan. Green synthesis approaches such as have tremendous advantages over chemical method that involved the use of potential toxic agents [7]. Among the chemical approaches, the use of inorganic reducing agents and radiolysis are extensively used for nanoparticles synthesis. Several chemicals methods are still in developmental stages because they face aggregation problems of nanoparticles, as well as growth control, morphology and size distribution. Moreover, nanoparticles extraction and purification for further application are still imperative issues [13].

In Pakistan, chemical methods have not been extensively used for the production of nanoparticles [14, 15]. In physical methods the most widely used are evaporation-condensation and laser ablation for synthesizing nanoparticles. The absence of solvent contamination, size uniformity and distribution are the advantages over chemical approach. Physical methods also withstand potential disadvantages such as large space requirement and high energy consumption [16, 17]. Non-biodegradable compounds produced by the widely used physiochemical methods, (i.e. photochemical reduction, UV irradiation and ultrasonic fields); are potentially hazardous to the environment and biological systems [17].

1.1. Biological Synthesis

Medicinal plants are predominantly utilized in green synthesis methods as they have potential advantages [18]. Researchers are screening plants for the purpose of identifying new compounds of medical importance [19]. Contribution of Pakistani

Sr. No	Year	Number of Publications	Key Search Terms
1	2019	15	• Green Synthesis of Nanoparticles • Biosynthesis • Green Synthesis • Nanosynthesis
2	2018	29	
3	2017	21	
4	2016	19	
5	2010-2015	14	
	Total	98	

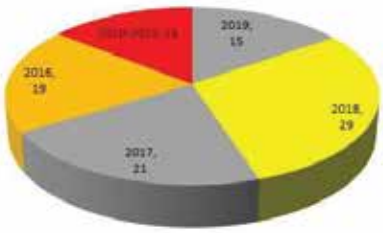

Fig. 2. Number of publication in the line of Bioinspired synthesis of nanoparticles from 2010 to mid of 2019 (Source: Web of Science).

Table 1. Green synthesis of nanoparticles from various plants extracts. All these studies are performed in Pakistan.

References	Plant species	Common name	Part used	Physical properties of NPs		
				Shape	Characterization	Size (nm)
Tahir et al. [23]	*Taraxacum laevigatum*	Dandelion	---	Spherical	UV, XRD, TEM, SEM, EDX, DLS and FTIR	2–7
Zia et al. [24]	*Cydonia oblong*	Quince	Seed	Cubic	UV, FTIR, XRD and SEM	38
Tahir et al. [25]	*Salvadora persica*	Toothbrush tree	Stem	Spherical	UV, XRD, FTIR,HRTEM, SEM and EDX	1–6
Tahir et al. [26]	*Sapium sebiferum*	Vegetable Tallow	leaf	Spherical	UV, FTIR, XRD, HRTEM, SEM, TGA and DLS	5
khan el al. [27]	*Sueda fruciotosa*	Shrubby sea blight	Whole	Spheroid	UV, XRD, SEM HRTEM and FTIR	6-8
Ahmad et al. [28]	*Fagonia indica*	Dhreima	Whole	hexagonal	UV, SPR, DLS, HRTEM, XRD, FTIR and EDX	15-20
Javed et al. [29]	*Stevia rebaudiana Bertoni*	Candyleaf	Shoot		UV, XRD, FTIR	34
Zia et al. [30]	*Lycopersicon esculentum, Vitis vinifera*	Tomato, Grape	Fruit	Cubic	FTIR, XRD, SEM,	10-30
Phull et al. [31]	*Bergenia ciliata*	Zakham-e-Hayat	Whole	Spherical	UV, SEM, FTIR	35
Zia, F. et al. [24]	*Cydonia oblong*	Quince	Seed	Cubic	UV, FTIR, XRD	38
Khan et al. [32]	*Citrus sinensis var. Kozan yerly*	Citrus	Fruit	Spherical	UV, XRD, EDX, HRTEM and FTIR	4-10
Ullah, I. et al. [33]	*Teucrium stocksianum Boiss.*	Togreyern	Shoot	Cubical	UV, XRD, SEM, DLS and FTIR	< 100
Hameed, S. et al. [34]	*Silybum marianum*	Milk thistle	Shoot	Plate and Spherical	UV, HR-SEM, HR-TEM and FTIR	~26-27

Abbasi, B. H. et al. [35]	*Cannabis sativa*	Marijuana	Leaf	Face centered cubic	UV, XRD, FTIR, EDX and SEM	38.94 [29], 45.3 (Ag)
Nazir, S. et al. [36]	*Silybum marianum*	Milk thistle	Seed	Spherical, Triangular	XRD, FTIR, SEM and EDX	64-70 (C), 63-65 (WPE)
Hassan, D. et al. [37]	*Callistemon viminalis*	Bottlebrush	Flower	Spherical	UV, HR-SEM HR-TEM, XRD, FTIR and EDS	32, 26, 22
Khalil, A. T. et al. [38]	*Sageretia thea*	Bird plum	leaf	Face centered cubic	XRD, FTIR, Raman, EDS, SAED, HR-SEM and HR-TEM	~ 27
Afridi, M. S. et al. [39]	*Verbena officinalis, Verbena tenuisecta*	Verbena, Moss verbena	leaf	Rod, Flower shaped	UV, FTIR, XRD, SEM and TEM	65–75, 14–31
Anjum, S. et al. [40]	*Linum usitatissimum L.*	Linseed	Seed	Face-centered cubic	UV, XRD, FTIR, SEM and EDX	19–24
Hassan, D. et al. [41]	*Callistemon viminalis*	Bottle Brush	Flower	Rhombo-hedral	UV, XRD, FTIR, HR/ SEM, TEM, EDX and SQUID	15, 17
Anjum, S. et al. [42]	*Phlomis bracteosa*	Jerusalem sage	Seed	Face-centered cubic	UV, FTIR, XRD, SEM and EDX	22.41
Khalil, A. T. et al. [43]	*Sageretia thea*	Bird plum	leaf	Face-centered cubic	XRD, HR-SEM, HR-TEM, EDS and ATR-FTIR	20.03
Khalil, A. T. et al. [44]	*Sageretia thea*	Bird plum	Leaf	Tetragonal	XRD, FTIR, Raman, EDS, HR-SEM/TEM and SAED	~30
Riaz, H. R. et al. [45]	*Catharanthusroseus-var. Alba*	Rosy periwinkle	Calli	Triangular	XRD, FTIR and SEM	77–79
Nasar, M. Q. et al. [46]	*Ephedra Procera C. A. Mey.*	Pinellia	Shoot	Spherical	UV, FTIR, XRD and SEM	17.2
Khalil, A. T. et al. [47]	*Sageretia thea*	Bird plum	Leaf	Hexagonal	XRD, FTIR, HR-SEM/ TEM, EDS, Raman, SAED and UV	12.4
Khalil, A. T. et al. [48]	*Sageretia thea*	Bird plum	Leaf	Spherical	XRD, FTIR, HR-SEM/ TEM, ATR- EDS, SAED and Raman	~18
Ovais, M. et al. [49]	*Olax nana Wall. ex Benth.*	Conchidium	Whole	Spherical/ nanorods	XRD, FTIR, SEM, TEM, DLS, EDX, and SAED	26 (Ag), 47 [29]
Ullah, I. et al. [50]	*Teucrium stocksianum*	Mekhzani	Shoot	Face-centered cubic	UV, XRD	10–15 (Che), 10–40 (Bio)
Shinwari, Z. K. et al. [51]	*Sageretia thea*	Bird plum	Leaf	Hexagonal	XRD, TGA/DSC, FTIR, HR-TEM and Raman	28.09 ± 5

Abbreviations: UV; Ultra violet, EDS; Energy-dispersive X-ray spectroscopy, SPR; surface plasmon resonance, SAED; selected area (electron) diffraction, FESEM; field emission scanning electron microscope, SEM; scanning electron microscopy, TEM; Transmission electron microscopy, FTIR; Fourier Transform Infrared Spectroscopy, NMR; Nuclear Magnetic Resonance, XRD; X-Ray Diffractometer, AFM; atomic-force microscopy, XPS; X-ray photoelectron spectroscopy, TGA; thermogravimetric analysis, FFT; fast fourier transform, WPE; Whole plant extract, Bio; Biological method, Che; Chemical method.

researchers in the field of bioinspired synthesis of nanoparticles has been illustrated in Table 1 and Figure 2. Redox potential of phytochemicals confer the antioxidant activity in plant extracts [20], due to which plants perform vital activities such as neutralizing the free radicals and oxygen quenching. The higher antioxidant potential of nanoparticles is assumed to be due to nanoparticles that has antioxidant material from the plant extract upon its surface. Particle size, surface area and surface reactivity are the factors that determine the toxicity of the nanoparticles [21].

2. APPLICATIONS OF NANOPARTICLES

Nanotechnology has many applications in the field of medicines, drugs delivery, drugs analysis, catalytic activity, waste water treatment, cancer treatment, antimicrobial, antibacterial, and antifungal activities. Latest studies revealed that AuNPs are used to achieve functional electric coating. AuNPs are best biosensors, used for diagnosing of medicinal problems inside the body. Nanoparticles are now used as antiviral against HIV, Hepatitis-B, monkey pox virus and many other viral diseases. Nanosilver can act as viricidal agent by inhibiting the initial stages of HIV-I cycle [22].

2.1. Antibacterial Activities of NPs Reported from Pakistan

In Pakistan, various studies have been conducted confirming antibacterial activities of Biologically synthesized nanoparticles. *V. vinifera and L. esculentum* derived AgNPs were used against *S. typhi, E. aerogenes*, *M. luteus*, *P. septica, S. aureus* and *B. subtilis* for their antibacterial assessment. It revealed its highest Zone of Inhibition (ZOI) of 26±1 mm against *S. aureus* [30]. *Bergenia ciliate* pharmacological assessment revealed broad spectrum antibacterial potential against multiple bacterial species [31]. AgNPs of *D. mucronata* revealed prominant antibacterial potency against *E. coli* and good it showed good potency against *P. vulgaris, P. aeruginosa, S. aureus, M. morganii*, *A. baumannii* and *VRSA* [52]. PtNPs of *Taraxacum laevigatum* showed substantial antibacterial potency against *B. Subtilis* and *P. aeruginosa* [23]. Palladium NPs (PdNPs) of *Sapium sebiferum* revealed substantial antibacterial activity against *B. subtilis* and *S. aureus*. The ZOI were reported as 29 (± 0.8 mm) and 19 (± 0.6 mm), while the said NPs exhibited moderate potency against *pseudomonas aeruginosa* [26]. AgNPs of tomato and grape juice exhibited significant bactericidal activity against *Staphylococcus aureus, Pseudomonas septica*, *Bacillus subtilis, Micrococcus luteus*, *Enterobacter aerogenes and Salmonella typhi* [30]. The most and least effective antibacterial potential of *Silybum marianum* mediated ZnO–NPs was reported against *B. Subtilis* and *P. aeruginosa*, while the effect of Ag–ZnO heterostructures against *B. subtilis* was similar and comparatively higher against *P. aeruginosa* [34]. *Cannabis sativa* leaf extract mediated bimetallic NPs used against five bacterial species exhibit bactericidal potency [35]. ZnO of *Silybum marianum'*s callus extract used against *Klebsiella pneumonia* and *Bacillus subtilus* through well- diffusion method exhibited antibacterial potential of 13 mm and 15 mm respectively against the tested bacterial strains. The aforementioned results of the ZnO were somewhat similar to the ZOI of the standard antibiotic Cefixime [36]. Iron oxide nanoparticles (IONPs) of *Callistemon viminalis* floral extract were used against nine gram negative and three gram positive bacterial strains for its bactericidal assessment at various concentrations through microplate-based technique that reports percent inhibition of bacterial strains. IONPs exhibited maximum antibacterial potential against *S. aureus, S. typhi, S. enterica* and *K. pneumonia* and exhibited least antibacterial potential against *S. dysenteriae* among all tested bacterial strains [37]. PbO NPs of *Sageretia thea* on its bactericidal potential assessment against Staphylococcus epidermis, *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Klebsiella pneumonia* and *Pseudomonas aeruginosa* revealed maximum antibacterial potential against *Klebsiella pneumonia* among the tested bacterial strains with a MIC of 31.25 µg/mL and minimum antibacterial potential against *Pseudomonas aeruginosa* with a MIC of 250 µg/mL [38]. Callus extract (CE) mediated AgNPs of *Linum usitatissimum* L. were used for its antibacterial assessment against potent multi drug resistant species (*S. aureus, K. pneumoniae* and *E. coli*). The antibacterial potential of CE based NPs was found more than the NPs synthesized from whole plant extract [40], while AgNPs of *Phlomis bracteosa* exhibited 11.1±0.10, 10.3±0.11 and 13.2±0.12 mm ZOI against the aforementioned three pathogenic

bacterial strains. They reported that antibacterial potency of the AgNPs of the *Phlomis bracteosa* was almost similar with that of Ampiclox (Standard antibiotic) [42]. Chromium oxide NPs (Cr_2O_3 NPs) of *Callistemon viminalis* exhibited dose dependent bactericidal response against the tested bacterial strains in the microplate method. *K. pneumoniae, P. vulgaris, C. sakazakii* and *V. cholera* were found more susceptible to Cr2O3 NPs with MIC of 12.5µg/ml and two bacterial strains (*Citrobacter, S. Aureus*) were found less susceptible to Cr2O3 NPs with a MIC 50 µg/ml [41]. Cobalt oxide (Co3O4) NPs of *Sageretia thea* was used against six bacterial strains, that comprised three gram negative and three gram positive bacteria. The NPs exhibited more lethality against *E. coli* and *S. aureus* while the least lethality was recorded against *Pseudomonas aeruginosa* [43]. *Sageretia thea* mediated IONPs were used against five bacterial strains at various concentrations ranging from 1000–31.25 µg/ml. Maximum activity of biogenic IONPs was reported against *S. epidermidis* and *P. aeruginosa* with 7.8 µg/ml MIC for both of the strains [44]. *Ephedra Procera* mediated AGNPs on its antibacterial assessment revealed MIC of 11.33 µg/ml and 11.12 µg/ml against *E. coli* and *B. subtilis* respectively that was highest reported activity while against *P. aeruginosa,* moderate activity was observed. *S. aureus* and *S. epidermidis* were resistant to the EPNPs [46]. ZnONPs of *Sageretia thea* exhibited maximum and minimum activity against *K. pneumonia* and *P. aeruginosa* respectively that were used in different concentration ranging from 2000 to 62.5 µg/ml [46]. Nickle oxide (NiO) NPs of *Sageretia thea* showed its maximum efficacy against *Bacillus subtilis* and *E. coli*. The reported ZOI were 15.1 mm and 14.1 mm respectively against the said pathogens. The least efficacy was reported against *K. pneumonia* and *P. Aeruginosa* [48]. Silver and gold NPs of *Olax nana* were used against four bacterial strains at 62.5–2000µg/ml concentration range. AgNPs revealed strong antibacterial potency against *Staphylococcus epidermidis* and *Escherichia coli* with MICs of 7.14µg/ml and 8.25µg/ml respectively, while AuNPs were found active only against *Staphylococcus aureus* with MIC of 9.14µg/ml [49]. CE-mediated ZnONPs of *Catharanthus roseus* produced, ZOI of 7 ± 1.25 mm for *E. coli* and 13 ± 0.7 mm for *B. subtilis* and no ZOI was recorded against *P. aeruginosa.* Melatonin and NAA stimulated CE-mediated ZnONPs were more active against *E. coli, P. aeruginosa* and *B. subtilis* with ZOI of 10 ± 0.57, 13 ± 0.54 mm and 17 ± 0.76 mm, respectively [45]. The above discussion suggests antibacterial properties of several biosynthesized nanoparticles however; there is no commercialized product in the country-Pakistan. As these products have potentials to address the emerging issue of antibiotics resistance therefore, it can be developed and marketed.

2.2. Antifungal Activities of NPs from Pakistan

Antifungal assessment of *Bergenia ciliate'*s NPs revealed antifungal potential against various fungal species [31]. AgNPs of *D. mucronata* leaves were used for its antifungal assessment and it only showed its potency against *C. albicans* and *A. niger* [52]. *Silybum marianum*-mediated ZnO–NPs and Ag–ZnO heterostructures, used against four fungal pathogenic strains at a concentration of 1000–50µg/ml were considered insignificant in comparison with standard antibiotics [34]. The Iron oxide-NPs of *Sageretia thea* exhibited highest percent inhibition of 79.03% ± 2.90, 74.58% ± 3.15 and 74% ± 3.20 against *R. solanai* followed by *A. fumigatus* and *M. racemosus* at a dosage of 2 mg/ml [44]. Antifungal assessment of *Ephedra Procera*'s NPs revealed considerable potential against *A. flavus* and *A. niger* while moderate activity against *Mucor spp* [46]. ZnONPs of *Sageretia thea* were used against five fungal strains that revealed linear growth inhibition against all tested fungal strains. *M. racemosus* and *A. fumigatus* were inhibited at all tested concentrations of the said NPs [47]. NiO NPs of *Sageretia thea* exhibited maximum fungicidal potential against *M. racemosus* and *R. solani* with percent inhibition of 64% and 63.2% respectively at 2 mg/ml concentration while it exhibited minimum fungicidal potential against *A. flavus* [48]. Future research could be directed towards commercial production of nanoparticle-based antifungal agents that can replace or complement conventional pharmaceutics in Pakistan.

2.3. Antioxidant Activities of NPs Reported from Pakistan

Plants contain multiple chemical constituents that have extensively studied to find their pharmacological importance. Among these constituents, major chemical groups such as

flavonoids and polyphenols are reported as reducing agents. These chemicals have antioxidant potentials through which plant protect itself from oxidative stress. During NPs synthesis, the said chemical groups play important role in Ag +ions reduction [53]. Free radical scavenging potential of plant extracts and its derived AgNPs has been reported in various studies. AgNPs synthesized from the fruit extract of *Citrus sinensis var. Kozan yerly* has a good antioxidant potential, assessed through 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay [32].

The development of reducing power is considered as causative reason for the antioxidant potential and this phenomenon has been demonstrated in various studies [54]. AgNPs are supposed to be electron donors, thus convert the free radicals to more stable form through donation of electrons [32]. AgNPs synthesized from tomato showed 76% and 83% free radical inhibition at 20 and 30 mM concentration. NPs synthesized from grapes were also significant for the said property. AgNPs of *Bergenia ciliata* were synthesized that showed 59.31% free radical scavenging potential in DPPH assay. The antioxidant potential (60.48±2.2) ascorbic acid equivalent (AAE) was reported of *B. ciliate* AgNPs [31]. *Daphne mucronata* mediated AgNPs exhibited its highest antioxidant potential of (85.4% AAE) at 600 µg/ml concentration [52]. AgNPs of *Lycopersicon esculentum* fruit juice exhibited 83% free radical scavenging potential at 30 mM concentration of AgNO3 while the AgNPs of *Vitis vinifera* also exhibited prominent potential. The highest antioxidant potential of *Lycopersicon esculentum* fruit juice mediated AgNPs was 5.8 µg AAE/100 µg and that of *Vitis vinifera* was 8.3 µg AAE/100 µg, respectively [30]. *Silybum marianum* mediated ZnO and Ag–ZnO revealed highest antioxidant potential of 67.6±1.44 and 72.6±1.32 AAE respectively at 1000µg/ml concentration [34]. IONPs of *Callistemon viminalis* floral extract revealed its maximum free radical trapping potential of (55.16 ± 1.34% AAE) at the concentration of at 200mg/mL. The dose dependent antioxidant activity increased with an increase in the IONPs concentration and in antioxidant activities, analogous pattern was reported. The maximum power of reduction (51 ± 2.4% AAE) was reported at 200mg/mL concentration of IONPs [37]. PbO NPs of *Sageretia thea* exhibited 58 % AAE free radical trapping potential in DPPH assay. The scavenging potential and NPs concentration were found in direct relation. At 200 µg/ml, Moderate reducing power and moderate antioxidant capacity of 22 µg AAE/mg and 19.6 µg AAE/mg were reported respectively [38]. Co3O4 NPs of *Sageretia thea* exhibited 57 % free radical trapping potential in DPPH assay at 200 µg/ml concentration. At 200 µg/ml, maximum total reduction potential and total antioxidant potential of 19.8 and 23.6 µg AAE/mg was reported respectively [43]. The highest total antioxidant potential (38.21 ± 1.93 AAE) of *C. viminalis*'s α-Cr2O3 NPs was reported at 200µg/ml. The quenching ability of the said plant can be associated with phenolic compounds present in *C. viminalis* that might be involved in the capping of the α-Cr2O3 NPs. Maximum DPPH scavenging potential of 57.69 ± 2.19 AAE was reported at the concentration of 200µg/ml [41]. The highest total antioxidants potential of *Sageretia thea*'s ZnONPs was 25.6 ± 1.54 AAE/ milligram for the tested concentration of 200 µg/ml. In DPPH assay moderate radical scavenging (63.5 ± 2.4) was observed at 200 µg/ml and minimum scavenging was reported at 25 µg/ml [47]. NiO NPs of *Sageretia thea* revealed 65% to 3.27% DPPH radical quenching potency at a concentration range of 200lg/ml to 1lg/ml and 11.3lg AAE/mg antioxidant potential was observed at 200lg/ml [48]. These studies suggest that bioinspired NPs have potential for antioxidant activities that can be manipulated for formulation of different commercial products.

2.4. Cytotoxic Activities Assessed for NPs Reported from Pakistan

Daphne mucronata mediated AgNPs revealed a higher cytotoxicity of 73.33% and LD^{50} of 5.074 µg/ml [52]. AgNPs of *Bergenia ciliate* revealed enhanced cytotoxicity with a LD50 of 33.92µg/ml in brine shrimp lethality assay. The cytotoxic behavior of NPs can be associated with anticancer potential [31]. Cytotoxic potential of *Silybum marianum* mediated ZnO–NPs and Ag–ZnO heterostructures used against *Artemia salina* larvae revealed its highest activity of 80% and 70% (at 1000 µg/ml), respectively [34]. AgNPs of *Teucrium stocksianum* leaf extract revealed cytotoxicity against J774 macrophage cells. The NPs of the aforementioned plant extract successfully inhibited cells propagation with inhibitory concentration (IC50) of 110.98 µg/ml [33]. The PbO of *Sageretia*

thea exhibited dose dependent cytotoxic response against *Artemia salina* (Brine shrimp) with the median lethal dosage 27.74 µg/ml [38] while the median lethal dosage of cobalt oxide NPs of the said against the said species (Brine shrimp) was reported 19.18 µg/ml [43]. IONPs of *Sageretia thea* revealed mortality of 100% against brine shrimps at 200 µg/ml concentration [44]. *Ephedra procera* mediated AgNPs on its cytotoxic assessment revealed cytotoxic potential against HepG2 Cells with 61.3 and 247µg/ml as inhibitory concentration (Median) [46]. Zinc oxide nanoparticles (ZnO NPs) of *Sageretia thea* revealed its IC_{50} as 21.29µg/ml with 80% cytotoxic response against *Artemia salina* [47]. Nickel oxide nanoparticles (NiO NPs) of *Sageretia thea* has shown IC50 value as 42.60l g/ml against brine shrimp [48]. Silver nanoparticles (AgNPs) of *Teucrium stocksianum* were assessed through MTT assay that revealed 80% growth inhibition at 266µg/ml [50]. Further research can be directed towards understanding of molecular pathways (of different NPs) that are involved in the mechanism of NPs-mediated cytotoxicities. This will help to differentiate the products/NPs that have anticancer potential.

2.5. Anticancer Activities of NPs Reported from Pakistan

AgNPs of *Teucrium stocksianum*'s aqueous extract revealed dose dependent upsurge in its anti-oncogenic potential against Michigan Cancer Foundation-7 (MCF-7), a breast cancer cell line. Additional killing of the aforementioned cancer cell line was observed when the cell line was exposed for longer duration [33]. Bioinspired IONPs of *Callistemon viminalis* used against HepG2 cells for its cytotoxic assessment through MTT assay in multiple concentrations ranging from 500 to 7.8mg/ml. IONPs exhibited substantial cytotoxicity against the said cell line with 80% mortality of oncogenic cells at 500mg/ml. The anticancer potential of the NPs reported was in direct relation with the concentration of the NPs as the activity increased with increase in the concentration of *Callistemon viminalis*'s NPs [37]. *Callistemon viminalis* mediated α-Cr2O3 NPs used for the assessment of its anticancer activity against HepG2 revealed dose dependent inhibitory potency with a lethal concentration (median) of 46.32µg/ml. The results revealed that the said NPs were found effective even with a low dosage of 7.8µg/ml [41]. *Olax nana*'s silver and gold NPs were used against HepG2 cell line with multiple dose concentrations (3.9–500µg/ml). Preferential dose dependent cytotoxic potency of the NPs was revealed. IC_{50} values of AuNPs was 2.97 µg/ml, followed by AgNPs (14.93 µg/ml). IC50 value of crude extract of the said plant was > 200µg/ml [49]. Bioinspired-NPs could be developed as a low cost therapeutic option against different kinds of cancers. However, it needs further research and experimental trials.

2.6. Photo Catalytic Activities of NPs Reported from Pakistan

The living environment sustains damage from industries that leave untreated hazardous soluble organic and inorganic pollutants. Synthetic dyes are considered more toxic for living organism due to their carcinogenic potential [55]. Methylene blue (MB) is used in textile industries can cause a list of disorders such as skin irritation, eye burns and gastrointestinal complication and many more. Photo degradation is extensively used waste water processing [56, 57].

Multiple approaches are employed for organic dyes removal from polluted water [58, 59] but they sustain limitations such as high cost and energy consumption [60]. Biologically synthesized AgNPs using *Salvadora persica* stem extract was used as a photo degradative tool for MB as the method is comparatively energy and cost effective. The photo degradation of MB by AgNPs in the presence of light was reported by the sharped decline in the absorption peak compare to controlled sample [25]. PdNPs of *Sapium sebiferum* leaf extract exhibited prominent outcome in the decomposition of MB. Significant photocatalytic potency of 90% was reported after 70 minutes at a concentration of 10 ml [26], while the Gold NPs (AuNPs) of *Fagonia indica* revealed 80% photocatalytic activity of the said chemical in a time span of 80 minutes. The AuNPs also exhibit photocatalytic activity against an organic pollutant, nitro phenol [28]. *Sueda fruciotosa* AuNPs also exhibit significant photocatalytic potency against MB [27]. As most of the commercial dyes from industries are left untreated that pollutes our environment. Research should be directed towards NPs-mediated treatment of these pollutants on mass scale.

3. CONCLUSION

The applications of nanotechnology are extended to multiple scientific fields; ranging from drug development, drug delivery, gene delivery, diagnostics and energy and other fields. These nanostructures have triggered a significant attention towards its use due to their unique characteristics such as small size and shape that make these nanoparticles competent for a wide range of medical and pharmacological applications. There are several reports from Pakistan on the multidisciplinary basics concepts of biosynthesized NPs however; there is a tremendous potential for commercial production of these NPs-based products.

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Proceedings of the Pakistan Academy of Sciences:
B. Life and Environmental Sciences 56 (3): 49–56 (Special Issue, 2019)

ISSN: 2518-4261 (print), ISSN 2518-427X (online)

Pakistan Academy of Sciences

Review Article

Elicitation strategies of *in-vitro* cultures for the sustainable use of medicinal plants

Tariq Khan[1*], Bilal Haider Abbasi[2], and Mubarak Ali Khan[3]

[1]Department of Biotechnology, University of Malakand
[2]Department of Biotechnology, Quaid-i-Azam University Islamabad
[3]Department of Biotechnology, Faculty of Chemical and Life Sciences,
Abdul Wali Khan University Mardan (AWKUM), Mardan 23390, Pakistan

Abstract: Medicinal plants are highly traded for its promising potential against different types of diseases including cancers. Development of elicitation strategies for increased production of important anticancer compounds from *in vitro* cultures of medicinal plants has proved very productive. For this purpose, different stresses are applied to *in vitro* cultures to produce increased amounts of the compounds. For instance, cell cultures are produced via stem explants in the Murashige & Skoog basal medium supplemented with different concentrations of plant growth regulators (PGRs). The extracts from samples are then subject to flavonoid and phenolic content assessment, antioxidant quantification and Chromatographic analysis. In our experiments, among the many PGRs, Thidiazuron (TDZ) triggered higher quantities of biomass and total flavonoid & phenolic content (191.03 µg quercetin/mg and 202.8 µg gallic acid equivalent/ mg, respectively) through cell cultures of *F. indica*. Similarly, sucrose induced the maximum biomass among the different carbon sources (fructose, glucose, maltose, and sucrose) given in different concentrations to cell cultures of *F. indica* while glucose produced the maximum phenolic content followed by fructose when harvested after 42 days. Manipulation in the supply of light to the cultures with a combined effect from other chemicals, a significant effect was seen on growth and secondary metabolism such that dark-grown cell cultures treated with Methyl Jasmonate (Me-J) gave the highest TPC. High-performance liquid chromatography analysis revealed an increased quantity of secondary metabolites. In conclusion, cell cultures of *F. indica* treated with Thidiazuron and grown in dark in the presence of glucose as a sugar source and Me-J as elicitor gives enhanced quantities of important anticancer secondary metabolites.

Keywords: *F. indica, Callus*, Thidiazuron, *Anticancer, in vitro*

1. INTRODUCTION

Herbal medicine is the primary source of medical treatment in developing countries. Infact, the world health organization (WHO) describes herbal medicine as the major source of medicine (80%) in the world [1]. Asian countries such as Pakistan are rich in diverse medicinal plants [2, 3]. Plants that have been used for anticancer, anti-fever, anti-hyperglycemic, anti-inflammatory, antiseptic, antiviral, hepato-protective, ischemic, immune-stimulating, sedative, and spasmolytic properties [4-7]. Therefore, research has been conducted in-depth on the investigation of compounds in the extracts of different parts from many different medicinal plants. The components of medicinal plants harbor a wide range of medicinal compounds that have promising healing as well as preventive properties. It is, however, asserted that individual plants grown naturally do not contain enough medicinal compounds. These need to be concentrated in extracts which in turn need harvesting a huge amount of plant biomass to extract only ample quantities of medicinal compounds. This causes overharvesting and thus a threat to the existence of important plant species. Further to endangerment, seasonal and geographic dependence, climate dependence, and non-uniform metabolic profile of wild-grown plants calls are some limitations in the use of wild-grown medicinal plants [8]. This calls for the need of *in-*

Received: February 2019; Accepted: September 2019
* Corresponding Author: Tariq Khan; tariqkhan@uom.edu.pk

vitro cultures to grow important medicinal plants that are independent of season, geography, climate and have a uniform and novel medicinal compounds expressed through manipulations [9]. Apart from these advantages, *in-vitro* cultures offer room for strategies that can enhance the production of these medicinal compounds. This type of manipulation is known as elicitation [10].

An example of such an important plant that we work on in our lab is *Fagonia indica*. *F. indica*, commonly known as "true herb", is famous for its variety of medicinal activities especially anticancer potential. It is a member of the genus *Fagonia* and family *Zygophyllaceae*. The species of the genus *Fagonia*, especially, *F. indica* is famous for its diverse class of medicinally important compounds including terpenoids, flavonoids, and other polyphenolic compounds. The medicinal activities of *F. indica* especially antioxidant and anticancer activities may be attributed to its phenolic compounds. However, isolation only from wild-grown *F. indica* does not guarantees sustainable production of these metabolites. This is because of limitations with wild-grown plants such as over-harvesting, endangerment, seasonal and geographic dependence and variations in metabolic profiles of the plant. *In-vitro* cultures promise to deal with these limitations as they are independent of seasons and geography. Especially, cell cultures promise sustainable, uniform and homogeneous production of secondary metabolites. The current study highlights the various strategies for the enhancement of phenolic compounds through the establishment of feasible cell cultures of medicinal plants.

2. MATERIALS AND METHODS

This is a research review article that mainly focuses on strategies used with different plants for the elicitation of phenolic compounds. For reporting the data as secondary research, a thorough search was performed through scholarly databases including google scholars, PubMed, the web of science and Scopus. The search terms used alone or in combination were "medicinal plants", "herbal medicine", "plant metabolites", "plant *in-vitro* cultures", "elicitation", "and phenolic compounds".

3. RESULTS

3.1. Strategies for Production of Higher Biomass and Secondary Metabolites

Manipulations during *in-vitro* growth of plant tissues and cells bring changes to the accumulation of biomass and secondary metabolites in the cultures. Such manipulations are done at various levels with the aim of increasing biomass accumulation and production of medicinally important secondary metabolites. The many different strategies to manipulate *in-vitro* growth of plant cultures for increased yield can be classified into two broad categories: Manipulations in the medium composition, and environmental conditions.

3.2. Manipulations in the Medium Composition

The composition of the media has a prominent effect on the growth of *in-vitro* cultures and the production of secondary metabolites. The standard medium used for *in-vitro* cultures is MS medium. MS medium is comprised of MS salts (4 g/L of water) mixed with carbohydrate in the form of sucrose (30 g/L) and a gelling agent such as agar (8 g/L) [11]. Any type of manipulations in the basic composition of medium or addition of extra ingredients will affect the growth of cultures and production of secondary metabolites directly. For instance, changing the type and concentration of carbohydrate, mineral salts and introduction of several types and concentrations of PGRs such as auxins and cytokinin will drastically affect the growth parameters and secondary metabolites *in-vitro* [12, 13]. The most important media manipulations can be further classified as follows.

3.2.1 Manipulations of Carbohydrate Type and Concentration

In-vitro cultures require a continuous carbohydrate source in the media. As discussed above, the standard carbohydrate used in MS medium is sucrose at the ratio of 30 grams per liter of the medium. Sucrose is preferred because it is easily transported across the membranes and unlike monosaccharides, it is not rapidly metabolized and thus available for growth for a longer time [14]. Sucrose acts both as a metabolite and signaling molecule in plants and thus altered levels change the quantity of sucrose derived metabolites and sucrose-specific signaling

that in turn affects the plant growth, development, and physiology [15]. Carbohydrates like glucose, fructose, and maltose have been employed in media for *in-vitro* cultures to enhance the yield of secondary metabolites. For instance, callus cultures of *Gossypium hirsutum* L. (cotton) when grown in the presence of different sugars, showed that increasing the sucrose concentration increased the secretion of phenolic compounds. Similarly, increased biomass accumulation was observed in the presence of 3% maltose compared to other types of sugars [16]. Plantlets of *Metroxylon sagu* also showed better results in response 3% sucrose supplementation compared to other types of sugars. Therefore, the alteration in the sugar type and concentration from the generally optimized protocol results in the higher secretion of phenolic compounds and other secondary metabolites.

3.2.2 The Effects of Changes in Mineral Composition

Mineral salts in the media play a pivotal role in the growth and metabolism of the plant *in-vitro* cultures. MS medium is comprised of major and minor salts i.e. macro and micronutrients and they make an indispensable part of the medium [17]. These salts contain different elements notably nitrogen, magnesium, phosphorus, calcium, sodium, zinc and iron. All these are used as salts in the medium at different ratios, some being higher and some in minute quantities [11]. Changes in the ratio of these elements affect the ratio of mineral salts which in turn affect the growth and secondary metabolism of the plants. For instance, changes in nitrogen level directly affect the ratio of nitrate to ammonia which alters the course of secondary metabolism *in-vitro* [18]. Numerous studies have shown that increasing the levels of NO_3^- in proportion to NH_4^+, results in increased metabolite content. Increased levels of NO_3^- has a stimulatory effect on the yield of with anolide A and gymnemic acid in hairy root cultures of *Withania somnifera* [19]. The effect of nitrogen levels on cell and tissue cultures of different plants such as *Capsicum annuum*, *Solanum laciniatum*, *Artemisia annua*, and *Morinda cetrifolia* have been evaluated and optimized long ago [20]. Another essential macronutrient, phosphorus in the form of phosphates is also a vital component of the medium required for plant growth. The concentration of phosphorus affects the growth of plant cultures and influences the production of secondary metabolites. For example, Abdolzadeh, Wang [21] showed that deficiency of phosphorus resulted in the death of leaves in *Lupinus* species while excess or higher concentration inhibited cluster root formation in the plant. Furthermore, altering the levels of phosphates changes the production of secondary metabolites. For example, studies have reported elevated levels of different metabolites such as phenolics and alkaloids in *Gymnema sylvestre* [22], *Catharanthus roseus, Peganum harmala* and *Nicotiana tabacum* [23].

3.2.3 Effects of Plant Growth Regulators

PGRs are signaling molecules, actively involved in the regulation of growth and metabolism of plants. The two main classes of PGRs; auxin or cytokinin, are widely studied for their effects on plant growth, development, and secondary metabolism. Auxins are either natural such as indole-3-acetic acid (IAA), PAA, and indole-3-butyric acid (IBA) or synthetic such as NAA, 2,4-). Auxins play a vital role in cell division, elongation, and differentiation, and substantially influence the structure and function of cells and tissues [24]. Similarly, cytokinin such as BA, and Kinetin (Kn) are also reported to influence plant growth and development greatly [25, 26]. Different studies have demonstrated the role of cytokinin in the regulation of many aspects of plant growth and development including embryogenesis, root, and shoot branching, meristematic activity, phyllotaxis, and vascular development [27]. For example, studies have shown BA as the most suitable PGR for somatic embryogenesis in *Hygrophila spinosa*, *Sapindus mukorossi*, *Albizia lebbeck* [28, 29]. The role of auxins and cytokinin in secondary metabolism has been studied in many different plants [20]. For instance, NAA or IAA has a stimulatory effect on secondary metabolites production [23]. Similarly, Saeed, Ali [30] found that the administration of exogenous PAA to adventitious root cultures enhances the production of important phenolic compounds. PGRs other than auxin and cytokinin, such as Gibberellins, is also known to enhance the production of secondary metabolites [31]. Other studies have reported the combined use of auxin and cytokinin [32, 33]. One important PGR is TDZ, which is classified as cytokinin but believed to play roles both as auxin and cytokinin [34]. This PGR has a significant effect on *in-vitro* morphogenesis with roles in the initiation of callus cultures, shoots, somatic

embryos [34] and regeneration [35]. TDZ has been found very effective in yield-enhancement in secondary metabolism [36]. For instance, TDZ based enhancement of phenolic compounds has been reported in callus cultures of *Artemisia absinthium* [37] and *in-vitro* grown plantlets of *Cucumis anguria* [38]. Similarly, application of TDZ to callus and suspension cultures of *Salvia fruticose* resulted in the enhanced production of rosmarinic acid [39].

3.2.4 Chemical Elicitation of Secondary Metabolism

Secondary metabolism in plants is basically its defense system, producing chemical compounds that cope with damage induced by external and internal stresses. This means that application of any exogenous stress agent will stimulate the secondary metabolism of plants leading to the generation of chemical compounds, called secondary metabolites [40]. Secondary metabolites, because of their antioxidant nature, are important medicinal compounds. Strategies are applied during *in-vitro* cultures to enhance the production of these valuable metabolites. The process of triggering the metabolic pathways to produce metabolites in higher amounts is called as elicitation and the agents used for the process are called elicitors. Elicitors may be biotic or abiotic compounds [41]. Biotic elicitor may come from fungi, bacteria, animals and the same plant when it acts on invading pathogens or other plants while abiotic elicitors may be inorganic chemical compounds, metallic ions and very recently metal nano products [42] and environmental stresses. Elicitation through environmental stresses such as irradiation is discussed in the next section (1.9.2). There are many reports available on the effects of different biotic elicitors such as the plant hormone, jasmonic acid and its derivatives [43]. Jasmonic acid (JA) and Me-J belong to a family of cyclopentanone compounds that show a variety of responses in plant systems. Me-J is involved in elicitation of secondary metabolism to produce a diverse number of different metabolites [44]. For example, it enhanced the production of important phenolic compounds in adventitious roots cultures of *Ajuga bracteosa* [30]. Other studies have shown the stimulatory effect of Me-J on cell suspension [45], adventitious roots [46] and hairy root cultures of *Panax ginseng* [47]. Other types of biotic elicitors include Salicylic acid, bacterial extracts, fungal extracts, chitosan, and plant cell wall derivatives [44].

3.2.5 Environmental Manipulations

Culture environment is a key player in the growth and development of *in-vitro* plant cultures. Culture condition such as air supply, temperature, irradiation, and medium pH, etc. directly affect the accumulation of biomass and secondary metabolites from *in-vitro* cultures [23]. Manipulations in any of these culture conditions can be exploited as a process of elicitation of secondary metabolites. For example, numerous studies have reported the use of different temperature regimes on the accumulation of important secondary metabolites. For example, during *in-vitro* cultures of *Hypericum brasiliense*, temperature regimes either lower (17ºC) or higher (36ºC) than the normal (25ºC) induced higher phenolic compounds [48]. Similarly, Zobayed, Afreen [49] found that increasing the temperature during *in-vitro* growth of St. John's wort reduced the photosynthetic capacity and increased the accumulation of important secondary metabolites such as hypericin, pseudo-hypericin, and hyperforin in shoot tissues. Other conditions such as aeration (exchange of gases like carbon dioxide and oxygen) and pH drastically affect the growth and secondary metabolism of plants *in-vitro* [20]. Other abiotic elicitors include chemicals such as acetic acid, CO_2, ethanol, mercuric chloride ($HgCl_2$), copper sulfate ($CuSO_4$), metal ions and physical factors such as drought, extreme temperature shock, high-pressure inorganic salts, and irradiation [41].

3.2.6 The Effects of Light on Secondary Metabolites Accumulation

Light has been studied extensively for its effects on *in-vitro* growth and secondary metabolites accumulation in plants. Light is an important parameter that affects plant cultures in an array of ways ranging from its effect on growth and development [50] to primary and secondary metabolism [51]. Manipulations in light regimes are considered a very effective way of eliciting secondary metabolites in different *in-vitro* cultures. Light is the most important environmental factor affecting the biosynthesis of phenolic compounds [52]. Khan, Abbasi [53] reported enhanced silymarin content in plantlets of *Silybum marianum* grown under the effect of 2 weeks dark and 2 weeks

light. Similarly, Shohael, Ali [51] reported a high total phenolic and flavonoid content in somatic embryos of *Eleutherococcus senticosus* under the effect of fluorescent light. Manipulations in light regimes have been shown to enhance the production of caffeic acid derivatives in hairy root cultures of *Echinacea purpurea* [54], total phenolic production and total secondary metabolites in cell suspension cultures of *Artemisia absinthium* [55], lignans and neolignans in cell cultures of *Linum usitatissimum* [56] and piperine production in *Piper nigrum* [57]. Besides duration of light, intensity and wavelength have also been found to significantly affect the production of secondary metabolites. For example, changing the wavelength of light enhanced antioxidant secondary metabolites in callus cultures of medicinally important *Prunella vulgaris* [58] and *Artemisia absinthium* [59].

4. CONCLUSIONS

In conclusion, elicitation of *in-vitro* cultures to produce commercially and medicinally important phenolic compounds is an important and promising strategy for enhancement. It has the potential to control the overexploitation of medicinal plants as well as regulating the costs associated with producing effective herbal medicine. Elicitation produces uniform metabolic profile as well as can be geared to produce novel secondary metabolites in *in-vitro* cultures of medicinal plants. Diverse and comprehensive studies are needed for the elicitation of the different plant *in-vitro* cultures to build a profile of different effective elicitors for commercially enhanced production of medicinal compounds from plants.

5. ACKNOWLEDGEMENTS

The authors acknowledge the role of higher education commission in funding the Ph.D. studies of the principal author and the Pakistan Academy of Sciences for providing the platform to present this study.

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Proceedings of the Pakistan Academy of Sciences:
B. Life and Environmental Sciences 56 (3): 57–67 (Special Issue, 2019)

ISSN: 2518-4261 (print), ISSN 2518-427X (online)

Pakistan Academy of Sciences

Research Article

Comparative Analgesic Activity of Selected Medicinal Plants from Pakistan

Huma Shareef[1*], Sadaf Naeem[2], and Erum Zaheer[3]

[1]Department of Pharmacognosy, Institute of Pharmaceutical Sciences,
Jinnah Sindh Medical University, Karachi
[2]Department of Pharmacy, Institute of Pharmaceutical Sciences,
Jinnah Sindh Medical University, Karachi
[3]Department of Pharmaceutical chemistry, Institute of Pharmaceutical Sciences,
Jinnah Sindh Medical University, Karachi

Abstract: Pain is a natural self-protective system which is always unpleasant both mentally and physically with probable tissue harm. Analgesics are the medicinal agents which relief the pain without losing consciousness. Medicinal plants are being widely used all over the world for human healthy life form. We selected ten Pakistani plants which play an important role as traditional medicine in the prevention and management of acute and chronic diseases. All plants were extracted with ethanol and have been investigated for analgesic effects using hotplate and writhing reflex tests in rats. Phytochemical screening revealed the presence of polyphenols, saponins, sugar, flavonoids, and alkaloid. In our research, the analgesic effect of selected medicinal plants shows significant effect like standard aspirin and diclophenic. The intake of these aforementioned phytochemical flavonoids and polyphenols are considered to be responsible for pain management because they may contain many powerful antioxidant and free radical scavenging capabilities which are the backbone of other bioactivities such as anti-inflammatory action, anticancer, anti-aging, and protective action for cardiovascular diseases, diabetes mellitus, obesity and neurodegenerative disorders. In future, these plants may be utilized as a health benefit in view of their potential research in the prevention and management of pain.

Keywords: Analgesics, Medicinal plants, Non-steroidal anti-inflammatory (NSAIDs), Pain, Phytochemicals, Side effects.

1. INTRODUCTION

1.1. Pain & its Types

Pain is described as a condition of discomfort and anxiety. It is an indication of some trauma, injury, diseased state or even emotional misery. Pain is a vital feature of the body's defense mechanisms & it provides an urgent warning to transmit instructions to the motor neurons of the central nervous system to reduce and prevent further physical damage. Pain functionally is of two broad types' i.e. acute pain and chronic pain. Acute pain is for the short span of time and its causes are easily recognizable. It is alertness to a sudden damage to a tissue or any disease. It is quite rapid and sharp immediately followed by throbbing pain. Chronic pain is the pain which persist for far longer duration than the acute pain. Chronic pain can be mild or severe, continuous or intermittent and is usually difficult to subside than acute pain. Pain can also be divided into different groups on the basis of its source as well as associated neurons such as somatic pain, referred pain, visceral pain, neuropathic pain, cutaneous pain, phantom pain and central pain [1]. Pain is an immense problem as evaluations indicate that 20% of adults experience pain globally and further 10% are diagnosed every year which suffer chronic pain. Pain is considered as a medical problem but is given less attention by the stake

Received: February 2019; Accepted: September 2019
* Corresponding Author: Huma Shareef; huma.phr77@gmail.com: huma.shareef@jsmu.edu.pk

holders of public health. Pain is not having same prevalence in all countries although it can affect all populations. Pain is an unpleasant experience which can be faced by people of any sex, age group, race and geographical region. It can be recurrent, acute, chronic or combination of all.

1.2. Causes & Consequences

Major causes of pain are spinal injuries, arthritis (Rheumatoid and osteo both), different types of cancers and postoperative conditions. Several consequences of pain have also been identified which are troublesome such as disturbed concentration, inability to perform any physical tasks, anxiety and depressed personality, disturbed social behavior and suicidal views **Fig. 1** [2] shows some typical causes & consequences of chronic pain. Pain has gained great level of impact among general public because of its high rate of occurrence. Pain is a multifaceted experience which is complicated to determine and quantify. About 10% of the total world's population is a victim of chronic pain that means 60 million people around the globe [3-8]. Prevalence of chronic pain in different countries is shown in **Fig. 2**. The burden of pain can be realized by assessing its level of severity as well as disabilities accompanied with pain. It is, therefore, justified to regard pain as public health priority as it is linked with social and economic aspects of life [9-10].

1.3. Pain Etiology

Pain receptors called nociceptors have free nerve endings. As soon as they are stimulated by chemical, thermal and mechanical means, they send impulses to central nervous system through sensory neurons and perception of pain is being occurred **Fig. 3** [11] shows various steps in the perception of pains. A large number of somatic and visceral pain receptors are activated by various stimulants and inflammatory mediators like bradykinin, prostaglandins, leukotrienes, serotonin, histamine, glutamate, substance p, nervous growth factor (NGF), adenosine and adenosine phosphate capsaicin and free radicals [12-15].

1.4. Analgesia

Analgesia means removal of pain without the loss of consciousness. Generally the condition of pain is treated with several over the counter (OTC) analgesic drugs. These are extensively researched

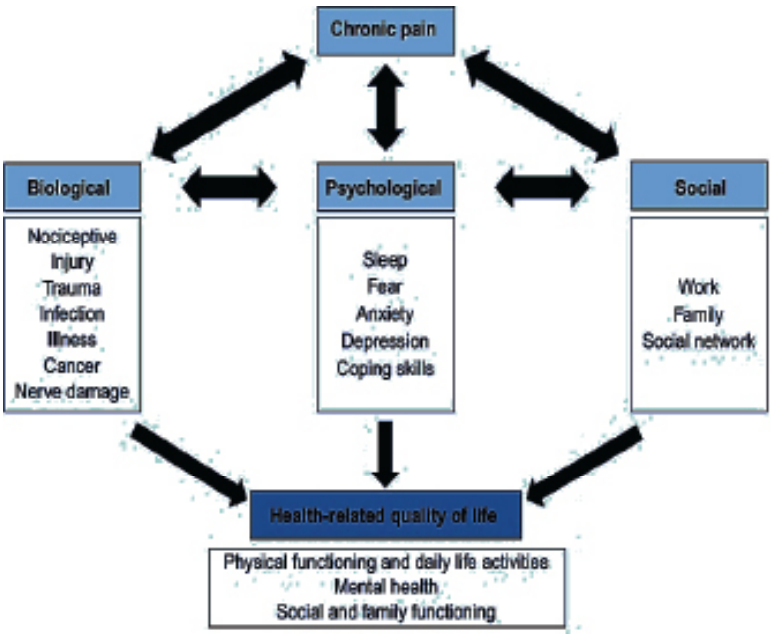

Fig. 1. Causes and Consequences of Chronic pain

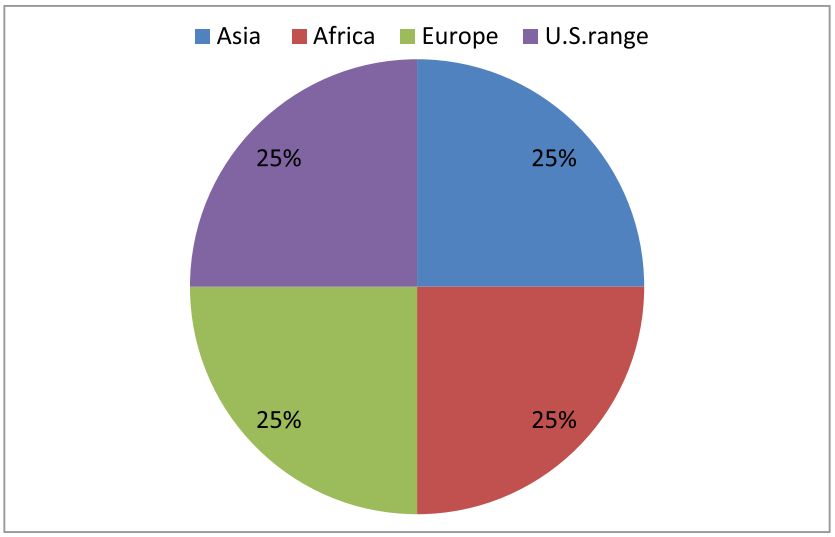

Fig. 2. Epidemiology of pain around the Globe in primary care setting

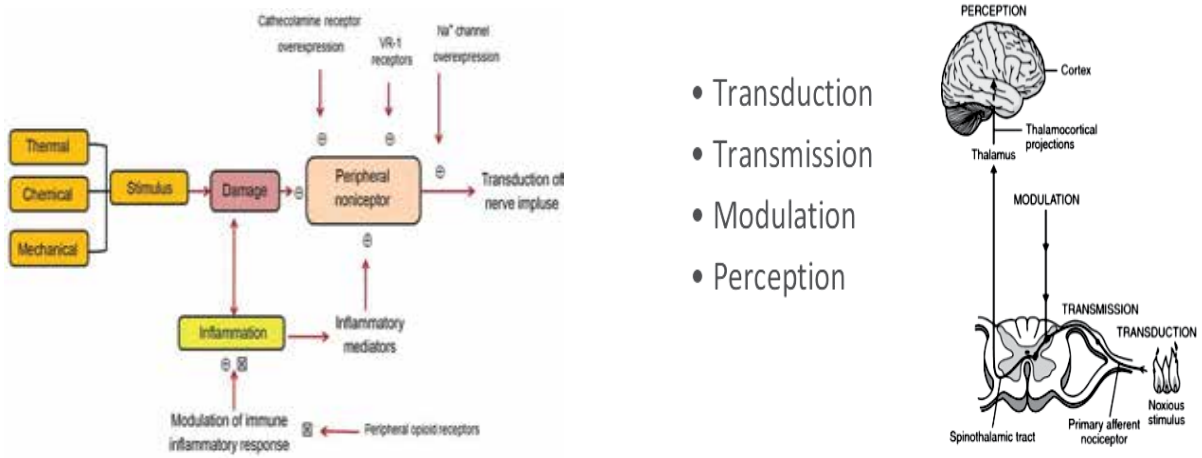

Fig. 3. Steps in perception of pain

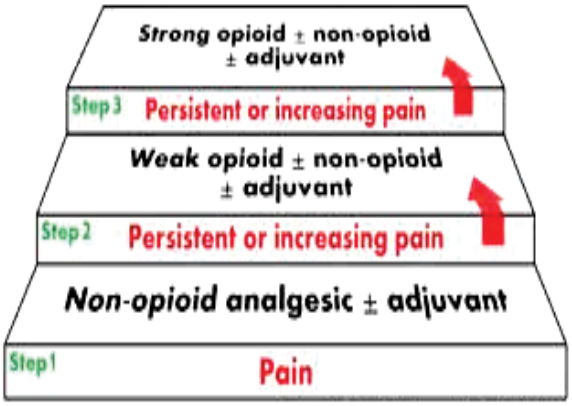

Fig. 4. WHO Treatment Ladder of pain

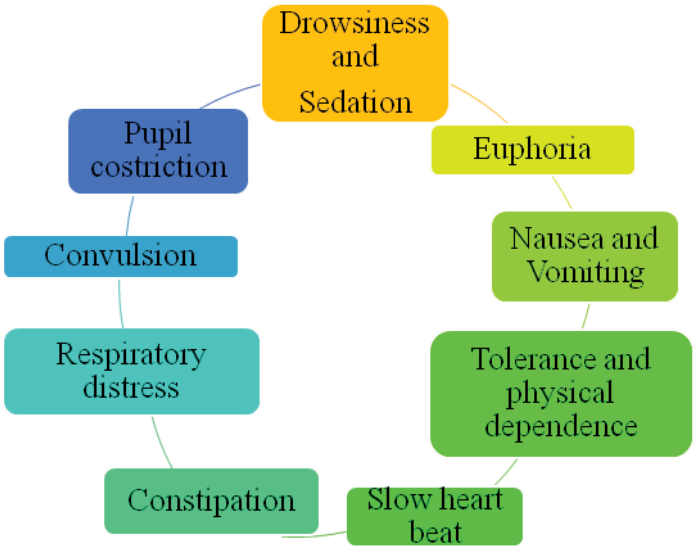

Fig. 5. Common side effects of Opioid analgesics

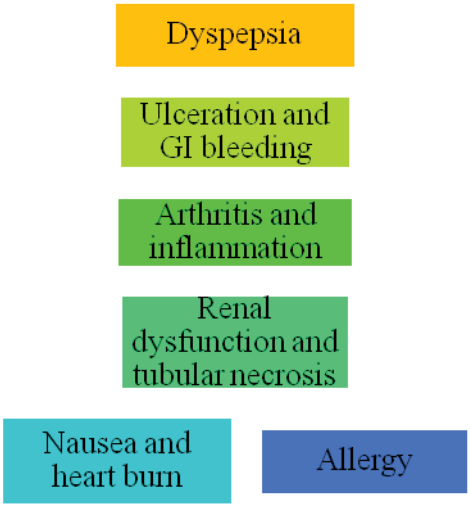

Fig. 6. Common side effects of NSAIDs

but are bound to have several side effects. Approximately 70% of the western population is habitually using analgesics to diminish headache, to treat dysphoric states of mood, to reduce sleep disturbances and for other pains and illnesses. **Fig.4** shows the WHO pain ladder which starts with common OTC drugs at lower levels and reaches to strong opioids at higher levels [16]. However, long term or excessive use of analgesics is considered as abuse [17].

1.4.1. Side Effects of Synthetic Analgesics

Any injury or trauma leads to pain accompanied with swelling, burning sensation and erythema. Opoids and Non-steroidal anti-inflammatory drug (NSAIDs) are the drugs of choice to subside pain in joint and spine associated pains. They are known to have many undesirable side effects. **Fig. 5 and 6** represent the common side effects of opioids and NSAIDs analgesic [18-19].

1.4.2. Medicinal Plants as Analgesics

Natural resources specifically plants are continuing their role in drug development, in order to discover the safe, effective, and reasonable treatment for the rising spectrum of human ailments. People experiencing different types of disorders and related pains desire to find drugs with lesser side effects [20]. Various medicinal plants have been advised for prevention and cure of certain pain related conditions. Drugs of herbal origin have received considerable attention of researchers because of possessing low or no side effects [21-22].

Therefore, ten Pakistani medicinal plants have been selected for their phytochemical screening and analgesic activities by tail flick and writhing reflex method. Their name, family, parts used and tradition uses are summarized in **Table 1** [23-33].

2. MATERIALS AND METHODS

2.1 Collection of plants

The rhizome, fruits and seeds of plants were procured from local market of Karachi, Pakistan. Leaves of plants were collected from the garden of University of Karachi. The identification of leaves of plant species was done by Botany department of University of Karachi, Pakistan.

2.2 Extraction

For extraction, leaves stem, and fruits of the selected plants were cut into thin slices and dried at room temperature. The dry plant materials were ground into powder and percolated in ethanol (Merck, Germany) in a separate container for one week at room temperature. The ethanol extract was evaporated via rotary evaporator at 40^{o}C under reduced pressure.

Table 1. Selected Pakistani Medicinal Plants

S. No.	Botanical name & parts used	Family	Chemical Constituents	Traditional medicinal uses
1.	*Zingebar officinale* Roscoe. Rhizhom	*Zingiberaceae*	sesquiterpenoids, with (-)-zingiberene, Sesquiterpene Lactones, carbohydrates, lipids, terpenes, and phenolic compound including gingerol, paradols, and shogaol	Acute and chronic cough, common cold, fever, allergic rhinitis, sinusitis, acute chronic bronchitis, respiratory troubles, pain, headache, backache
2.	*Cllistemon viminalis* L.Seeds	*Myrtaceae*	Phenolics, triterpenoids, flavonoids,saponins, steroids, alkaloids, tannin, carbohydrates, amino acids and proteins compounds	Gastroenteritis, diarrhea and skin infections
3.	*Citrullus lanatus* Fruit	*Cucurbitaceae*	Alkaloids, Amino acids, Saponins , Flavonoids, Glycosides , Steroids, Oils Tannins, Carbohydrates, Proteins and phenolics	diuretic and tonic. used in the treatment of the urinary passages, a good vermifuge and has hypotensive action.
4.	*Trachyspermum ammi* L.Seeds	*Apiaceae*	Carotenoids, Lutein and Zeaxanthin, p-cymene, γ-terpinene, α-pinene, β-pinene and α-terpinene	Paralysis, weakness of limbs, chest pain, liver disease, hiccups, kidney, spleen problem, carminative, diuretic and decreases the pain, in the acute phase of common cold or migraine
5.	*Adansonia digitata L. Leaves*	*Malvaceae*	Terpenoids, alkaloids, Flavonoids, Glycosides, Sterols, vitamins, Amino acids, minerals, carbohydrates, phenols, and lipids	Malaria, tuberculosis, fever, microbial infections, diarrhoea, anaemia, dysentery, toothache, immune stimulant
6.	*Murraya paniculata* L. Leaves	*Rutaceae*	Alkaloids, prenylatedcoumarins, polymethoxyflavones and flavonoids , sesquiterpenes (l- cadinene), a sesquiterpene alcohol and methyl anthranilate	Stimulant, Astringent, diarrhoea, dysentery, management of pain and inflammatory conditions. Diseases of teeth and gum, useful against rheumatism, useful against rheumatism, coughs and hysteria
7	*Holoptelea integrifolia* (Roxb.) Planch Leaves	*Ulmaceae*	Terpenoids, sterols, saponins, tannins, proteins, flavonoids, phenols, cardiac glycosides, coumarins, quinines carbohydrates, and alkaloid	inflammation, gastritis, dyspepsia, colic, intestinal worms, vomiting, wound healing, leprosy, diabetes, hemorrhoids, dysmenorrhea, and rheumatism
8	*Nepeta adenophyta* Hedge Leaves	*Lamiaceae*	Diterpenes, sesquiterpenes, and triterpenes, flavonoids	Abdominal pain, kidney pain, menstrual pain, and headache and also to control bleeding disorders
9	*Hibiscus schizopetalus* (Mast.) Hook Flower / leaves	*Malvaceae*	Alkaloids, steroids, anthocyanin and triterpenoids	Cold, Cough and to reduce fever analgesic and antipyretic diabetes, menstrual disorder and piles
10	*Sesamum indicum* L. seeds	*Pedaliaceae*	50-60% oil, 18-25% protein, 13.5% carbohydrate and 5% ash	Anti oxidative, anticancer, anti hypersensitive and anti immunoregulatory actions

2.3 Preliminary Phytochemical Screening

The presence of secondary metabolites including polyphenols, alkaloids, saponins, sugar and flavonoids was analyzed in the following procedures described by Shareef et al., 2010 [34].

2.3.1 Analgesic Screening

2.3.1.1 Hot plate Test

Rats weighing 100-120 g of both sexes were taken for the mentioned study. The Ethanolic extracts (200-400 mg/kg) of the all extracts were administered to rats, which were divided in 13 groups (n=6 in a group). Aspirin and Diclofenac Sodium (5 mg/kg) were selected as reference drugs. Hot plate technique was employed and reactions were assessed one hour before treatment (control) and at different time interval treatment. The rats were placed on a Techno hot plate maintained at 56°C, and the time between placement of the rat on the platform and shaking or licking of the paws or jumping was recorded as the hot plate latency. Rats, which showed a pre-treatment time of greater than 15 seconds in hot plate test, were not included in the study. The pre- treatment time must not exceed 25 seconds in the test. To overcome chances of tissue damage it was ensured that [35] the percentage protection against thermal stimuli was calculated as follows:

% Thermal stimuli latency = [(Treatment – Control) /Control] x100

2.3.1.2 Acetic acid induced writhing response

The rats of either sex, weighing 180-200 g were selected for the study. The Ethanolic extracts (200 -400 mg/kg) of the plant extracts were administered to rats, which were divided in 13 groups (n=6 in a group). Aspirin and Diclofenac Sodium (5 mg/kg) were used as reference drugs. The rats were treated with extracts of medicinal plants and reference drugs daily for a week. After 30 minutes of the final administration 0.6% acetic acid (0.1 ml/10 g) was injected intra peritoneal. After administration of acetic acid, the number of writhes was evaluated continuously for 20 minutes, by placing rats in transparent boxes. The control group only received saline. The number of writhes of the test group rats was compared with number of writhes of the control group and inhibition rate was determined as follows [35]:

% Percent inhibition of writhes= [(Control mean - Test mean)/Control mean] ×100

2.4 Statistical Analysis

The results were presented as mean ± SEM and calculate their percentages. One way Analysis of Variance (ANOVA) was used to considered the values of $p < 0.05$ statistically significant.

3. RESULTS & DISCUSSION

Natural resources specifically plants are continuing their role in drug development, in order to discover the safe, effective, and reasonable treatment for the rising spectrum of human ailments. People experiencing different types of disorders and related pains desire to find drugs with lesser side effects [36]. Various medicinal plants have been

Table 2. Preliminary phytochemical analysis of selected medicinal plant species

S.No.	Plants name	Polyphenols	Reducing sugar	Saponins	Flavonoids	Alkaloids
1	*Zingebar officinale*	+	+	+	+	+
2	*Cllistemon viminalis*	+	-	+	+	-
3	*Citrullus lanatus*	+	-	-	+	+
4	*Trachyspermum ammi*	+	+	+	+	+
6	*Adansonia digitata*	-	+	-	+	-
7	*Murraya paniculata*	-	+	+	+	+
8	*Holoptelea integrifolia*	+	+	+	+	+
9	*Nepeta adenophyta*	+	+	-	+	-
10	*Sesamum indicum*	+	+	+	+	+

(+): Presence (-): Absence

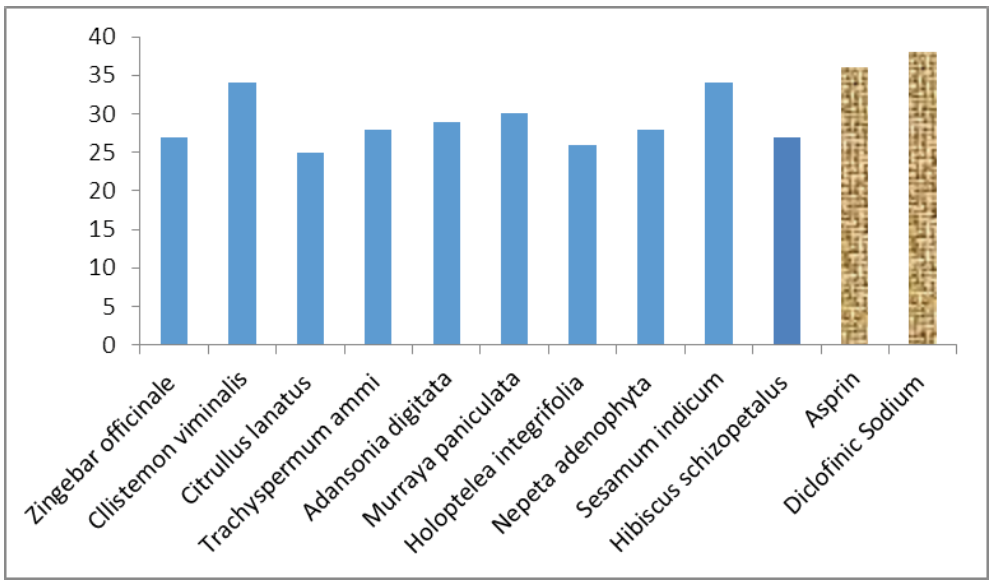

Fig. 7. % inhibition Writhing effect of medicinal plants

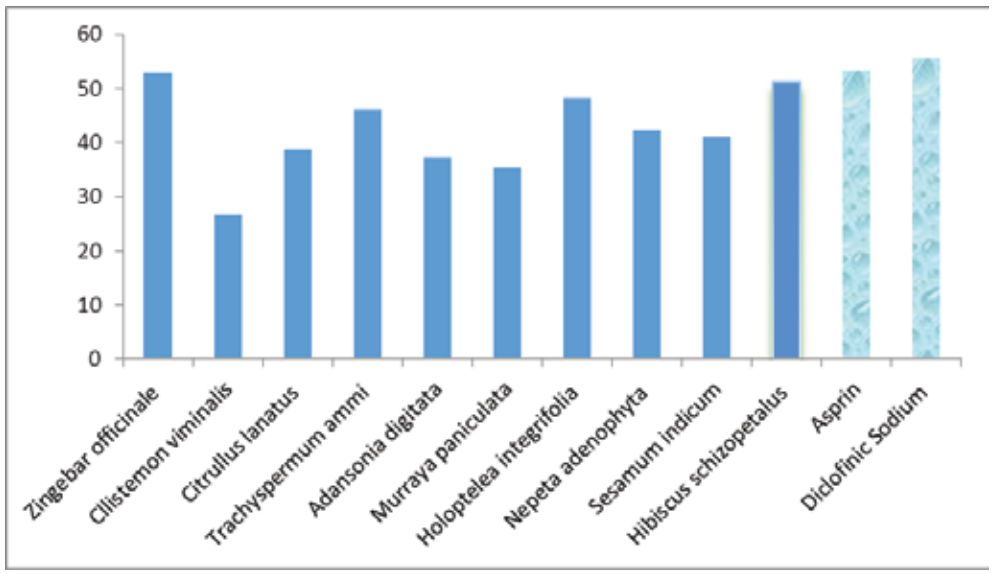

Fig. 8. % Protection in latency time of medicinal plants as compare to standards

advised for prevention and cure of pain and its related conditions because of possessing low or no side effects.

In the current research, we collected 10 Pakistani medicinal plants and performed their preliminary phytochemistry (**Table 2)** and analgesic activity by hot plate and writhing reflex methods. All selected Pakistani medicinal plants have showed dose dependent significant analgesic activity in both tests (**Fig. 7 & 8)**. As in writhing reflex test, the number of writhes was decreased after administration of all selected plants. Moreover, in hot plate test, the pain latency time was significantly increased in *Callistemon viminalis*, *Citrulus lanatus*, *Zigebar officinale* extracts. Hot plate method is used generally for centrally acting analgesic [37], while peripherally acting drugs are ineffective in these tests but sensitive to acetic acid induced writhing test. It was observed that all of the medicinal plants significantly ($P < 0.05$) reduced the abdominal contractions induced by acetic acid even after 30 min of administration. Similarly, in the hot plat tests all of the plant extracts showed significant effect up to 120 minutes just as standard aspirin and diclofenic sodium drug.

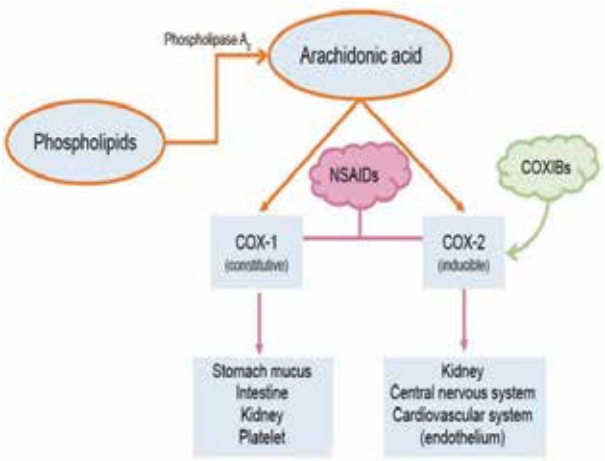

Fig. 9. Mechanism of action of NSAIDs

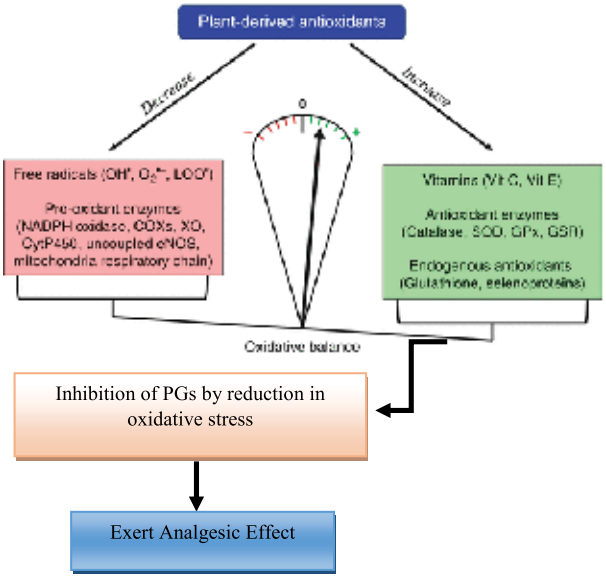

Fig. 10. oxidative balance of Analgesic from plants

We found different phytochemicals such as flavonoids, phenols, alkaloids, tannins and saponins in all medicinal plants extracts. Six medicinal plants gave positive test for alkaloids as alkaloids basically affects the central nervous system, reduces pain perception and produces good analgesic activity in all the different models of analgesia as reported by different scientist [38-39]. Furthermore, the presence of flavonoids occurred in eight plant extracts. Flavonoids have analgesic activity that prevents oxidative cell stress [40-41]. Seven plants showed positive test against phenols. Phenols are bioactive polyphenols because these may also be helpful in prevention of oxidative stress diseases including cardiovascular disorder, cancer etc. and also used as antioxidant and analgesic [42-43]. Plant provides various promising medicinal agents as their phytochemical components, which may use to prevent various diseases. Flavonoid, poly phenols, saponins, alkaloids, terpenoids etc. are important phytochemical constituents that are used as antioxidant and work as analgesics and anti-inflammatory agents etc. These phytochemical constituents may play important role also in the formation of crude drugs that contribute for development of new drugs at pharmaceutical industries to cure pain and its complications.

Non-steroidal anti-inflammatory (NSAIDs) drugs are first choice of drugs for treatment of pain and inflammatory conditions (**Fig. 9**). Reported side effects of NSAIDs such as gastrointestinal bleeding and declined function of the immune system has shifted the attention of researchers to alternative pharmacotherapies which are rich in antioxidant agents [44-46]. The bioactive components such as phenolic compounds, flavonoids, saponins, and alkaloids of plants possess multiple therapeutic activities such as antioxidant, anti-inflammatory, antimicrobial and anticancer activities [47-48]. The mechanism followed for the analgesic activities via free radical scavenging or inhibition of major anti-inflammatory enzymes like cyclo-oxygenases (COX) and lipoxygenases (LOX) enzymes are shown in **Fig. 10** [49-50]. Moreover, the possible antioxidant involved mechanisms is the major connection for their analgesic effects and our current findings demonstrated scientific rationale for the folk use of these selected medicinal plants as analgesic via antioxidant potential.

4. CONCLUSION

Pain is a neglected health issue affecting millions of people globally. Incidence of pain has been increased due to certain life threatening and degenerative disorders, such as Cancer, Arthritis, and Diabetes, or due to tissue damage. Chronic Pain could be a result of the imbalance between reactive oxygen species and naturally producing antioxidant supplementation is prescribed treatment. In conclusion, all the medicinal plant extracts demonstrated presence of different phyto chemicals and exhibited significant analgesic activity by writhing reflex and hot plate test.

Analgesic activity may be linked with their antioxidant potential due to the presence of phenols and flavonoidal contents that boost physiological defense mechanism by decreasing free radicals and oxidative markers during injury through inhibition of prostaglandin pathway. However, more work is required in the isolation and characterization of the bioactive compound(s) and determination of Reactive Oxygen Species (ROS), Inducible Nitric Oxide (iNOS) and glutathione levels to determine the exact antioxidant mechanism.

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Proceedings of the Pakistan Academy of Sciences:
B. Life and Environmental Sciences 56 (3): 69–74 (Special Issue, 2019)

ISSN: 2518-4261 (print), ISSN 2518-427X (online)

Pakistan Academy of Sciences

Research Article

Antidiabetic and Antimicrobial Properties of Some High Altitude Medicinal Plants of Nepal

Rachana Regmi[1], Mitesh Shrestha[2], Megha Raj Banjara[1], and Deegendra Khadka[3*]

[1]Central Department of Microbiology, Tribhuvan University, Kirtipur, Kathamandu, Nepal
[2]Research Institute for Bioscience and Biotechnology, Ekantakuna, Lalitpur, Nepal
[3]Nepal Academy of Science and Technology, Khumaltar, Lalitpur, Nepal

Abstract: Regarding severe side effects caused by synthetic drugs, studies were carried out taking high altitude medicinal plants to evaluate antidiabetic and antimicrobial activities. Antidiabetic property was measured by the inhibition protein tyrosine phosphatase 1B (PTP1B) enzyme with plant extract taking *p*-nitrophenyl phosphate (*p*NPP) as a substrate in the assay. For antimicrobial activity, plant extracts were tested against four pathogenic bacteria; *Staphylococcus aureus, Escherichia coli, Klebisella pneumonia* and *Pseudomonas aeruginosa*. BA17 appeared to be more effective for the inhibition PTP1B enzyme (95-100%) at various concentrations. BU17 showed the maximal zone of inhibition of 16 mm on three microbes in *S. aureus, E. coli*, and *K. pneumonia* at 100 mg/mL.

Keywords: Medicinal plants, Antimicrobial, Protein Tyrosine Phosphatase 1B, High altitude, Type 2 Diabetes Mellitus.

1. INTRODUCTION

Nepal is well known for its enriched biodiversity and of course, herbal plants affluent. Solely on Nepal's alphine zone, more than ten thousand medicinal plants have been covered. In the range of 1,792 to 2, 331 numbers of both aromatic and medicinal plants have been recorded based on their utilization in healing human ailments. Local people have practiced such plants for livelihood, conventional therapies and home remedies since ancient times [1]. Several findings unveiled that the plants are the robust sources of drugs. Hence, medicinal plants are pillar in both traditional and modern medical interventions, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs [8]. Compounds having antimicrobial activities are found abundantly in several medicinal plants and used medicinally around the globe as a source of potent drugs [15]. In drug extraction process, various parts of plants are used which include root, stem, flower, fruit, twigs exudates and modified plant organs. Local citizens collect raw drugs material in limited quantities for self-therapeutic purposes while pharmaceutical companies need huge quantity for producing medicine in bulk.

The cause and cure of type 2 diabetes mellitus (T2DM) are still mysterious; however, genetic factors and sedentary living style play crucial role in developing T2DM. Regular monitoring blood glucose level is the appropriate way to get informed before being life threatening. Synthetic drugs for diabetes are scattered in the global market. Among them, drugs like metformin, alpha-glucosidase, thiazolidinediones, and sulfonylurea are currently available in the market. Despite having side effects, people have no other options but rely on these medicines [2]. Mainly the family like Compositae, Berberidacae, Liliaceae and Papaveaccae are renowned for their medicinal value. An alternative of synthetic drug would be natural product which is relatively safer.

The antibiotics typically used in the treatment of human diseases are produced from the bacteria as a mechanism of competition to ensure their

Received: February 2019; Accepted: September 2019
* Corresponding Author: Deegendra Khadka; deegendra.khadka@nast.gov.np

own survival. Resistivity of microbes in natural environment like soil is developed either by specific mutation or exchanging genetic information (including resistance genes). So, it is easy going to get permission while transmitting the resistance to other microbes [9]. One of the major causes of becoming infectious diseases treatment less effective in the world is the emergence of bacterial strains that exhibit resistance to a variety of antibiotics. A bacterium has also called "superbug" because merely a few antibiotics are available to cure bacterial infected diseases. Using antibiotic widely both for human consumption and animal feed facilitates in development of resistance to a variety of gram-negative and gram-positive pathogenic bacteria [7].

To beat the risk factors generated by the consumption of synthetic drugs in the treatment of diabetes and bacterial infections, natural products would be the effective way for the health management. Our team selected high altitudinal medicinal plants for this study. It is believed that the plants grown up in harsh climatic conditions can produce relatively different bioactive compounds which may be the target molecule for the drug development. The main aim of the study is searching a novel natural compound that possesses antidiabetic property and also finding potent natural products having antibacterial characters.

2. MATERIALS AND METHODS

2.1. Study Area

The study was conducted at Molecular Biotechnology Laboratory, Nepal Academy of Science and Technology, Khumaltar, Lalitpur, Nepal. Details of ten selected high altitude medicinal plants with different parts like stem, leaves, roots and their collection are described in **Table 1**. Chemicals were purchased from Sigma-Aldrich (Merck).

2.2. Plant Extract Preparation

Plants including leaves, stem and roots were air dried in shade place for a month. The dried plant materials were ground into the fine powder using grinding machine. For the preparation of plant extract, 21 gram of fine powdered plant matter was dissolved in 150 mL of absolute methanol at room temperature for two successive days. Each day, the dissolved parts were filtered using the Whatman no. 1 filter paper and stored in glass bottle. The final collection of dissolved parts was then evaporated at reduced pressure at 50°C using a rota-evaporator. These obtained solid mass was weighed carefully to express the gram of extract per 100 g of the plant powder. For each sample, extract was prepared individually. Similarly, the extracts were obtained

Table 1. Description of high altitude medicinal plants used in the experiment

S.No.	Scientific name	Vernacular name	Code number	% Yield (MeOH)	Collection sites	Used plants part
1.	*Berberis asiatica*	Chutro	BA17	35	Low camp, Mardi Himal	Leaves and stem
2.	*Betula utilis*	Bhojpatra	BU17	45	Low camp, Mardi Himal	Leaves and stem
3.	*Cassia fistula*	Rajbriksha	CF17	38	Dhangadi, Kailali	Leaves and stem
4.	*Cassia spp.*	-	CS17	30	Dhangadi, Kailali	Leaves
5.	*Murraya koenigii*	Curypatta	MK17	36	Dhangadi, Kailali	Leaves
6.	*Nardostachys grandiflora*	Jatamashi	NG17	30	Low camp, Mardi Himal	Leaves
7	*Neopicrorhiza scrophulariiflora*	Kutki	NS17	43	Mai Pokhari, Ilam	Leaves
8	*Rheum austral*	Padamchal	RA17	35	Dhangadi, Kailali	Leaves and stem
9	*Rhododendron anthopogen*	Laliguras	RhA17	55	Low camp, Mardi Himal	Leaves and stem
10	*Macropanax undulates*	Chenday	MU17	54	Low camp, Mardi Himal	Leaves and stem

from the solvent hexane and ethyl acetate. The extracts were kept at 4°C for further analysis [16].

Percentage Yield (%) of extract = (Dry weight of extract/ Dry weight of plant material) × 100

2.3. *In vitro* PTP1B Enzyme Assay

In the enzyme assay, the PTPase activities were calculated by *p*NPP assay. In this assay, buffer A is composed of the mixture of HEPES (100 mM) and EDTA (5 mM) maintaining pH 7.0. The reaction is carried out at 37 °C using *p*NPP of 1M concentration. The enzyme is diluted with enzyme dilution buffer (25 mMHepes, 5 mM EDTA, 1 mM DTT, 1 mg/ mL bovine serum albumin, pH 7.3). Inibihitors that is plant extract of concentration 250, 500, 750 and 1000 µg/ mL were dissolved in Dimethyl sulfoxide (DMSO). The absorbance at 405 nm was measured to calculate the quantity of *p*-nitro phenol expelled out.

2.4. Inhibition Studies

For inhibition studies, enzyme was diluted in the enzyme dilution buffer (2.2µL of enzyme and 107.8µL of enzyme dilution buffer). Reaction mixture i.e. bulk was prepared which contain 250µLof water, 100µL of 5x reaction buffer with DTT and 50µLof diluted enzyme. PTP1B activity was measured by the addition of 5 µL of 1M *p*NPP (as substrate) in the reaction mixture along with or without different concentrations i.e. 250, 500, 750 and 1000 µg/mL of inhibitors. After incubation for 10 min at 37°C, the reactions were halted with 950 µL of 0.5M Sodium Hydroxide (NaOH). The amount of *p*-nitro-phenol formed was measured by UV absorbance at 405 nm [3]. The percentage inhibition of PTP1B was calculated as follows:

$$\% \text{ inhibition} = [(Abs_{blank} - Abs_{sample})/Abs_{blank}] \times 100$$

Where, Abs_{blank} is absorbance of the blank and Abs_{sample} is absorbance of the sample [17].

2.4.1 Determination of Antibacterial Activity

2.4.4.1 Preparation of the standard bacterial culture inoculums

Four active bacterial strains namely *Staphylococcus aureus (ATCC 25923), Escherichia coli (ATCC 25922), Klebsiella pneumoniae (ATCC700603) and Pseudomonas aeruginosa (ATCC 27853*) were used. Three or four isolated colonies of each strain were inoculated in the 5 mL nutrient broth and incubated till the growth in the broth was equivalent with Mac-Farland standard (0.5 %). The inoculated culture bottles were kept in the incubator at 37 °C for 3-4 hours. The turbidity of the sub-cultured bacterial suspension was adjusted at 0.5 % McFarland standards (freshly prepared a day before the experiment). These bacterial inoculums were used for the swabbing on the Muller Hinton agar (MHA) plates to test the antimicrobial effects of plant extracts.

2.4.4.2 Antibacterial Activity Assay

For antibacterial activity assay, the well diffusion method on MHA media procedure was used to evaluate effectiveness of plant extracts against bacterial activity [3]. While making wells on MHA media, a cork borer having 6 mm in diameter was used. Bacterial inoculums with the concentration of 10^6 CFU/mL were spread on the solid media with a sterile cotton swab. 20 µL of the working solution of plant extract with the concentration of 100, 50, 25 and 12.5 mg/mL and same volume of extraction solvent (methanol and DMSO) was used as negative control, whereas 1mg/mL streptomycinas positive control was filled in the wells with sterile micropipette. Plates were left for some time till the extract diffused into the medium with the lid closed and incubated at 37°C for 24 h. After overnight incubation the plates were observed for the zone of inhibition (ZOI) and the diameter of the inhibition zone were measured using scale.

3. RESULT

BA17 and MK17 have high inhibitory potential against PTP1B. This plant extract has a potential property to be used against the treatment of the disease associated with the over activity of the PTP1B. The BA17 and MK17 can be the possible sources for the treatment of T2DM in traditional medicine. Among ten medicinal plants sample, BA17 showed promising result against the inhibition of PTP1B as shown in **Fig. 1. Table 1** shows summary of plant sample with their code numbers.

Methanolic plant extracts showed the zone of inhibition against microbes ranged the concentration

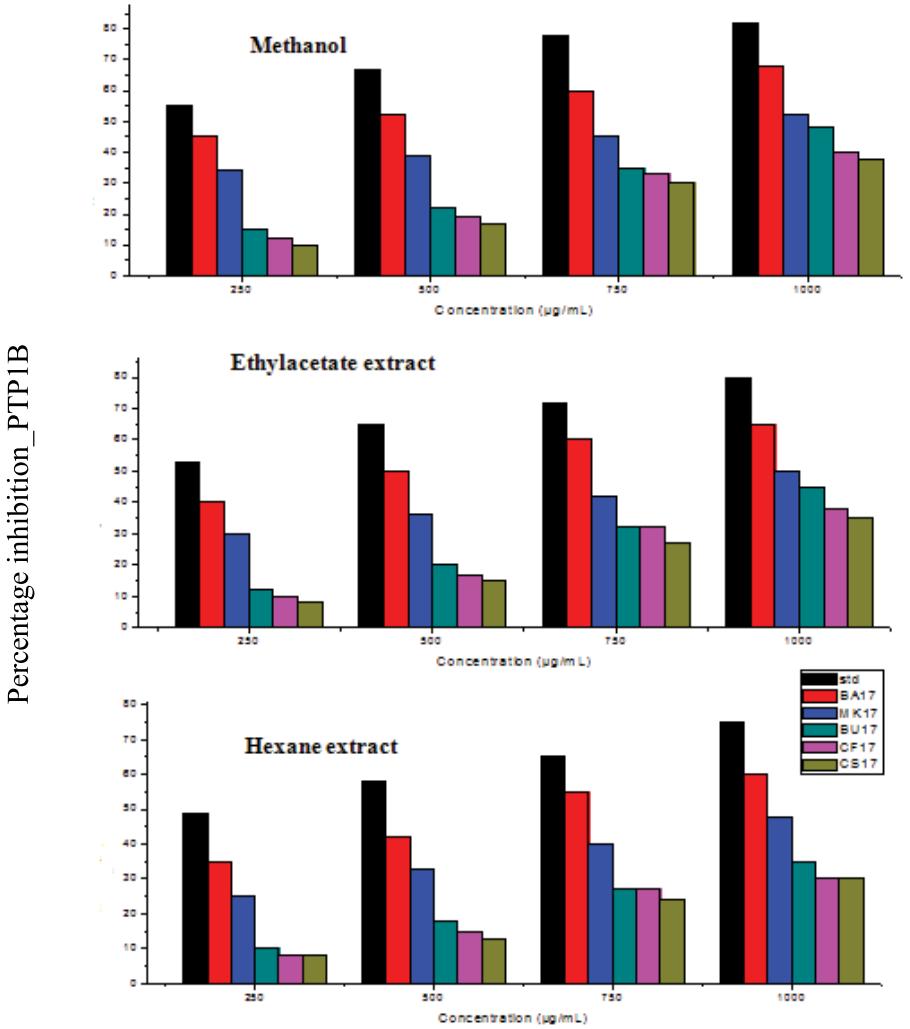

Fig. 1. Inhibitory effects of extracts of the selected plants on PTP1B activities

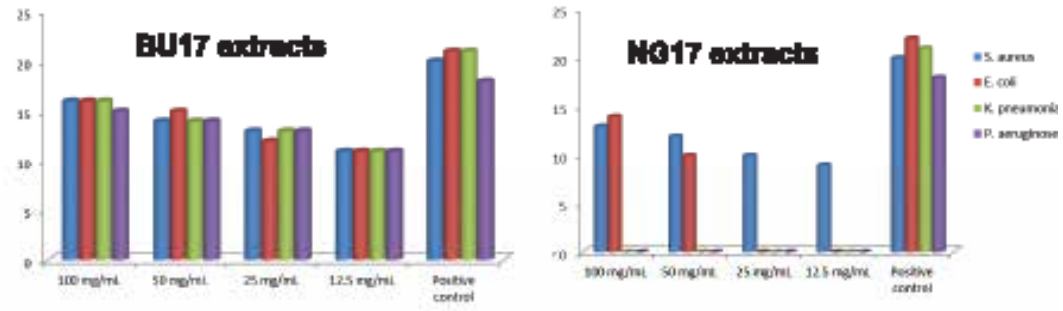

Fig. 2. Zone of Inhibition (ZOI) of BU17 and NG17 plant extracts against four different bacterial strains

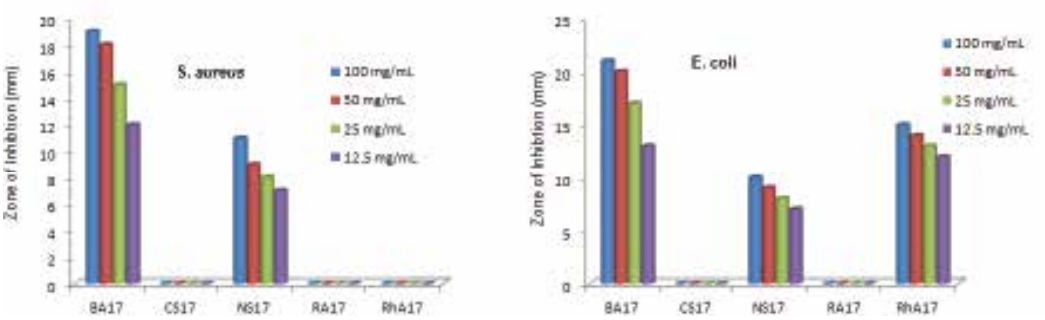

Fig. 3. Zone of Inhibition (ZOI) of five plants extracts against S. aureus and E. coli bacterial strains

6.25 mg/mL to 100 mg/mL. BU17 plant extract showed the highest zone of inhibition of 16 mm, 14 mm, 13 mm and 11mm on *S. aureus* at 100, 50, 25 and 12.5 mg/mL concentration respectively and the detailed on other bacterial strains are depicted in the **Fig.2** and **Fig. 3.**

4. DISCUSSION

In this study, plants having medicinal values were selected from higher altitudes of Nepal. Higher altitude plants which are locally used as medicine are selected because they are rich sources of the bioactive compounds in the view of their growth in stressful conditions [11]. Climatic conditions, parts of the plant used, extraction time, and temperature and extraction procedure (including the solvent selection) plays a vital role in isolating bioactive compounds that have pharmacological activity [10]. In this study, selected plants were freshly collected and shade dried to avoid the loss of the bioactive compound. Drying in the artificial environments at low temperature reduces the loss of large moisture content and prolonged storage time [6].The dried sample were ground mechanically and subjected to solvent for extraction. Methanol is the solvent used for the extraction because alcohol solvent presumably ruptures the cell membrane and extracts greater amount of endo-cellular materials [12].

Overexpression of PTP1B is associated with the resisting insulin molecule to bind on insulin receptor and eventually glucose molecule are unable to get inside the cells which could lead elevating blood glucose level, called T2DM. PTP1B enzyme has been identified as one of the primary drug targets for treatment of T2DM [13]. It has been reported that natural inhibitors like berberine, and iso-quinoline alkaloid possess potent antidiabetic properties to inhibit PTP1B enzyme [5]. Papaverine, a structural analog of berberine, which belongs to member of iso-quinoline alkaloids have also exhibit potent PTP1B inhibitory activity thereby lowering fasting blood glucose level *in vivo* [4]. BU17 plant contains alkaloids like berbamine and berberine which may be responsible for the highest inhibitory activity. Although hypoglycemic effects of some of the plants have been reported, the mechanism of action has not been fully elucidated [14]. BU17*,* MK17 and NS17 have greater inhibitory effect on PTP1B. This study showed that BU17and MK17 can be better inhibitors of PTB1B in the future for the treatment of T2DM.

BA17, NS17 and RhA17plant extracts showed higher zone of inhibition for *S. aureus* and *E. coli.* But they did not display any inhibition zone on *K. pneumoniae* and *P. aeruginosa*. The CS17 plant extract showed nearby zero inhibition zones on *S. aureus, E. coli*, *K. pneumonia* and *P. aeruginosa*. Gram-negative bacteria (*E. coli*, *K. pneumoniae* and *P. aeruginosa*) had been found to be less susceptible to plant extracts than Gram positive (*S. aureus*). Gram-positive bacteria are highly sensitive in comparison to gram-negative bacteria because of possessing less effective permeability barrier of the outer peptidoglycan layer.

5. CONCLUSION

Methanolic extract of different higher altitudinal plants possess inhibitory potential against the PTP1B, especially the extract of BA17 and MK17 possess significant inhibition which indicates possible option for the treatment of the diseases associated with the over activity of the PTP1B

for example T2DM. BU17 showed the promising antibacterial activity against *S. aureus*, *E. coli, K. pneumoniae* and *P. aeruginosa* revealing as possible alternative to antibiotics.

6. ACKNOWLEDGEMENT

This research was supported by Nepal Academy of Science and Technology. Authors thank Dr. Ram Chandra Poudel and Dr. Rosa Ranjit for the supports during research period.

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Proceedings of the Pakistan Academy of Sciences:
B. Life and Environmental Sciences 56 (3): 75–82 (Special Issue, 2019)

ISSN: 2518-4261 (print), ISSN 2518-427X (online)

Pakistan Academy of Sciences

Research Article

Anti-oxidant and Aldose Reductase Inhibitory Activity of *Piper betle* Extracts

Sri Fatmawati[1*], and Kuniyoshi Shimizu[2]

[1]Department of Chemistry, Faculty of Science, Institut Teknologi Sepuluh Nopember, Surabaya 60111, Indonesia
[2]Department of Agro-environmental Sciences, Faculty of Agriculture, Kyushu University, Fukuoka 812-8581, Japan

Abstract: *Piper betle*, known as daun sirih, is one of popular jamu ingredients which could be consumed freshly from the natural product resources by Indonesian people as traditional medicine. The present study is the primary article on human aldose reductase inhibition and also antioxidant activity of *P. betle* leaves extracts. The ethanol extract of *P. betle* exhibited the most inhibitory activity of the aldose reductase enzyme among extracts. It was discovered to present an IC50 value of 18.8 µg/mL for in vitro human aldose reductase and showed antioxidant activity by ORAC assay with value of 3861.2 ± 451.0 µmol Trolox Equivalent/g extract. Further investigation on the chemical components of the ethanol extract showed a total of 14 compounds by GC-MS analysis. The major compounds were bisphenol A (13) (34.4%) isoxylic acid (3) (13.8%), *trans*-phytol (11) (6.6%) and octadecyl aldehyde (14) (6.4%). These results implied that *P. betle* leaves should be prospective as an aldose reductase inhibitor.

Keywords: *Piper betle,* Jamu, Natural products, Aldose reductase, Antioxidant.

1. INTRODUCTION

Aldose reductase (alditol: $NAD(P)^+$ 1-oxidoreductase) is recently known to work as a key player in the polyol signalling pathway. The enzyme converted the reaction of glucose to sorbitol, while sorbitol leads to the development of long term diabetic complications [1]. To overcome this phenomenon, several potential aldose reductase inhibitors have been practiced both from natural [2, 3] and synthetic one [4, 5].

The use of Indonesian traditional medicines has been expanded recently. Some of our papers related with biological activities of some Indonesian traditional medicines have been reported [6, 7, 8]. In some region in Indonesia, people have used *P. betle* as a health supplement for avoiding from obesity, ulcer, toothache, as well as dental healthy [9]. In addition, the leaf of *P. betle*, also known for having a strong pungent aromatic flavour, is the best traditional medicines for female health and vitality [10].

P. betle leaves are credited with many properties. In the past few years, *P. betle* was also reported for its biological activity such as antigiardial [11], antibacterial [12, 13, 14], antifungal [15, 16], cytotoxic [17], antifertility [18], antibiofilm [19], anti-atherogenic [20], anti-inflammatory [21], also antidiabetes [22, 23], and antioxidant [24, 25, 26]. The phenolic compounds, for instance allyl pyrocatechol, from the leaves prevented halitosis activity [27]. *P. betle* ethanol extract decreased both histamine and GM-CSF by a hypersensitive response significantly. Besides, the ethanol extract inhibited secretion activity by a TNF-α and IL-4-induced allergic reaction [28]. *P. betle* leaves also demonstrated the effect hepato-protective significantly and upgraded the tissue antioxidant activity by rising the non-enzymatic antioxidants levels. In addition, free radical-detoxifying enzymes activity of ethanol-treated rats in liver was also increased [29]. The other report presented

Received: February 2019; Accepted: September 2019
* Corresponding Author: Sri Fatmawati; fatma@chem.its.ac.id

that both hot water and cold ethanol extracts of leaves of *P. betle* reduced the blood glucose level significantly by oral administration of diabetic rats [23]. Furthermore, Siddiqui, *et al*., (2012) reported that *P. betle* extracts could be a potential agent of membrane bio-fouling aspect [30].

In the few past decades, there were studies about antioxidant and anti-diabetes also. However, this study reported from a different new approach. The previous study reported that *P. betle* has a good antioxidant activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical [14, 16, 24, 25, 26] together with the other reactive radicals such as thiobarbituric acid reactive substances (TBARS), nitric oxide (NO), hydroxyl or superoxide radicals. However, there is no report about free radical scavenging activity by ORAC yet. In addition, there is a little report about anti-diabetes of *P. betle*. The previous report showed that *P. betle* aqueous and ethanol extracts have an inhibitory activity on streptozotocin (STZ)-induced diabetic rats [22, 23]. From these report, *P. betle* is a potent to do further investigations regarding its biological activity as an aldose reductase inhibitor. In this study, aldose reductase inhibition of *P. betle* leaves extracts together with free radical scavenging activity of these extracts had been reported.

2. MATERIALS AND METHODS

2.1 Sample and Chemicals

P. betle leaves were purchased from Pasar Genteng, one of traditional markets in Surabaya, Indonesia. The dried powder leaves of *P. betle* were extracted for 24 hours using *n*-hexane, dichloromethane, methanol, and ethanol at 25 °C. The dissolved solvent was evaporated by rotary vacuum evaporator to afford each extracts. β-NADPH was purchased from Oriental Yeast Co., Ltd. DL-glyceraldehyde was obtained from Wako Pure Chemical Industries, Ltd. Human recombinant aldose-reductase (HRAR) was purchased from AT Gen Co., Ltd. All other chemicals are analytical grade or high purity commercially obtainable.

2.2 The Extraction with Solid Phase Micro (SPME)

We purchased polydimethylsiloxane fibers that have been coating with length of 1 cm and 100 **µm** film thicknesses from Supelco (Bellefonte, PA, USA). At 250 **°C the** fibers were prepared for 1 hour in the gas chromatograph inlet before use. By using a manual SPME container, the fibers were positioned for ready to use. By injecting the SPME penetrating needle across the foil and subjecting the headspace of fibers over the sample, the adsorption of the chemical components was obtained for 30 minutes. Once sampling was done, for desorption and analysis process, the fibers were directly transported to gas chromatograph's port of inlet.

2.3 Chemical Constituents Identification

The identification of chemical constituents was accomplished by means of Shimadzu QP-5050 gas chromatograph/mass spectrometer (GC/MS) from Kyoto, Japan. We used DB-5 with a fused and attached silica capillary column, with film length, 30 m; thickness, 0.25 µm; i.d., 0.25 mm; which produced by Agilent. Helium was used as carrier gas, with a 100 kPa column head pressure. The chemical constituents were desorbed on SPME in a split-less injector at 250 °C. The program of oven temperature was initiated for 5 min at 40 °C and enlarged with a slope of 3 °C/min until 300 °C continue by 300 °C for 10 min. Finally, The MS data were linked and comparing with the NIST62 MS library to identify the chemical constituents.

2.4 Aldose Reductase Assay

The activity of Human Recombinant Aldose Reductase (HRAR) was examined on a UV/VIS spectrophotometer, JASCO V-530 - Japan. The HRAR activities were determined conferring to our previous method [3]. The percentage of inhibitory activity (%) was calculated as this equation: [1-(Δ*A* sample/min - Δ*A* control/min)]×100. Δ*A* sample/min exposed a diminution of absorbance with a sample for 1 min and Δ*A* control/min with dimethyl sulfoxide (DMSO) instead of a sample. The determination of reaction were started with mixture of 10mM dl-glyceraldehyde, 0.15mM β-NADPH, 100 µl of tested sample solution on DMSO and 5 µl of HRAR, 100mM sodium phosphate buffer (pH 6.2) in a total volume 1.0 ml. Afterward the reaction mixes, the incubation at 25 ºC were performed for 5 min, then the reaction was initiated by adding HRAR, and later the reduction of absorbance at λ

340 nm was examined using a JASCO V-530 UV/VIS spectrophotometer for 10 min. Each plant extract was liquefied in DMSO at less than a 1% concentration which have no enzyme activity.

2.5 Oxygen radical absorbance capacity (ORAC) Assay

The ORAC assay was conducted based on previously described procedures [31, 32] but with slight modifications. *P. betle* extracts were pre-treated with DMSO with concentration of less than 0.1 % then dissolved in 75 µM phosphate buffer in pH of 7.4. After that, 20 µL of sample, buffer, and trolox solutions were added into tube wells, respectively. Next, 200 µL of fluorescein solution was added. After 10 min incubation at 37 °C, 75 µL of 2,2'-azobis(2-amidino-propane) di-hydrochloride (AAPH) working solution was also injected. Finally, fluorescence degradation was measured over 90 minutes. Every 30 second interval was measured by using Molecular Devices Flex Station 3 microplate reader. The excited and emission wavelengths were 485 nm and 535 nm, respectively. The result data were managed by Soft Max Pro 5.4.1. The minimum and maximum concentrations of extracts in buffer were 6.25 and 50 µg/mL, respectively. In our assay system, trolox solutions with concentration of 6.25, 12.5, 25, and 50 µM were used to make the standard curve.

3. RESULTS

The extracts were prepared from the dried of *P. betle* leaves with maceration process for 24 h to yield *n*-hexane, dichloromethane, methanol and ethanol extract. The inhibitory activity of HRAR of each extracts is shown in Fig.1.

Ethanol extract of *P. betle* showed the highest inhibition among extracts at concentrations of 100 µg/mL. The methanol extract exposed some inhibition, but it was fewer than that of the ethanol extract. The dichloromethane was discovered to be somewhat more effective than that of the *n*-hexane extract. In the present study, quercetin was used as a positive control which is known as a naturally occurring HRAR inhibitor, and in our assay system exhibited an IC_{50} of 2.9 µg/mL. We determined the inhibitory activity of HRAR of the ethanol extract of *P. betle* (Fig. 2) and it displayed the dose dependently (IC_{50} = 18.8 µg/mL) inhibitory activity. These results indicated that ethanol extract of *P. betle* can constrain the progression of *in vitro* HRAR.

The results of ORAC assays of *P. betle* extracts are shown in Table 1. ORAC values (µmol TE/g

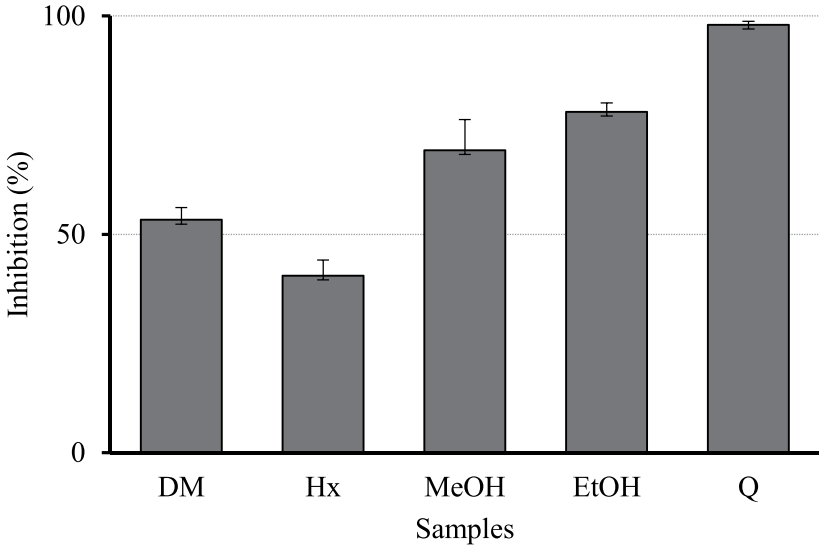

Fig. 1. Aldose reductase inhibitory activity of extracts of *P. betle* at a concentration of 100 µg/mL. DM is dichloromethane extract; Hx is *n*-hexane extract; MeOH is methanol extract; EtOH is ethanol extract; and Q is quercetin (positive control). Each column represents the mean ± SD, n = 3.

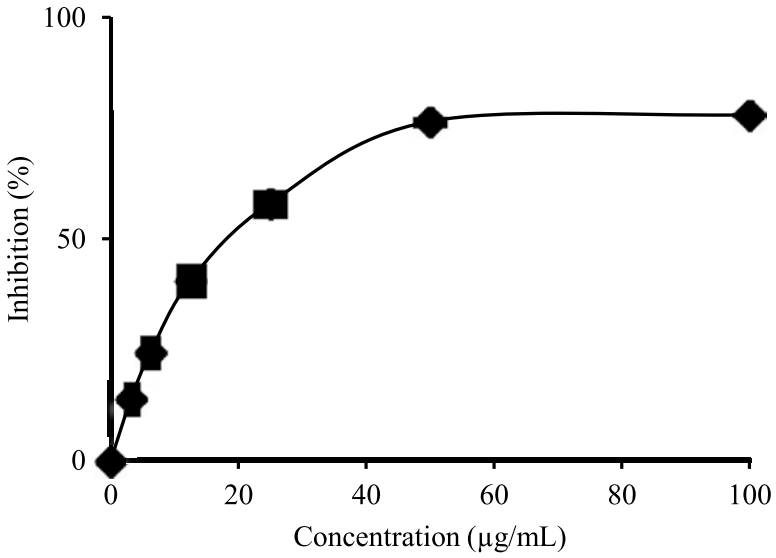

Fig. 1. The effect of *P. betle* ethanol extract on aldose reductase.

Table 1. ORAC values of *P. betle* extracts.

Samples	ORAC Values (µmol TE/g extract)
n-Hexane extract	832.4 ± 244.1
Dichloromethane extract	2343.2 ± 421.4
Ethanol extract	3861.2 ± 451.0
Methanol extract	4107.3 ± 487.6

Table 2. Chemical composition of *P. betle* ethanol extracts with SPME.

Components	R.T (min)	%*
Chavicol(1)	28.1	2.3
Isoeugenol(2)	34.2	2.0
Isoxylic acid (3)	38.9	13.8
α-curcumene(4)	39.2	3.5
1,1'-[1-(2,2-Dimethylbutyl)-1,3-propanediyl]biscyclohexane(5)	39.9	1.4
Cinnamyltiglate(6)	46.6	2.8
(3E,7E)-10-Isopropenyl-3,7-cyclodecadien-1-one (7)	47.8	1.3
Scobenol(8)	50.3	2.7
Hexadecanoic acid (9)	57.2	1.8
Ethyl pentadecanoate(10)	58.3	1.7
trans-Phytol(11)	62.1	6.6
13-Tetradecenal (12)	63.8	1.1
Bisphenol A (13)	63.9	34.4
Octadecyl aldehyde (14)	65.3	6.4

*area percentage

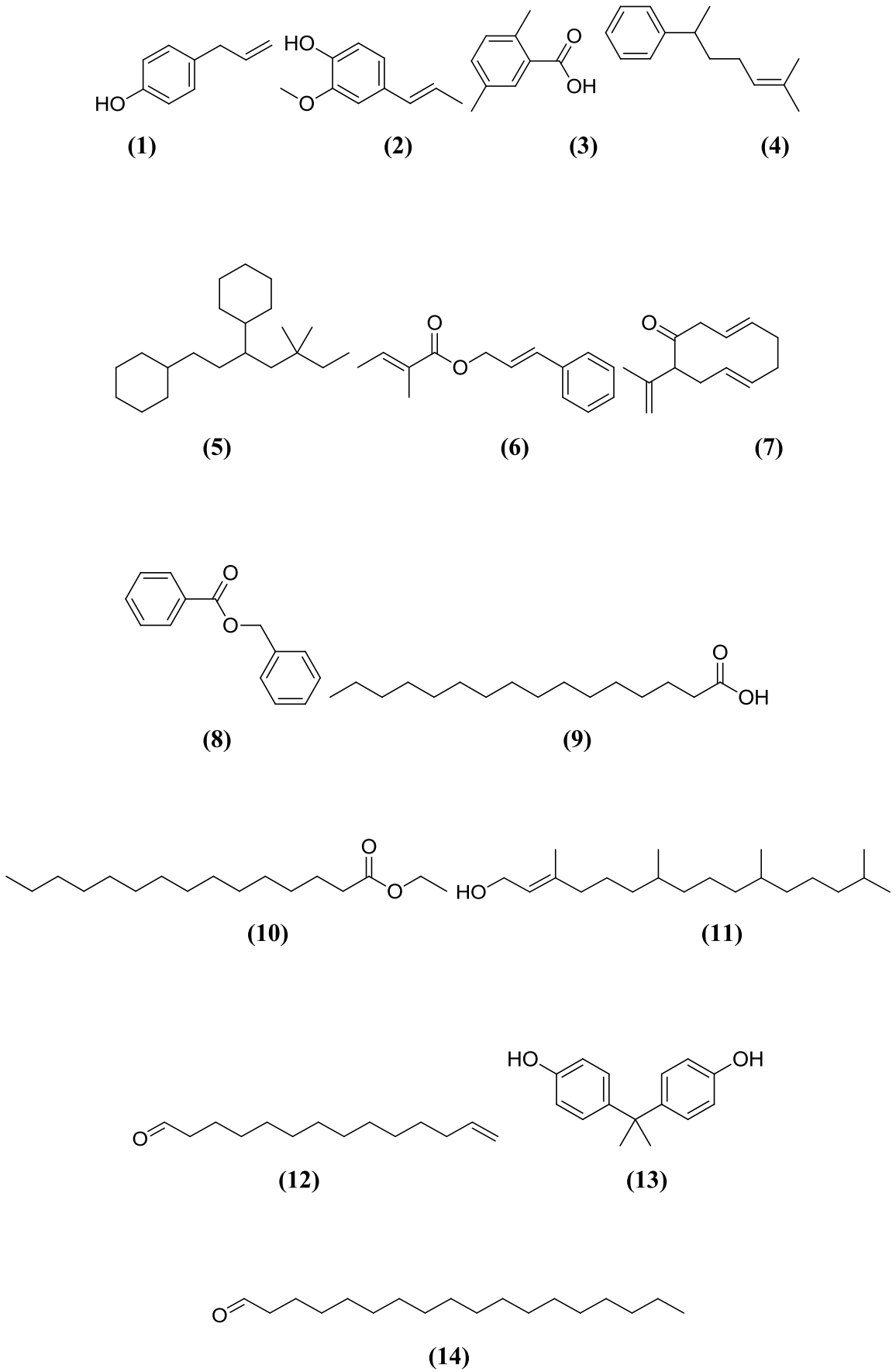

Fig. 3. GC-MS based chemical composition analysis of the *P. betle* ethanol extracts showed a total of 14 compounds

extract) of ethanol and methanol extract were 3861.2 and 4107.3, respectively. Solid-phase micro extraction was used to identify chemical composition in ethanol extract of *P. betle*. GC-MS based chemical composition analysis of the *P. betle* ethanol extracts showed a total of 14 compounds presented in Figure 3 and summarized in Table 2. The major compounds were bisphenol A (13) (34.4%) isoxylic acid (3) (13.8%), *trans*-phytol (11) (6.6%) and octa-decylaldehyde (14) (6.4%).

4. DISCUSSION

This present report confirmed the HRAR inhibitory activity of *P. betle* extracts for the earliest time. *P. betle* is one of Piperaceae famili mostly consumed in Asia [33]. This plant, known as daun sirih, could be found in some Indonesia traditional markets. Related to this study, the four extracts of *P. betle* were founded for their inhibitory activity on HRAR at a minimum concentration of 100 µg/mL for each of the extracts. In our assay system, quercetin, as known as a potent aldose reductase inhibitor, was used as a standard. The results showed that the ethanol extract is the most effective to inhibit aldose reductase enzyme. As a *like dissolved like* concept, the ethanol has a polar side to extract the polar compounds from *P. betle*. They might be called as aldose reductase inhibitor. On the other hand, an ethanol extract of *P. betle* also reported significantly lowered the blood glucose level on STZ-induced diabetic rats [23, 34]. Further investigation of ethanol extract *P. betle* also was reported by ORAC values. The results presented the ethanol extract has a fine amount of µmol TE/g extract. It should be notable that both HRAR inhibition and the ORAC value of the ethanol extract of *P. betle* were almost the alike as that of the methanol extract. Furthermore, these results indicated that here gave a linear correlation among aldose reductase inhibitory activity and free radical scavenging.

Aldose reductase enzyme is frequently used to *in vitro* antidiabetic assay model [3, 6, 7]. This enzyme, as the first enzyme in polyol pathway, is catalysed glucose to sorbitol. For diabetic disorders, a hyperglycaemic condition will be activated the polyol pathway highly. These conditions made more sorbitol's produce. Unfortunately, a high accumulated sorbitol caused the diabetic complications [1] such as cataracts, neuropathy and nephropathy. Literally, sorbitol could be converted to fructose by sorbitol dehydrogenase then to be fructose-6-phosphate catalysed by hexokinase. Thus, fructose-6-phosphate could be used for further metabolism circle to produce an energy namely glycolysis. But these metabolism circles do not work as simple process as well, when a hyperglycaemic condition caused a high affinity of aldose reductase. However, a hyperglycaemic will inhibit the activity of sorbitol dehydrogenase, hexokinase as well as NADPH as the main body cofactor. Certainly, many aspects in the metabolism system concern for more investigation.

Based on aldose reductase inhibition and ORAC values results, further experiments were focused on chemical composition of ethanol extract. The chemical compositions were determined by SPME connected to GC-MS identification. SPME is a solid phase extraction method [35]. This is a recommended method for extraction because it is simple, fast and solvent less [31]. After extraction, the SPME fiber is transferred to the inlet port of GCMS instrument. In addition, the GCMS is a good choice instrument for the chemical identification of *P. betle* because most of chemical compositions of this plant are the volatile oil components. The major components of ethanol extract of *P. betle* typically have hydroxyl group and/or carboxyl group which are important group as aldose reductase inhibition [36].

5. CONCLUSIONS

In conclusion, this paper presents a primary study on *P. betle* leaves for the inhibition of HRAR and free radical scavenging by using ORAC assay. Among extracts, ethanol extract showed the uppermost inhibitory in contradiction of HRAR activity, and it was applicable in reducing free radical scavenging by the ORAC assay. Advance examinations will emphasis on the isolation of the bioactive constituents dependable for the HRAR inhibitory effects and antioxidant of *P. betle* ethanol extract.

6. ACKNOWLEDGEMENTS

The authors gratefully acknowledge the financial support provided by Directorate General of Higher Education (DGHE), Ministry of Education and Culture, Republic of Indonesia.

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Proceedings of the Pakistan Academy of Sciences:
B. Life and Environmental Sciences 56 (3): 83–96 (Special Issue, 2019)

ISSN: 2518-4261 (print), ISSN 2518-427X (online)

Pakistan Academy of Sciences

Research Article

Extraction, Phytochemical Screening and Wound Healing Activity of Herbal Formulation of *Saussurea lappa*

Aitazaz Ahsan*, G. A. Miana, Humaira Naureen, Masood Ur Rehman, Kamil Anum, and Imran Malik

Riphah Institute of Pharmaceutical Sciences, Riphah International University, Islamabad, Pakistan

Abstract: The aim of present research work was to develop emulgel and *In-situ* gels of methanolic extract of *Saussurea lappa* with the purpose to determine their wound healing and anti-bacterial properties. Phyto-chemical analysis of extract was also performed. Emulgel was prepared by using carbopol 940, Span 80, tween 80, polyethylene glycol (PEG 4000) and methyl paraben while in-situ gel was prepared by using polaxomer (P407) as thermo-sensitive and carbopol (934P) as pH sensitive polymers. All formulations were maintained at pH 6-7 and stored at 4 ◦C. Lyophilized extract was added in solution form to enhance the solubility as well as the stability. In-vitro release profile was performed by using Franz diffusion method and data was plotted in different pharmacokinetic models like first order, higuchi and hixon-crowell models. All formulations followed first order release mechanism. Hemolytic activity of extract was performed at concentration of 100µg/ml through heat induced hemolysis of erythrocyte membrane model system while anti-bacterial activity was determined by using agar well diffusion method. Acute toxicity assay of crude extract showed that 1000 mg/kg was safe dose with no toxic symptoms. Excision wounds were induced and wound healing potential of all formulations was determined. Results were compared and expressed as mean ± SEM, and data was analyzed by one way analysis of variance (ANOVA) with $p<0.05$.

Keywords: *Saussurea lappa,* Wound healing, Emulgel, *In-situ gel,* Herbal formulation

1. INTRODUCTION

Natural products are very diverse and almost 35,000 - 70,000 plants species have been screened till date. Ethno pharmacological use of crude drugs provided a major clue in drug discovery. Data suggested that more than 50% of medicines used during last 30 years were of natural origin [1]. *Saussurea lappa*, commonly known as costus or kuth occur in South East Asia and Pakistan having 400 species with long and rich use in local traditional herbal products to treat internal heat or fever, menstruation, wound healing purposes, unbalanced blood circulation, unwanted bleeding, body pain and rheumatic arthritis [2]. *S. lappa* contains variety of phytochemicals like sesquiterpene lactones that have anti-inflammatory and wound healing potential. Gastric ulcers are inhibited by costunolide and Saussure amines while cyanopicrin is potent immunosuppressive agent [3]. Herbal formulations are becoming popular as they are considered safe due to their natural origin. Herbal medicines are used in variety of health ailments like liver problems, diabetes and heart problems etc.

Wound healing is a normal physiological process that involves hemostasis, inflammation, proliferation, and remodeling which highly programmed phases. All these phases are necessary for proper wound healing in a proper sequence and time frame [4]. Topical drug delivery system is becoming popular but conventional topical drug delivery systems like ointments and creams have a drawback of low bioavailability and poor retention [5]. Emulgels are the recent formulations in novel drug delivery systems that are combination of emulsion and gel. When applied topically they provide dual control release in the form emulsion

Received: February 2019; Accepted: September 2019
* Corresponding Author: Aitaza Ahsan; aitazazahsan@gmail.com

as well as gel [6].

In-situ gelling systems involve use of polymers that have phase transition from solution to gel upon alterations in physico-chemical properties of drug [7]. In-situ gels effectively overcome the drawbacks of conventional topical dosage forms and drug is released in controlled manner because they have better stability and release profile upon gelation [8].

The purpose of present study was to formulate and evaluate anti-microbial and wound healing activity of herbal formulations of *S. lappa* for the cost effective treatment. Results were compared with standard formulations. The study was divided into three parts. First part the extraction and phytochemical evaluation of crude extract of *S. lappa*, Second part the designing of dosage forms i.e Emulgel and *In-situ* gels along with their post formulations studies to determine the *in-vitro* release profile, stability, sterility and other physico chemical properties and the third part acute toxicity study of crude extract in mice, wound healing activity of formulations in rats and anti-bacterial activity by agar well diffusion method.

2. MATERIALS AND METHODS

2.1 Plant collection and extraction

The roots of *S. lappa* were collected from the wild cultures growing in and around Swat in April, 2018. Roots were washed thoroughly with water and shade dried. Fine powder (750g) of dried roots was prepared by using pestle and mortar. Powder was macerated with 3 liters of methanol for 14 days and extract was dried by using rotary evaporator. Chemicals were purchased from Sigma Co., Aldrich, Merck of Germany and BDH Lab Supplies of England.

2.2 Thin Layer Chromatography

Analytical thin layer chromatography (TLC) was performed for the detection of sesquiterpene lactones. Analytical TLC plates (TLC Silica gel 60 F254 20x20 cm Merck KGaA, Darmstadt, Germany) were spotted with 5-10 µg of each extract and placed in glass chamber containing chloroform and methanol (9:1). After development all the plates were dried and placed in glass chamber containing Iodine. After complete sublimation of iodine, plates were examined.

2.3 Phytochemical Screening of Extract

The root extracts of *S. lappa* were analyzed for the presence of alkaloids, terpenoids, carbohydrates, proteins, Flavonoid, glycosides, phenolic compounds, saponins and tannins as described [9].

2.4 Hemolytic Activity of Crude Extract

The blood was collected from healthy human volunteer and centrifuged at 3000 rpm for 10 min which was then washed three times with equal volume of normal saline. The volume of blood was measured and re-constituted as 10% v/v suspension with normal saline. The reaction mixture (2ml) consisted of 1 ml test sample of different concentrations (100 µg/ml) and 1 ml of 10% RBCs suspension. Aspirin was used as a standard drug and normal saline was used as control. All the centrifuge tubes containing reaction mixture were incubated in water bath at 56ºC for 30min. The reaction mixture was centrifuged at 2500 rpm for 5 min and the absorbance of the supernatants was taken at 560 nm. The percentage inhibition of hemolysis was calculated as:

$$\text{Percentage inhibition} = (Abs_{control} - Abs_{sample}) \text{ X } 100 / Abs_{control} \quad [10]$$

2.5 Animal Studies

Male Balb-C mice (20-30 gm) and Sprague-Dawley rats (180-220 gm) of local breed were housed in at the Animal House of Riphah Institute of Pharmaceutical Sciences, Islamabad, in plastic cages (47x34x18) cm^3 at 23-25 ^{0}C. All the animals were maintained under standard husbandry environment with food and water *ad libitum*. Experiments were performed and compiled following rules of institute of Laboratory Animals Resources, Commission on Life Science University, National Research Council (1996) and approved by Research Ethical Committee of Riphah Institute of Pharmaceutical Sciences (Ref. No: REC/RIPS/2017/00).

2.6 Acute Toxicity Study

Twelve Male Balb-C mice (20-30g) were divided into two groups (n=6). Experimental group was

orally treated with 1000 mg/Kg dose of methanolic extract dissolved in normal saline while control group was administered with normal saline (10ml/kg). Mice were strictly monitored for 24 hours with free access to food and water *ad libitum* to observe any lethal effects [11].

2.7 Formulation of Extract into Emulgel

For topical application 0.5%, 1%, 2%, 3% ,4% and 5% of emulgel of crude extract was formulated (F1 to F6) as described by [12]. First of all, 30g of carbopol 940 was dissolved in 1000ml of distilled water which have pH 6.2 and pH was adjusted with few drops of NaOH upto 7.1. Mixture was left for overnight. Oil phase was prepared by mixing 1.8ml of span 80 and 10ml liquid paraffin while aqueous phase was prepared by mixing 2ml of tween 80 with 10ml of distilled water separately for each formulation. 0.5g, 1g, 2g, 3g, 4g and 5g of extract was taken and mixed with 10ml PEG, 0.12g methyl paraben and 0.1ml of eucalyptus oil for fragrance. Aqueous phase was added in oil phase followed by mixing extract phase. Stable emulsions were formulated and further formulated into emulgel by mixing with gel made with carbopol 940 (100g for each formulation).

2.8 Formulation of Extract into *In situ* Gel

2.8.1 Lyophilization of Extract

Lyophilization of extract was done for its better reconstitution. 14g of extract was dissolved in 20ml distilled water and was frozen at -40 ^{0}C for 10 hours. Primary drying was done by increasing temperature to -10 ^{0}C at 0.6 ^{0}C/min for 5 hours and further up to 20^{0}C at 0.25 ^{0}C/min with pressure range of 1000 to 5 pascals [13].

2.8.2 Preparation of Gel

15% solution of polaxomer P (407) was prepared and refrigerated for 24 hours. 2% Carbopol 940 was dissolved in phosphate buffer and left for 24 hours to become hydrated. 15% of hydroxy propyl methyl cellulose (HPMC) and poly vinyl alcohol (PVA) were also dissolved in phosphate buffer separately. All the polymers were then mixed and gel was formulated with 3%, 4% and 5% of lyophilized extract. Benzyl alcohol was used as preservative [14].

2.9 Characterization of Formulations

2.9.1 Appearance

Formulations were visually examined for color, texture and grittiness [15].

2.9.2 Viscosity

Viscosity was determined by brookfield viscometer with spindle 61 at various speeds and results were recorded as centipoises (cp).

2.9.3 pH

pH was determined by using pH meter as described by Sultana et al., 2016 [12].

2.9.4 Spreadability

350 mg of each formulation was placed over a glass slide and second slide was dropped from distance of 5cm over the top of first slide. Diameter of the circle was determined [15].

2.9.5 Swelling Index

1g of each formulation was taken on porous aluminum foil and placed in beaker containing 10ml 0.1N NaOH. Samples were removed and dried at various time intervals. After drying, samples were reweighed and % swelling index was calculated by [16].

$$\text{Swelling Index (SW) \%} = [(Wt - Wo) / Wo] \times 100$$

2.9.6 Centrifugal Test

5g of formulations were centrifuged at 3750 rpm for few minutes at room temperature [17].

2.9.7 Accelerated Stability Test

Formulations were stored at 40 ^{0}C and physical parameters like pH and viscosity were determined at every week for the period of 4 weeks [17].

2.10 *In vitro* Permeation Study

Emulgel with 5% extract and *in situ* gel with 5% extract of *S.lappa* were used for *in vitro* permeation studies. Donor compartment of Franz diffusion cell was filled with formulation while the recipient compartment was filled with phosphate buffer (pH 6.0). 0.22 micrometer pore size dialysis membrane was used to separate both compartments. Outer jacket of the cell was filled with water and maintained at 37 ^{0}C with magnetic stirring at 50

rpm. Samples were taken at 0, 1, 2, 3, 4, 5, 6, 7 and 8 hours and UV absorbance was determined at 332nm. For UV analysis, phosphate buffer was used as blank. Sample was prepared after withdrawn from the cell and dilution with 10ml phosphate buffer. 150**µl sample was** collected from the solution and again diluted with 10ml phosphate buffer and UV absorbance was determined [18]. Percentage absorbance was determined by:

UV absorbance %= [(absorbance of sample / absorbance of standard) x 100]

After the UV analysis, results were fitted into various models to determine release rate pattern of formulations.

2.11 Pharmacokinetic Models

Drug diffusion and polymer chain relaxation are two parameters that determine drug release at a particular time. Pharmacokinetic models were developed and data obtained from *in vitro* release was fitted into each model. Various mathematical models were used to correlate drug permeation profile with drug release kinetics [19].

2.11.1 First order model

It establishes relationship between drug release verses time. Integration and rearrangement of equation is as follows:

$\log C_t = \log C_{0-K1t/2.303}$

K_1= first order rate equation expressed in time-1 or per hour,

C_0= initial concentration of the drug,

C_t=remaining percent of drug at time t

2.11.2 Higuchi Model

It is most prominent pharmacokinetic model that involves drug dissolution and diffusion which depends on drug concentration. Simplified form of Higuchi equation is as follows:

$Q = K_H \times t^{1/2}$

Q = Cumulative amount of drug released at time t

K_H = Higuchi release rate constant

$t^{1/2}$= square root of time

2.11.3 Hixson-Crowell Model

It mainly describes the release of drug from the system that involves change in surface area and diameter of drug particles. Simplified relationship between drug release and time is as follows:

$W_0^{1/3} - W_t^{1/3} = K_{Hc}\, t$

$W_0^{1/3}$ = cube root of initial amount of drug present in the matrix.

$W_t^{1/3}$= cube root of remaining amount of drug in matrix at time t.

KHc = release rate constant.

t= time

2.12 Wound Healing Study

36 male albino rats were divided into nine groups (n=4). Animals were closely monitored and infectious rats were excluded from the study. During wound healing study, no other topical or systemic treatment was given. Group I was untreated group while group II was treated with crude methanolic extract and group III received pyodine gel treatment. Group IV, V and VI were treated with 3%, 4% and 5% herbal emulgel. Group VII, VIII and IX were administered with 3%, 4% and 5% herbal *in-situ* gel. All the animals were given free access to food and water ad libitum. All the doses were administered topically. For wound healing study, dorsal skin of each rat was depilated and marked on the back of the rat by a standard ring. Rats were then anesthetized with ketamine (25 mg/kg intraperitoneally and excision wound of 380 mm^2 was induced). Full thickness of the marked skin was cut carefully. Wound was cleaned and kept open. 0.5g of each formulation was applied once daily from the day 1 to day 20 of wounding. 200mg/10ml extract was also applied topically. Wound size was measured after every 4 days for 21 days [20].

% wound contraction = [(initial wound size - specific day wound size) / initial wound size] x 100

2.12.1 In-vitro Antibacterial Activity

Rats were anesthetized with chloroform and excision wound was induced with sterile needles. Sterile cotton was placed over the wounds and exudate was collected. Exudate was cultured by using LB broth media [21]. After 48 hours, bacteria were streaked on LB agar plates. After growth of bacteria they were again streaked on separate agar plate for biochemical analysis like gram staining, coagulase test and catalase test. Zone of inhibitions of formulations and extract were determined by using agar well diffusion method [22].

2.12.2 Statistical Analysis

All values were expressed as mean ± SEM, and data was analyzed by one way analysis of variance (ANOVA).

3. RESULTS

TLC of extracts was performed by using cholorform and methanol (9:1). Plates were analyzed by using iodine. 1g of iodine was placed in closed chamber along with plate. Sublimation of iodine left spots on the plates that appeared brown with sespuiterpene lactones. Ethyl acetate and choloform extracts showed yellow spots of flavanoids. Hexane showed no significant spots, while methanolic extract showed brown spots that were our desired sesquiterpene lactones and other terpenoids which have significant *in vivo* activities.

3.1. Phytochemical Screening

Preliminary phytochemical analysis of crude extract was shown to contain alkaloids, glycosides, flavanoids, terpenoids, saponins, tannis, phenols and carbohydrates but no proteins were detected in the extract. Results are shown in table 1.

3.2. Hemolytic activityof crude extract

Methanolic extract of *Saussurea lappa* was shown to be effective in inhibiting heat induced hemolysis at concentration of 100 µg/ml. *S. lappa* efficiently protect the membrane against hemolysis induced by heat . Percentage inhibition of *S. lappa* was 42.8% while aspirin was 51% at 100 µg/ml. Results were reported in the form of graph as shown in figure 1.

3.3. Acute Toxicity Studies

Twelve male Balb-C mice weighed between (20-25g) were divided into groups (n=6) with free access to food and water *ad libitum.* Control group was given normal saline (10mg/kg) while experimental group was given crude extract (1000mg/kg) orally. Weight over fixed dose approach was followed. Extract was dissolved in normal saline. Each mouse weighed between (20-25g). All mice were monitored strictly for 24 hours and symptoms of toxicity were observed. After 24 hours, no toxic symptoms were observed in any of the mice [11].

Table 1. Phytochemical analysis of *S.lappa* extract

No	Phytochemical	Indication	Results
1.	Alkaloids	Reddish brown pecipitates	+
2.	Glycosides	Brown color	+
3.	Saponins	Stable froth for 10 minutes	++
4.	Phenols and tannins	Blue green color	++
5.	Proteins	No change	_
6.	Carbohydrates	Brick red color	+
7.	Flavanoids	Intense yellow color	++
8.	Terpenoids	Gray color	++

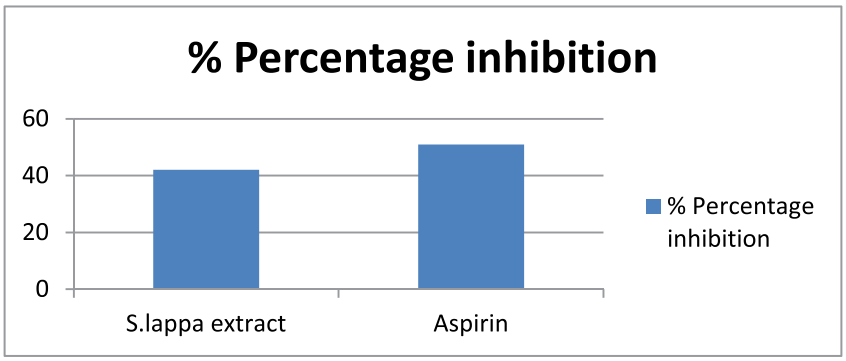

Fig. 1. Hemolytic activity of *S.lappa* extract

Table 2. Formula for emulgel preparation

No	Ingredient	Quantity/ 37g	
1.	Crude extract	**F1**	0.5g
		F2	1g
		F3	2g
		F4	3g
		F5	4g
		F6	5g
2.	Carbopol	3g	
3.	Span 80	1.8ml	
4.	Paraffin oil	10ml	
5.	Tween 80	2ml	
6.	Distilled water	10ml	
7.	Methyl paraben	0.1g	
8.	PEG 4000	10ml	
9.	Eucalyptus oil	0.1ml	

3.4. Formulation of Extract into Emulgel

Six formulations were prepared F1 to F6. Each formulation contained variable amount of extract ranging from 0.5% to 5% but amount of emulsion and other ingredients were not changed significantly [12] . Emulgel was formulated as shown in table2.

3.5. Formulation of Extract into *In situ* Gel

14g extract was dissolved in 20ml distilled water and lyophilized for 24 hours. 2% carbopol 940, 15% hydroxy propyl methyl cellulose (HPMC), 15% of polaxomer P (407) and 15% poly vinyl alcohol (PVA) solutions were prepared in phosphate buffer and left for 24 hours. After 24 hours, all the polymers were mixed and 3%, 4% and 5% (G1 to G3) *in situ* gels were formulated as shown in table 3. [14].

Table 3. Formula for in situ gel preparation

No	Ingredient	Quantity/ 37g	
1.	Crude Lyophillized extract	**G1**	3g
		G2	4g
		G3	5g
2	Carbopol	5g	
3	HPMC	5g	
4	PVA	5g	
5	Polaxomer P (407)	5g	

3.6. Characterization of Emulgels

Formulations F1 to F6 were characterized for appearance, viscosity, pH, spreadability, swelling index, centrifugal test and accelerated stability test. Results are shown in table 4.

3.7. Characterization of *in situ* Gels

Formulations G1 to G3 were characterized for appearance, viscosity, pH, spreadability and results

Table 4. Characterization of emulgel

Formulation	Color	Grittiness	pH	Viscosity (cps)	Centrifugation (Phase separation)	Swelling Index (%)	Spreadability (cm/sec)
F1	Cream	No	6.83	15678	No	56.2	18
F2	Pale yellow-brown	No	6.92	15750	No	69.3	20
F3	cream	No	6.35	15890	No	88.5	16
F4	Pale yellow-brown	No	6.79	15960	No	85.4	16
F5	Pale yellow-brown	No	7.12	16123	No	89.6	18
F6	Pale yellow-brown	No	7.33	16190	No	97.6	20

Table 5. Characterization of emulgel

Formulation	Color	Grittiness	pH	Viscosity (cps)	Spreadability (cm/sec)
G1	Brown	No	6.73	16650	17
G2	Brown	No	6.62	16825	19
G3	Brown	No	6.89	16960	16

are shown in table 5.

3.7.1. In vitro Permeation Study

In vitro permeation study was carried out by using franz diffusion cell of diamter 20 mm. The recipient compartment was filled with phosphate buffer surrounded by water jacket to maintain temperature at 37^0C and stirred at 50 rpm. A 0.22 µm pore size dialysis membrane was used to separate the donor and recipient compartment. Donor compartments were fillled with 5% emulgels and 5% *in-situ* gels. 150 µL of samples were collected at 0, 1, 2, 3, 4, 5, 6, 7 and 8 hours, diluted with phosphate buffer and absorbance was checked at 332 nm using phosphate buffer as blank. After each collection, the compartment was filled with equal volume of phosphate buffer [18]. Cumulative release of formulations over time was determined by fitting the data into various models.

3.8. Models

Various mathematical models were used to correlate drug permeation profile with drug release kinetics [19]. The results showed that formulation released drug constantly over the period of time and better realease rate was observed with *in-situ* gels as compared to emulgels.

3.8.1. First Order Model

It establishes relationship between drug release verses time. For both emulgel and *in-situ* gel formulations log cumulative percentage release was plotted against time as shown in figure 2.

3.8.2. Higuchi Model

It is most prominent pharmacokinetic model that involves drug dissolution and diffusion which depends on drug concentration. For both emulgel and *in-situ* gel formulations, cumulative percentage

Fig. 2. First order release pattern of emulgel and *in situ* gel containing 5% *S.lappa* extract

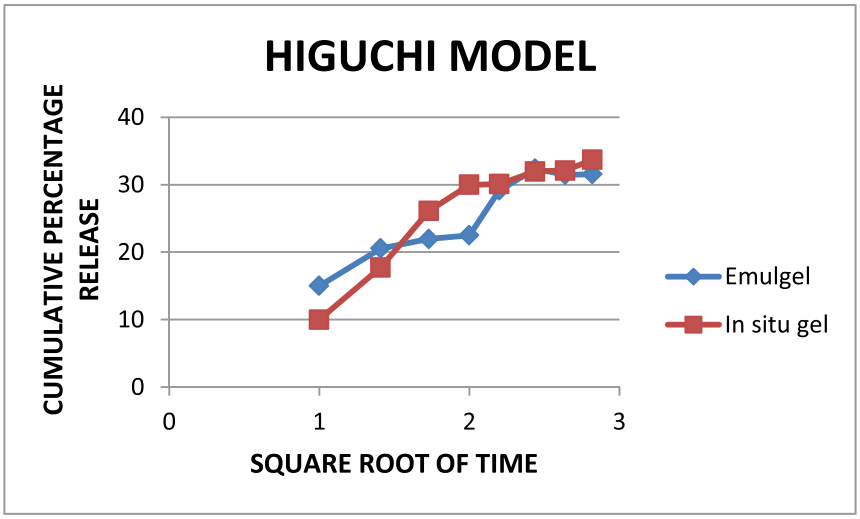

Fig. 3. Higuchi model for release pattern of emulgel and *in stu* gel containing 5% *S.lappa* extract

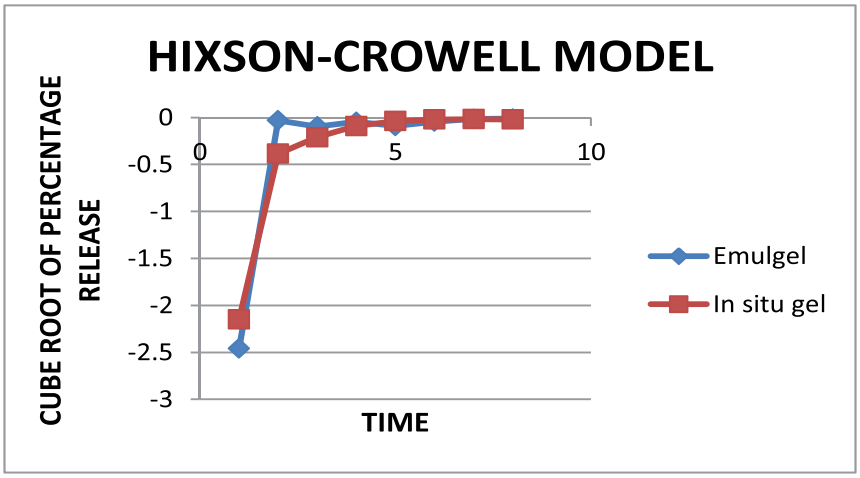

Fig. 4. Hixson-Crowell model for release pattern of emulgel and *in situ* gel containing 5% *S.lappa* extract

release was plotted against square root of time as shown in figure 3.

3.8.3. Hixson-crowell Model

It mainly describes the release of drug from the system that involves change in surface area and diameter of drug particles. For both emulgel and *in-situ* gel formulations, cube root of percentage released was plotted against time as shown in figure 4.

3.9. Wound Healing Activity

15 mm wounds were induced in 36 male albino rats divided into 9 groups (n=4). Group I was untreated group while group II was treated with crude methanolic extract and group III received pyodine gel treatment. Group IV, V and VI were treated with 3%, 4% and 5% herbal emulgel. Group VII, VIII and IX were administered with 3%, 4% and 5% herbal *in-situ* gel. All the animals were given free access to food and water ad libitum. Percentage wound contraction was detrmined by:

% wound contraction= [(initial wound size-specific day wound size) / initial wound size] x 100

All treatments were given for 20 days and wound sizes were determined after every 4 days. All treatments showed significant reduction in wound size as compared to control group. *In-situ* formulations showed faster and better wound healing potential as compared to emulgel formulations which were shown better than marketed formulation and extract. *In-situ* formulations also showed better permeation as compared to others. *In-situ* gel (G3) containing 5% extract of *S. lappa* was better than 3% (G1) and 4% (G2) formulation. 5% (F6) emulgel was also found effective but not as much better than *in-situ*. It was also shown that as the concentration of extracts in formulations increased wound healing activity was also increased. Percentage wound contraction was increased. All values were expressed as mean ± SEM, and data was analyzed by one way analysis of variance (ANOVA). Results are shown in table 6. Percentage wound contraction for all formulations is shown in figure 5 while for optimized formulation G3 and F6 these are shown in figure 6. Figures 7 through 9 highlight the wound healing images of rats treated with (a) herbal emulgels of *S. lappa* extract, (b) herbal *in situ* gels of *S. lappa* extract, and (c) pyodine gel, *S. lappa* extract and a control

Table 6. Wound healing activity assessment of herbal formulations of S.lappa

Formulation	Percentage wound contraction				
	Day 4	Day 8	Day 12	Day 16	Day 20
3% Emulgel	18 ± 0.829	42 ± 0.43	60 ± 0.707	85 ± 1.47	95 ± 0.43
4% Emulgel	22 ± 0.829	38 ± 0.829	63 ± 0.866	88 ± 1.08	98 ± 0.433
5%/ Emulgel	25 ± 0.829	47 ± 0.707	72 ± 0.829	93 ± 0.707	98 ± 0.433
3% In situ gel	27 ± 0.5	51 ± 0.829	73 ± 0.829	94 ± 0.707	100 ± 0
4% In situ gel	25 ± 0.43	53 ± 0.707	74 ± 0.707	95 ± 0.43	100 ± 0
5% In situ gel	30 ± 0.5	50 ± 0.54	87 ± 0.707	100 ± 0	100 ± 0
Pyodine Gel	13 ± 0.707	30 ± 0.5	57 ± 1.11	75 ± 0.829	91 ± 0.829
Extract	18 ± 0.82	40 ± 0.707	60 ± 0.707	81 ± 0.707	91 ± 0.43
Control group	12 ± 0.43	22 ± 0.43	37 ± 0.5	62 ± 1.08	82 ± 0.43

Values are mean ± SEM (n=4) P<0.05

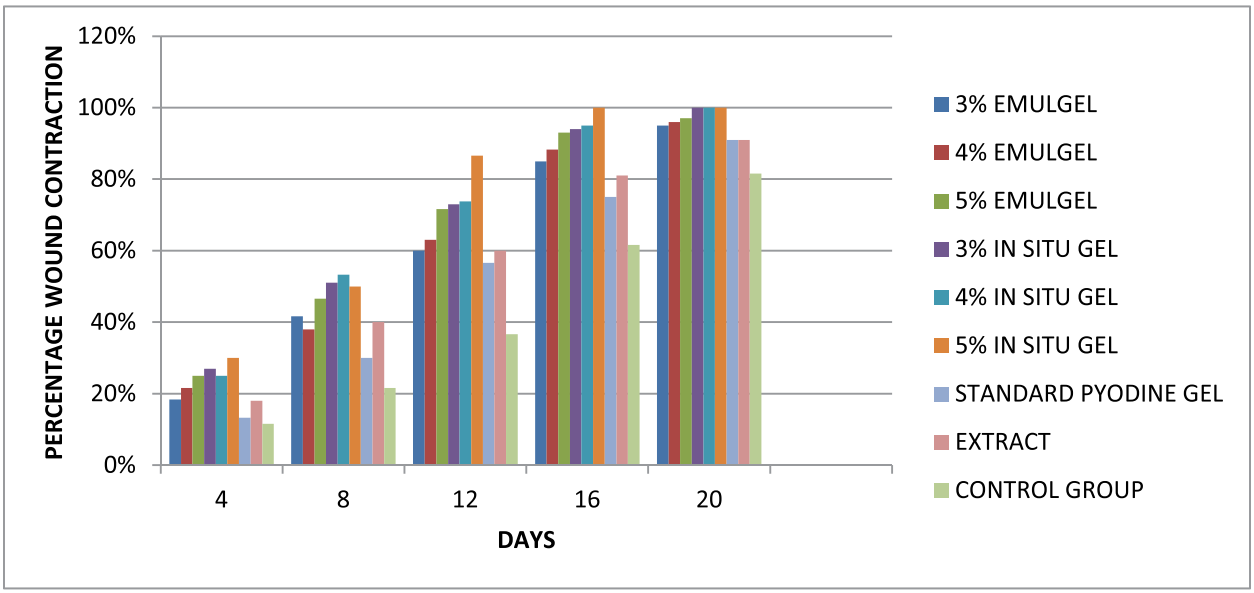

Fig. 5. Wound healing activity assessment for all formulations

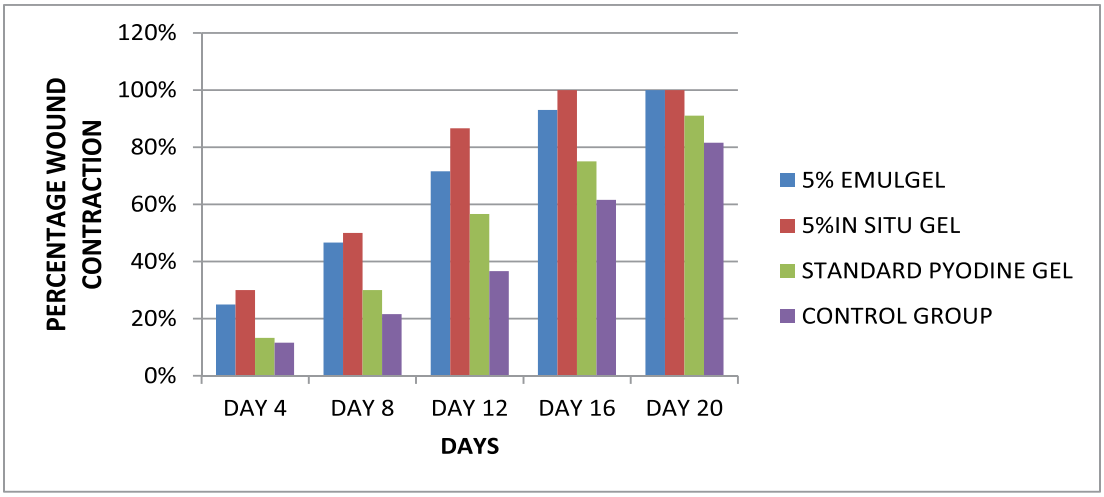

Fig. 6. Wound healing activity assessment of optimized G3 and F6 formulation

Fig. 7. Wound healing images of rats treated with herbal emulgels of *S. lappa* extract

Fig. 8. Wound healing images of rats treated with herbal *in situ* gels of *S. lappa* extract

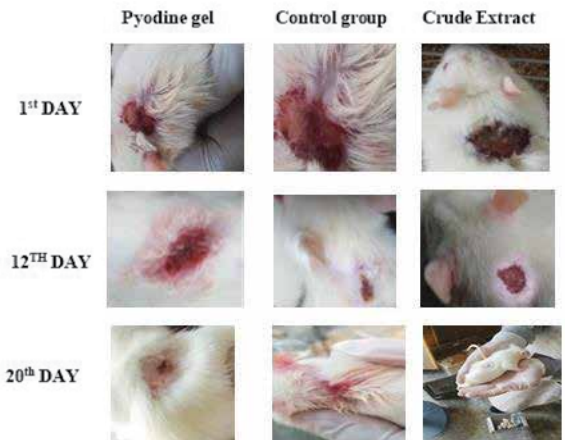

Fig. 9. Wound healing images of rats treated with pyodine gel, *S.lappa* extract and a control group

group, respectively.

3.9.1. In-vitro Antibacterial Activity

Rats were anesthetized with chloroform and excision wound was induced with sterile needles. Sterile cotton was placed over the wounds and exudate was collected. Exudate was cultured by using LB broth media [21]. After 24-48 hours bacteria were streaked on LB agar plates. After growth of bacteria they were again streaked on separate agar plate for biochemical analysis like gram staining, coagulase test and catalase test. Identification tests for bacteria are shown in table 7. *Staphylococcus aureus* was detected in pus culture.After identification *in vitro* anti-bacterial activity of formulations F3, F4, F5, G1, G2, G3, and crude extract was performed by using agar well diffusion method. Sulfazalazine was used as standardand different zones of inhibitons were determined which is reported in table 8. Results showed that sulfasalazine was having zone of inhibiton 31.3 mm, while extract was having 32.2 mm as compared to formulations and 33.1 mm in case of 5% formulation.

Table 7. Biochemical tests for wounded bacterial culture for identification of bacteria

Biochemical test	Observation
Coagulase test with plasma	Positive
Catalase test with hydrogen peroxide	Positive
Gram staining	Gram positive
Shape	*Cocci*
Type of microorganism	*Staphylococcus aureus*

Table 8. Zones of inhibitions of different formulations of *S. lappa* against standard

Formulation	Zone of inhibition (mm)
3% Emulgel	22.3 ± 4.5
4% Emulgel	19.6 ±5.85
5%/ Emulgel	25 ±3.15
3% In situ gel	*22.5 ±4.4*
4% In situ gel	*25 ±3.15*
5% In situ gel	*33.3 ±1.65*
Sulfasalazine cream	*31.3 ±0.55*
Extract	*32.2 ±0.55*

Values are mean ± SEM P<0.05

3.11. Statistical Analysis

All values were expressed as mean ± SEM, and data was analyzed by one way analysis of variance (ANOVA). The P value was found to be < 0.05 which showed that results were statistically significant.

4. DISCUSSION

Saussurea lappa contains multiple chemical constituents that are responsible for anti-bacterial and wound healing actvities. Phytochemical screening was carried out to evaluate the presence of these particular chemical constituents. *S. lappa* is shown to contain alkaloids, glycosides, flavonoids,

terpenoids, carbohydrates, saponins and taninns but no proteins have been detected [9]. In crude form, it is difficult to estimate which chemical is responsible for these particualr activities. So for, better estimation of these multiple phytochemicals subsequent fractionation of crude extract is better option.

In previous study it was found that 6 g extract of *S. lappa* when dissolved in 100 mL of water and applied topically to the rats significantly reduced the wound size [23]. In the light of this previous study we have tried to develop two topical dosage forms i.e. emulgels and *in-situ* gels in our present study. For further pharmacological studies acute toxicity studies also give estimation of dose that is considered to be safe. In mice, 1000 mg/kg dose of crude extract was administered and mice were strictly monitored for 24 hours to check if any death occurred. After 24 hours it was observed that no death or any toxic symptom occurred in any mice. These results revealed that no physiological or behavioral differences between any control group and treated group were observed [11]. So, the present study is a postive indication of lower toxicity profile of *S. lappa.*

Bioactivity of crude extract was assessed by *in-vitro* anti-hemolytic activity of crude extract at concentration of 100µg/ml through heat induced hemolysis of erythrocyte membrane model system. Percentage inhibition of *S. lappa* was 42.8% while aspirin was 51% at 100 µg/ml [10]. Although, *S. lappa* has little anti-inflammatory activity as comapred to aspirin but it can be a better option to use it as anti-inflammatroy agent as long term use of aspirin has a major side effect of gastric ulcer. Therefore, further work should be carried out to compare its effectiveness with other NSAIDS like declofenac sodium and naproxen etc. that can be beneficial for patients suffering from arithritis.

Conventional topical dosage forms like ointments and creams have low bioavailability and poor retention. After careful selection of drug carrier we have been able to design both formulations that have better bioavailability and improved retention [24]. Emulgels have better controlled release as well as increase drug loading capacity. When appliead topically, they also provide dual control release in the form of gel and emulsion [6]. Six formulations F1 to F6 were prepared with carbapol 940 which was found to be compatible with ingredients. Carbapol 940 is a cross-linked polyacrylate water soluble biodegradable polymer that provide controlled release and increased stability to our formulations. All the formualtions contained same amount of excepients and polymer but varying amount of crude extract. *pH* of all formulations was ranging from 6.3 - 7.3 that was also similar to physiologic *pH* of skin. All formualtions were found to be stable after stability testing of four weeks.

Keeping in view above mentioned properties of emulgels and crude extract another effort was carried out in present study to make thermo senisitive *in-situ* gel forming biodegradable topical system using carbopol 940, HPMC K 15 and polaxomer P407 that have better *in-vitro* release properties than emulgels. Polaxomer shows gelation at 37 degrees celsius and it also inhibit the effect of efflux pumps that cause drug to stay into the cells for a longer period of time [25]. *In-situ* gelling systems involves use of polymers that have phase transition from sol to gel upon alterations in physico-chemical properties of drug [7]. While designing emulgels and in situ gels factors like *pH* and viscosity should be kept in mind because these parameters should define the release pattern of formulations. Increasing temperature from 25 °C to 37 °C did not cause any significant increase in viscosity. However, too much increase in viscosity than required in case of *in-situ* gels will lead to delay release of formulation [26].

In the present investigations, *in-vitro* drug release study was performed by using Franz diffusion cell that is being used over centuries for diffuion study of semisolid preparations like gels and ointments etc. Formualtions F6 of emulgel and G3 of *in-situ* gel were used to determine drug release pattern. Dialysis membrane of 0.22 micrometre pore size was used for the determination of *in-vitro* release pattern and results showed that both formualtions followed first order release over the period of 8 hours. But in situ gel was having better and improved release pattern than emulgel [18].

Lyophillization of crude extract was done for *in -situ* gels formulation to minimize the moisture content and better stability of final products [13]. Lyophillization also helps in easy reconstitution of

freeze dried product with minimum contamination.

Wound healing activity was assessed by applying each formulation locally against excision model of rats. All treatments were given for 20 days and wound sizes were determined after every 4 days. Results showed that there was significant reduction in wound size in experimental groups as compared to control group. *In situ* gel (G3) with 5% crude extract of *S.lappa* showed excellent healing in 14 days as comapred to standard pyodine gel [20]. So, further work should be carried out on this formulation for more efficient wound healing because *S. lappa* extract has shown to accelerate wound healing. Although extact mechanism of wound healing is still unknown as crude extract contains multiple chemical constituents so fractionation of crude extract is necessary. Results also revealed that as the concentration of crude extract increases from 0.5% to 5% in all formulations, the wound healing activity also increases in similar manner.

In-vitro anti-bacterial activity was also performed by inducing pus in rats. Pus culture was prepared and bacteria were streaked on LB agar plates [21]. Biochemical analysis like gram staining, coagulase test and catalase test were performed for identification of bacteria. After identification *in vitro* anti-bacterial activity of formulations F4, F5, F6, G1, G2, G3 and extract agaisnt standard sulfazalazine was performed. Different zones of inhibitions were measured. Results showed that sulfasalazine was having zone of inhibiton 31.3 mm. While extract was having 32.2 mm as compared to formulations and 33.1 mm in case of 5% formulation. So, in future, it may be beneficial to develop cost effective and resistance free herbal formulation to prevent wound infections that is leading cause of illness in majority of world population. Morever, localized topical herbal formulaion will have less side effect and minimum chances of bacterial resistance as comapred to the systemic ones.

Extraction is the most important step in biological evaluation of medicinal plants, so it should be performed under controlled temperature. Choice of extraction method is very important as high temperature may lead to deterioration of heat sensitive constituents. Choice of solvent is also a critical step. Non polar solvent like n-hexane and ethyl acetate should be used for extraction of lipophillic compounds while solvent like methanol, ethanol and chloroform should be used for extraction of hydriphillic compounds [27].

5. CONCLUSION

The study reveals that *Saussurea lappa* has effective wound healing, anti-inflammatory and anti-microbial properties which are proved through various physical and biochemical parameters. Formulation of extract into *in-situ* gels enhances the wound healing potential of *S. lappa* because it provides controlled drug release pattern and greater stability to the extract. Further studies are required to confirm the main constituents responsible for wound healing properties.

6. ACKNOWLEDGEMENTS

The authors are thankful to the Higher Education Commission for providing financial support through Project # 20-686 under the National Research Program for Universities..

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Proceedings of the Pakistan Academy of Sciences:
B. Life and Environmental Sciences 56 (3): 97–112 (Special Issue, 2019)

ISSN: 2518-4261 (print), ISSN 2518-427X (online)

Pakistan Academy of Sciences

Research Article

Floristic Diversity, Ethnobotany and Traditional Recipes of Medicinal Plants of Maruk Nallah, Haramosh Valley, District Gilgit, Gilgit Baltistan

Qamar Abbas[1*], Adeeb Hussain[1], Sher Wali Khan[1], Alamdar Hussain[2], Shehla Shinwari[1], Azhar Hussain[3], Asad Ullah[4], Muhammad Zafar[5], and Karamat Ali[6]

[1]Biological Sciences Department, Karakoram International University Gilgit-Baltistan
[2]Biological Sciences Department, University of Baltistan, Skardu
[3]Department of Agriculture and Food Technology Karakoram International University Gilgit-Baltistan
[4]Centre of Plant Biodiversity, Peshawar University, Peshawar
[5]Department of Plant Sciences, Quaid-i-Azam University, Islamabad
[6]Environmental Sciences Department, Karakoram International University Gilgit-Baltistan

Abstract: Haramosh valley is one of the beautiful valleys located at 35°53'04" N latitude and 074°41'11" E longitude at elevation of 2500-5000 meters in district Gilgit. For the assessment of floristic diversity total 114 plant species were recorded at Maruk Nallah, out of which, 85 were herbs belonging to 34 families; 13 were shrubs belonging to 9 families; while 16 were trees belonging to 10 families. Results showed that, family Asteraceae was the most dominant family with 12 genera and 21 species while the genus Artemisia was the most dominant genera, with six species. Through semi structured questionnaire and interviews ethno botanical data was collected from the inhabitants of the area. Out of 114 plant species, People are habitual to use 65 plant species as a traditional medication for 45 different ailments. The plant parts used for medication include leaves (26%) followed by fruits (19.2%), seed and root 13.7%; aerial parts 12.3%; flower 5.48%, resin 4.11%; while the bulb contributes 2.74%. The inhabitants have a lot of cultural and mythical beliefs regarding some plant species. Some very important medicinal plants which have common use value as a local recipe include *Juniperus excelsa* M. Bieb, *Betula utilis* D. Don, *Delphinium brononianum* Royle., *Saussurea simpsoniana* Field & Garden, *Primula macrophyla* D. Don, *Pegnum harmala* L., *Geranium Pretense* L. *Saussurea simpsoniana* Field and Garden, and *Thymus linearis* Benth.. The natural resources are under pressure due to much grazing pressure, deforestation and over-exploitation need to conserve them for future generations.

Keywords: Ethnobotany, Floristic diversity, Maruk Nallah, Deforestation, Over-exploitation

1. INTRODUCTION

Gilgit-Baltistan (GB) is well known due to its unique natural beauty, snow covered mountains, pastures and dense forests patches. These northern mountainous regions of Pakistan located at 72°-75°East longitude and 35°-37° North latitude [1]. The junction of three great mountainous ranges located near to Gilgit city. Due to diverse topography, climate condition and different elevations, unique flora and fauna exist in these regions [2]. The three major mountainous regions contain about 10% of world flora and habitat of numerous medicinal plants [3]. GB is hub of medicinal and aromatic plants and people of the area have folk wisdom and dependent on their natural resources [4]. Approximately 3000 plant species have been reported in these areas, out of them about 200 plant species are used as medication among the inhabitants and nearly 80% flora of Pakistan is located in northern mountainous ranges [5, 6, and 7]. The population of GB is about 02 million with growth rate of 2.47% and hardly 1%

Received: February 2019; Accepted: September 2019
* Corresponding Author: Qamar Abbas; qamarabbaskiu@gmail.com

of area is used for agriculture while the rest 99% is covered by mountains, rivers rangelands, glaciers and forest [6]. The native people of these areas have strong cultural and traditional values. Most of the people in the area are dependent on their natural resources for food, medication and shelter either partially or completely [8, 9]. Aboriginal people are environmentally friendly most of the time, but due to dependency on fodder and forage as well as much consumption of fire wood for severe winter cause over grazing and deforestation respectively [10]. These areas are spread in different elevation and human settlements in these hard areas have no proper source of availability of daily requirements. So the rural communities are getting their all basic requirements from the natural resources. Even they have no proper planning for their sustainable utilization may cause destruction [11]. Infect the rural communities have much folk wisdom and they are still treating patients through traditional methods. The natural vegetation has maximum pressure because most of the communities of the rural areas have more than 80% dependency on their medicinal plants [12].

1.1. Haramosh Valley

Haramosh valley is located at the bank of the Indus River, boarder valley of district Gilgit links with the Rundo valley which is the first valley of GB. This valley has a unique potential diversity of flora due to high alpine pastures, glacier deposits, snow covered mountains, forest patches and diverse climatic conditions. Human population settlements are mostly in twelve major villages and living above 1500 to 2500m elevations. Most of the vegetation diversity is observed in the alpine and subalpine regions of this valley [13]. This area is also known as fruit basket of Gilgit-Baltistan. People of this area possess unique customs and majority of them are into agriculture and livestock.

Marukh Nallah has unique biodiversity and dense forest patches located at the elevation of 2500 meter to 5000 meters. Most of the low altitude area is used for the agriculture purpose; while the upper area consists of alpine pasture, and forest patches. Winter is very harsh in these valleys due to heavy snow fall, while summer season is very pleasant. The people of these areas are like the seasonal nomads and depend on their natural resources for food, fodder, shelter and fuel. The current study was conducted in this potential area of Haramosh valley, to discover the floral wealth, and to record the folk wisdom of the inhabitants and list down the recipes common in these areas.

2. MATERIALS AND METHODS

2.1. Field Survey

Different field visits were organized during 2017-18 to evaluate the floristic diversity, collect plant specimens, and record the ethno botanical data through semi structured questionnaires and interviews.

2.2. Specimen Collection & Identification

During the continuous field visits from March to October, proper plant specimens were collected, pressed, dried and mounted on standard herbarium sheets. The data was collected by using semi-structured questionnaire informants, mostly from indigenous peoples. We have frequently visited the area for specimen collection during fruiting and flowering season of the plants. A semi structured questionnaire was used to gather the folk inform and traditional application of medicinal plants especially the recipes details. All collected plant specimens pressed, dried and mounted on standard herbarium sheets according to herbarium techniques. All these specimens were identified with the help of Flora of Pakistan [14, 15] and finally deposited in the Biological Science Department Herbarium, Karakoram International University Gilgit, Pakistan.

3. RESULTS

The present study was carried out to check the floral diversity, ethno botanical studies and record the folk recipes of Maruk nallah Haramosh valley, Gilgit Baltistan (GB) Fig. 1. A total of 114 plants species were reported belonging to 45 families and 90 genera. Most of the identified species belong to angiosperms (dicots) while a few plants species monocot and gymnosperms. Out of 114 plant species 21 species belonged to Family Asteraceae, 7 species belong to each Labiatae and Rosaceae, 6 species belonged to family Umbelliferae, 5 species to Polygonaceae, 4 species each to Scrophulariaceae

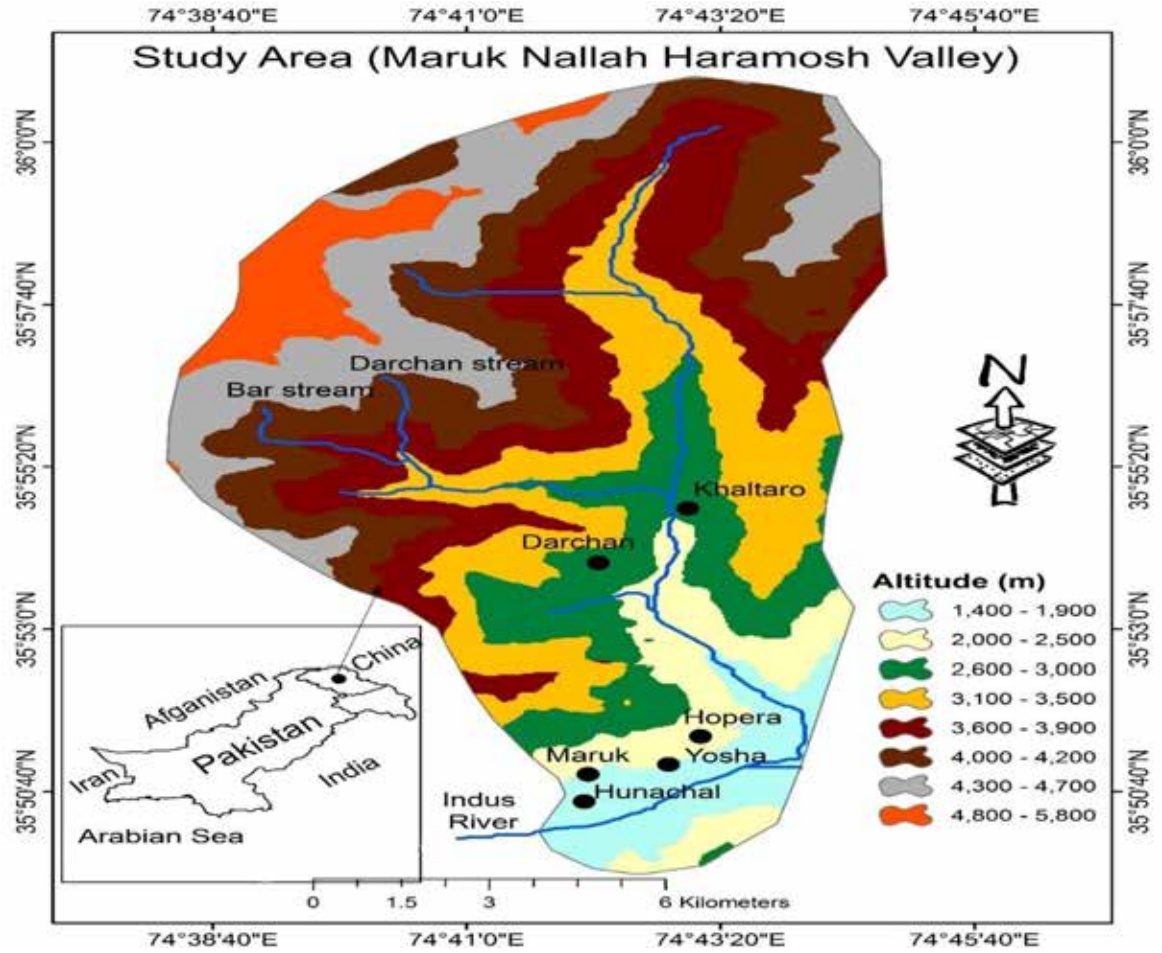

Fig. 1. Map of Maruk Nallah, Haramosh valley, District Gilgit

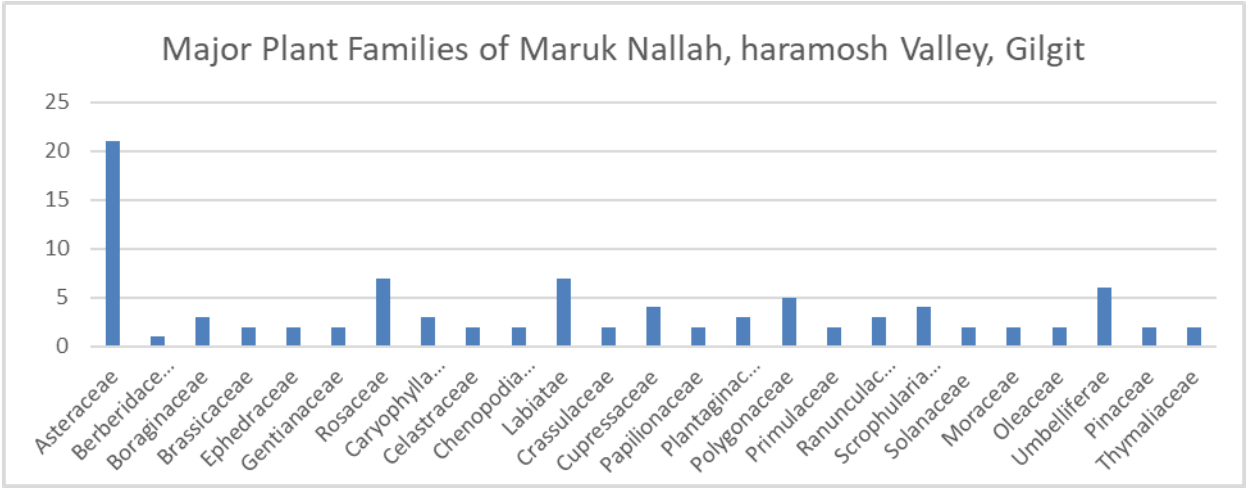

Fig. 2. Showing the major Plant Families of Maruk Nallah containing number of species

and Cupressaceae, while other families have less number of species (Table 1).

On the basis of habit, out of total 114 plant species 85 species were herbs, 13 species were shrubs while 16 species were trees. Results revealed that, the family Asteraceae is the most dominant family having 12 genera and 21 species while the genus *Artemisia* was the most dominant genus, contain six species (Fig 2).

Table 1. List of plant families and number of species reported from Maruk nallah, Haramosh valley, district Gilgit

S.No.	Family	No. of Species
1	*Asteraceae*	21
2	*Berberidaceae*	1
3	*Boraginaceae*	3
4	*Brassicaceae*	2
5	*Caryophyllaceae*	3
6	*Celastraceae*	2
7	*Chenopodiaceae*	2
8	*Crassulaceae*	2
9	*Cupressaceae*	4
10	*Ephedraceae*	2
11	*Gentianaceae*	2
12	*Labiatae*	7
13	*Moraceae*	2
14	*Oleaceae*	2
15	*Papilionaceae*	2
16	*Pinaceae*	2
17	*Plantaginaceae*	3
18	*Polygonaceae*	5
19	*Primulaceae*	2
20	*Ranunculaceae*	3
21	*Rosaceae*	7
22	*Scrophulariaceae*	4
23	*Solanaceae*	2
24	*Thymelaeaceae*	2
25	*Umbelliferae*	6

3.1. Ethno Botanical Studies

People of this valley have strong traditional and cultural values. Still they are much dependent on their natural resources. The results predicted that, about 65 plant species were commonly used for the cure of 45 different diseases. These 65 plant species were belonging to 37 families and 59 genera's, while on the basis of habit categories 43 species were herbs, 07 species were shrubs and only 15species were trees.

According to the use of plant parts for the treatment, most common used parts were the leaves (26%), followed by fruits (19.2%); seed and root were 13.7%; Arial part 12.3%; flower 5.48%; resin 4.11%; while the stem and bulb contribute 2.74% (Table 2). According to floristic diversity on the basis of habit category maximum identified flora of study area are herbs (66%) followed by the shrubs (11%), and tree (23%) as shown in fig. 3. The people of the area are very keen to use of medicinal plants for their medication (Table 3). Most common used part of plants is leaves, followed by fruits, seeds, roots, aerial parts (Fig. 4). According to the mode of use people are habitual to use direct method, either fresh of dry leaves intake are about 40.2 %, while second most common method of use is in powder form about 22% while decoction is about 27.3% (Table 4). Some plants species have more than one type of mode of use common among the inhabitants of the area.

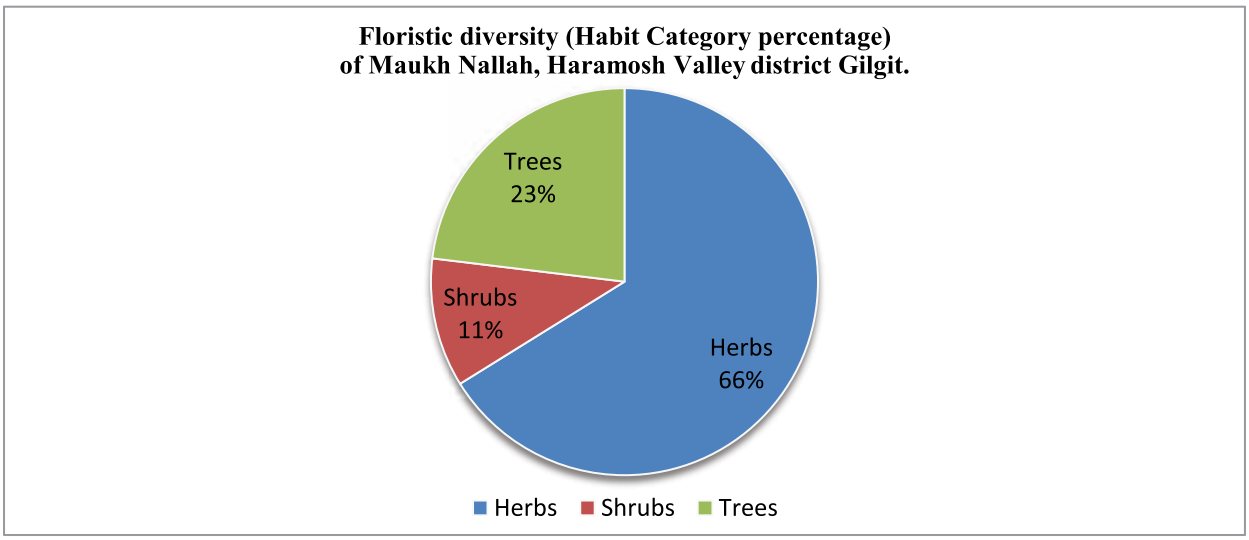

Fig. 3. Floristic diversity (Habit categorize percentage) of Maruk nallah, Haramosh valley, district Gilgit

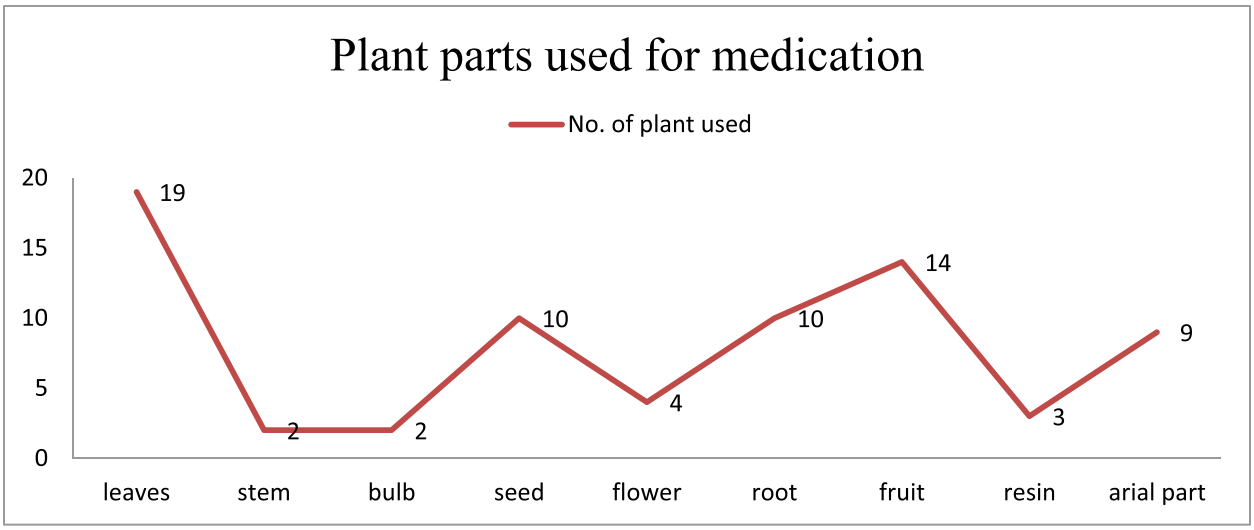

Fig. 4. Plant parts used for different ailments in Maruk nallah, Haramosh valley, district Gilgit

Table 2. List of plant parts used for the different ailments in Maruk nallah, Harmosh valley, district Gilgit.

Part used	No. of plant used	%age
Leaves	19	26
Stem	2	2.73
Bulb	2	2.74
Seed	10	13.7
Flower	4	5.5
Root	10	13.76
Fruit	14	19.2
Resin	3	4.5
Aerial parts	9	12.32

Table 3. Detailed list of medicinal plants common for medication in Maruk nallah, Haramosh valley, district Gilgit

S. No.	Family	Botanical Name	Vernacular Name	Habit	Part Used	Method of use	Purpose
1	Alliaceae	*Allium cepa* L.	Kashu	Herb	Bulb/ Seed	Remedy	The seed are taken for kidney problems while the bulb oil is used for cough and asthma
2	Alliaceae	*Allium sativum* L	Gokpah	Herb	Bulb	Direct	Used for Abdominal worms, dysentery and heart diseases. Also used as vegetable.
3	Anacardiaceae	*Pistacia khinjuk* Stocks	Khakawoo	Tree	Fruit	Direct	used for stomach problems, sour taste of mouth, vomiting and diabetes. The resin smoke is given for eye infection and as antiseptic. Plant parts smoke is used as mosquitoes repellent.
4	Asteraceae	*Saussurea simpsoniana* Field & Garden	Bushi Phunar	Herb	Flower	Decoction	Used for Pneumonia, Dysentery, joints pain, sore throat, fever, cough and Asthma.
5	Asteraceae	*Carthamus tinctorius* L.	Pong	Herb	Flower	Powder, decoction	The peoples make powder to give color to bread in traditional and cultural festivals. The decoction is used for cold fever, Pneumonia, vomiting and typhoid.

6	Asteriaceae	*Artemisia maritiama* L.	Paloyo Zoon	Herb	Aerial parts	Juice or Powder	The powder and juice is used for treatment of diabetes, cardiac problems, high blood pressure, dysentery, and to remove abdominal worms.
7	Asteraceae	*Artemisia absinthium* L.	Kakamoch	Herb	Leaves, Seed	Direct, decoction	The decoction of both leaves and seeds are used for diabetes, high blood pressure, stomach problems, dysentery, vomiting and to treat abdominal worms and pain also.
8	Asteraceae	*Datura stromonium* L.	Daturoo	Herb	Seed, Leaves	Direct, grind	The seed are used for retention of water in muscles (badi) while the fresh leaves paste is used for external injuries.
9	Asteraceae	*Lactuca sativa* L.	Salad	Herb	Leaves	Remedy	The fresh leaves are remedy of stomach acidity. The leaves are used as salad.
10	Asteraceae	*Cichorium intybus*	Ishinachii	Herb	Leaves	Remedy	Against weight loss and constipation
11	Berberidaceae	*Berberis orthobotrys* Bien ex Aitch.	Ishkeen	Shrub	Root	Decoction, Powder	The decoction/powder is used for backache, injuries, joint pain, infertility (females), weak uterus, jaundice and bone fractures.
12	Boraginaceae	*Myosotis alpestris* F.W. Schmidt	Heto Lelo	Herb	Flowers	Direct	It is used to treat throat problems and throat infection.
13	Boraginaceae	*Heliotropium dasycarpum* Ledeb.	Sabon Kach	Herb	Leaves	Decoction	The decoction is used for gastric problems.
14	Boraginaceae	*Onosma hispida* Wall.ex G. Don	Talcharong	Shrub	Leaves	Powder Decoction	The powder of leaves is mixed with oil which is used for long and silky hair. The decoction is given for fever, malaria, cough and heart problem.
15	Cannabaceae	*Cannabis sativa* L	Thunchi	Herb	Leaves Seed	Direct	The seeds are used to enhance milk in humans and animals. The leaves are used for Stomach problem, measles and chicken pox.
16	Chenopodiaceae	*Chenopodium foliosum* Asch., FI.	Shom Gurus	Herb	Berries	Direct	To remove the stain from skin.
17	Cucurbitaceae	*Cucurbita maxima* Duch, ex Lam.,	One	Herb	Fruit	Vegetable	Used for constipation and gastrointestinal problems.
18	Cucurbitaceae	*Cucmis sativus* L.	Law	Herb	Fruit, Leaves	Direct. paste.	The fruit is used for diabetes, stomach problem while the leaves are used for fever.
19	Capparidaceae	*Capparis spinosa* L.	Kavir	Herb	Seed, Roots	Decoction	The decoction of fruit seeds is used for obesity, cancer, joint pain and Ulcer. The decoction of roots is used for joint pain.
20	Cupressaceae	*Juniperus communis* L.	Mitthary	Shrub	Berries	Decoction	The decoction of berries is used for kidney stone and tuberculosis.
21	Cupressaceae	*Juniperus excelsa* M. Bieb	Chilee	Tree	Berries	Decoction	The decoction of berries is used for kidney stone and TB.
22	Euphorbiaceae	*Euphorbia cornigera* Boiss.,	Fotan	Herb	Aerial parts	Direct	The Aerial parts are used for constipation. Most in veterinary cases. (for Livestock)

23	Ephedraceae	*Ephedra intermedia* Schrenk & Meyer	Soom	Herb	Aerial parts	Decoction, grind Powder.	used for joint pain, for strong teeth and teeth pain. The fresh aerial parts are crushed well and used to stop bleeding during injuries. The powder is mixed with snuff for good taste. The decoction is used for bath which helpful in joint pain and backache.
24	Elaeagnaceae	*Elaeagnus angustifolia* L	Gunair	Tree	Fruit	Direct	The fruit is used for cough, dysentery and high blood pressure.
25	Ericaceae	*Rhododendron anthopogon* D. Don	Talachum	Shrub	Leaves	Decoction, Powder	The decoction used for Stomach problem, cough, and diabetes. The inhabitants used the leaves powder for silky-long hair and dandruff. Leaves are used in making tea, which helpful to maintain blood pressure and diabetes.
26	Geraniaceae	*Geranium pretense* L	Kuratkasho	Herb	Aerial parts	Powder crushed	The inhabitants used the powder to cure external injuries, urinary tract infection and the paste is also used to cure external injuries.
27	Grossulariaceae	*Ribes alpestre* Decne	Shumloo	Shrub	Fruit Root	Direct, Decoction	The fruit is best remedy for skin allergy (doosh) and hepatitis. The decoction of roots is used for backache.
28	Juglandaceae	*Juglan nigria* L.	Ashoo	Tree	Leaves, Root	Direct	Seed for used for high blood pressure and roots and root is applied for toothache. Use as miswak.
29	Labiatae	*Thymus linearis* Benth.	Tumuro	Herb	Aerial parts	Decoction	The decoction is used for abdominal pain, chest pain, weight loss and high blood pressure. The inhabitants also make tea which helpful to maintain blood pressure.
30	Labiatae	*Mentha royleana* Benth	Pheleel	Herb	Aerial parts	Direct, extract juice	This is best remedy for high BP, fever, dysentery stomach pain while the juice is used for abdominal pain and vomiting. Some people uses as salad as well.
31	Labiatae	*Mentha arvensis* L.	Pudina	Herb	Aerial parts	Juice	The juice is used for diabetes, diarrhea, dysentery, high blood pressure, stomach pain, vomiting, abdominal pain and abdominal worms. The leaves are also used as a salad.
32	Labiatae	*Isodon rugosus (Wall.ex Benth.)* Codd	Phaphus	Herb	Leaves	Powder	Powder of leaves are used for toothache.
33	Labiatae	*Salvia nubicola* Wall ex Sweet	Coropo	Herb	Leaves	Extract juice	The extract of leaves is given for treatment of asthma, cough and fever.
34	Malvaceae	*Abelmoschus esculentus* (L.) Moench,	Bindi	Herb	Fruit Root	Vegetable. Decoction	The decoction of fruit is used for diabetes and joint pain while the decoction of roots is used for kidney stone.
35	Moraceae	*Morus nigra* L.	Shatumaroch	Tree	Fruit	Juice	The juice is used for stomach problems, sore throat, constipation, ulcer and for weak bones.
36	Moraceae	*Morus alba* L.	Shaimaroch	Tree	Fruit	Direct	The fruit is used for stomach pain, constipation, anemia and weak bones.

37	Moraceae	*Ficus carica ssp. Carica* L.	Faag	Tree	Fruit	Remedy	The fruit is used against heart diseases and constipation.
38	Moraceae	*Ficus caria ssp. Rupestris* (Hausskn. Ex Boiss.).	Black Fig	Tree	Fruit	Decoction	The decoction of fruit is used for heart diseases.
39	Pinaceae	*Picea smithiana* Wall.	Cheenh	Tree	Resin	Direct, powder	The resin is used for blood clotting when cut.
40	Pinaceae	*Pinus wallichiana* A.B. Jacksn	Chachul	Tree	Resin	Direct	The resin is used for blood clotting when cut. Only external use.
41	Plantaginaceae	*Plantago major* L.	Khakhapai	Herb	Leaves	Powder, direct, Juice	The decoction of leaves is used for constipation, dysentery, blood pressure. The juice of leaves is given for dysentery.
42	Poaceae	*Hordium valgaris*	Joo	Serial	Grains	Make bread	The bread is used for heart, diabetes, high B.P and arthritis (joints).
43	Poaceae	*Zea mays* L.	Makai	Serial	Seed	Make bread	The bread is used for diabetes, dysentery and heart problems.
44	Polygonaceae	*Bistorta affinis* (D.Don) Green	Chumui	Herb	Seed	Direct	The fruit which contain large seeds are used for dysentery especially for infants.
45	Polygonaceae	*Rumex nepalensis* Spreng.	Obabal	Herb	Root	Decoction, powder	The decoction of roots is used for constipation while the powders of leaves are used for swelling and joint pain. The fresh roots are crushed to cure pimples (infection and pus)
46	Polygonaceae	*Rheum spiciforme* Royle.	Jaroo Chotal	Herb	Root	Powder,	The powder is used for joint pain and backache. The powder is also used for weak uterus.
47	Primulaceae	*Primula macrophyla* D.Don	Sujo Leloo	Herb	Leaves (Powder)	Use as dool	The lower side of the leaf contains a powder, which is used to eye infection and eye pain. This powder is also used to eye wash.
48	Punicaceae	*Punica granatum* L.	Danui	Tree	Fruit Cover	Powder, Direct, decoction	The decoction of fruit cover is used for cough, dysentery and pimples as well. The fruit is used for hepatitis and fruit cover is used to remove stains after injuries.
49	Ranunculaceae	*Delphinium brononianum* Royle.	Makhoti	Herb	Flower	Decoction	Decoction is used for heart diseases, high blood pressure, Pneumonia, cold fever and pain, dysentery, asthma and height.
50	Rosaceae	*Prunus armeniaca* L.	Juwi	Tree	Fruit	Juice	The fruit is used for heart problems, Stomach pain, Constipation, Diarrhea and anemia.
51	Rosaceae	*Prunas amygdalus* L.	Badam	Tree	Seed	Remedy	The seed oil or seed is used weak bones and against cold in winter.
52	Rosaceae	*Spiraea canesens* D.Don.	Dara	Shrub	Stem Oil	Heat the stem	When a dried stem is heated, oil is produced which is used for pimples, injuries infections and skin infection.
53	Rutaceae	*Haplophyllum gilesii* (Hemsl.) GC.	Sabon Char.	shrub	Leaves	Direct	Use as detergent. Used to wash hair and dandruff.
54	Salicaceae	*Salix denticulate* Andersson	Brauw	Tree	Leaves	Juice	Low blood pressure and fever

55	Saxifragaceae	*Bergenia stracheyti* Hook. & Thoms.	Safsar	Herb	Leaves, Root	Powder	The powder of roots is used for backache, asthma, and cough. The dry leaves are used for making tea.
56	Scrophulariaceae	Verbscum thapsis L.	Fundupal	Herb	Leaves	Decoction	The decoction of leaves is given for fever, constipation and cough.
57	Solanaceae	*Solanum nigrum* L.	Gabili	Herb	Berries, Leaves	Extract juice	The decoction of berries is used for Hepatitis and heart diseases. The leaves juice is given for fever.
58	Thymelaeaceae	*Daphne mucronata* Royle	Nirkoo	Shrub	Root	Decoction	The decoction of root is given for constipation.
59	Umbelliferae	*Ferula anthrax* Bioss.	Sab	Herb	Root	Powder	The powder of roots is used for serve cough and cold fever.
60	Umbelliferae	*Carum carvi* L.	Hayow	Herb	Seed	Direct	The seeds are used for abdominal worms, heart diseases, high blood pressure and pre-mature seed are used for dizziness.
61	Umbelliferae	*Pleurospermum candollei* (DG.) Clarke	Pucha Sing	Herb	Stem	Powder	The powder of stem is used for infertility (for both male and female), side pain and back pain as well.
62	Urticaceae	*Urtica dioca* L.	Jami	Herb	Aerial parts	Leaves extract	Young leaves used as vegetable and remedy for hepatitis, stomach problem and joint pain.
63	Vitaceae	*Vitis vinifera*	jach	Tree	Fruit	Direct	The fruit is used for fever and cough.
64	Zygophyllaceae	*Tribulus trestres* L.	Show Kono	Herb	Fruit	Decoction	The decoction of fruit is given for Cancer.
65	Zygophyllaceae	*Pegnum harmala* L.	Ispandur	Herb	Arial	Smoke	The smoke of leaves and seeds is given for eye pain and ear infection.

Table 4. Mode of utilization of Medicinal plants

Mode of utilization	No. of plant used	%age
Powder	17	22%
Remedy/Direct	31	40.2%
Decoction	21	27.3%
Juice	8	10.5%

Table 5. Mode of utilization of Medicinal plants

Diseases	No. of plants used	Diseases	No. of plants used	Diseases	No. of plants used.
Dysentery	12	Kidney Problems	4	Skin Problems	2
Stomach Problems	12	Eye Problem	4	Infertility	2
Constipation	11	Vomiting	4	T.B	2
Fever	11	Pneumonia	3	Typhoid	1
Heart Diseases	11	Eye Problem	3	Malaria	1
Blood Pressure	10	Hair Tonic/Dandruff	3	Chest Pain	1
Cough	9	Side Pain/ Kidney pain	3	Unary Tract Infection	1
Joint Pain	8	Sour Throat	3	Water retention in muscles.	1
Diabetes	8	Ulcer	2	Swelling	1
Abdominal Problems	7	Diarrhea	2	Urine problems	1
Backache	6	Weak Uterus	2	Bone Fracture/ Weak	1

Injuries	5	Jaundice	2	Arthritis	1
Asthma	5	Toothache	2	Ear Infection.	1
Pimples	5	Blood Clotting	2	Measles	1
Hepatitis	4	Anemia	2	Cancer	2

Medicinally important plants are used for the treatment of more than forty-five different disease types. In these areas most common diseases are digestive, respiratory, and heart diseases. The Table 5 shows that maximum numbers of plant species are applying for the treatment of these common diseases. Through the oral interviews and semi structured interviews a data gathered from the inhabitants of the Maruk Nallah, Haramosh Valley. About 59 respondents contributed the folk wisdom ranges in the age of 20 to 85. Respondents belongs both genders, and out of 59, 15 were females and 44 were males.

3.2. Traditional Recipes

People of the area are not only using the single plant species; they have some folk wisdom to make traditional recipes after mixing the more than two plant species and their parts for the treatment of different ailments (Table 6).

3.3. Cultural Myths and Believe

The people of these selected valleys have strong cultural belief and have unique myths as compare to other valleys of the area. Some special type

Table 6. Detail list of some important recipes commonly used in Haramosh valley district Gilgit

S. No	Family	Botanical name	Vernacular name	Part used	Method	Disease and Dose
1	Punicaceae	*Punica granatum* L.	Danu	Fruit husk	Each in equal quantity and extract juice	Juice is used for Diabetes and heart problems two tea spoon twice a day.
	Labiatae	*Mentha royleana*	Pudina	Arial part		
	Zingibraceae	*Zingiber officinalas*	Adrak,	Rhizome		
	Alliaceae	*Allium sativum* L.	Gokpah,	Leaves		
2	Ranunculaceae	*Delphinium brunonianum* Royle.	Makhoti	Flower	Decoction	Used for coldfever, cough, diabetes, heart, high BP, pneumonia two tea spoon twice a day.
	Compositae	*Saussurea simpsoniana* Field & Garden	Bushi phunar	Arial parts		
	Compositae	*Carthamus tinctorius* L.	Pong	Flower		
3	Labiatae	*Thymus linearis* Benth.	Tumuroo,	Arial parts	Juice	High Blood pressure, abdominal worms and abdominal pain a tea cup twice a day.
	Labiatae	*Mentha royleana* Benth	Pheleel	Arial parts		
4	Compositae	*Carthamus tinctorius* L.	Pong,	Flower	Decoction	Pneumonia, serve fever two tea spoon twice a day.
	Ranunculaceae	*Delphinium brononianum* Royle.	Makhoti	Flower		
5	Labiate	*Mentha royleana* L.	Pudina ,	Arial	Extract	Pneumonia, fever, abdominal pains two tea spoon twice a day.
	Alliaceae	*Allium sativum* L.	Gokpah	Leaves		
6	Alliaceae	*Allium cepa* L.	Kashoo	Leaves	Juice	Vomiting, dizziness, fever, dysentery a glass of Juice twice a day.
	Labiatae	*Mentha royleana* Benth	Pheleel,	Arial		
7	Polygonaceae	*Bergenia stracheyti* Hook. & Thoms.	Safsar	Root	Powder	Female (weak uterus) like a pill or capsule size twice a day.
	Polygonaceae	*Rheum spiciforme* Royle.	Jaroo chotal	Root		

8	Labiatae	*Mentha arvensis* L.	Pudina,	Arial	Extract juice	Dysentery, Diarrhea two tea spoon twice a day.
	Alliaceae	*Allium cepa* L.	Kashu	Leaves		
9	Labiatae	*Mentha arvensis* L.	Pudina ,	Arial	Juice	Dysentery, loosemotion, gastric issues, vomiting a glass of juice twice a day.
	Alliaceae	*Allium sativum* L.	Gokpah	Rhizome		
	Labiatae	*Mentha royleana* Benth	Pheleel,	Arial		
10	Polygonaceae	*Rheum spiciforme* Royle,	Jaro chotal	Root	Equal amount of all three plants and make smooth powder	Infertility like a pill, or capsule size twice a day.
	Umbelliferae	*Pleurospermum candollei (DG.)* Clarke	Pucha sing	Stem		
	Zygophyllaceae	*Tribulus terrestris* L.	Sow kono	Fruit		

of folk myths is, after burning the fresh leaves of *Juniperus* species smoke, when some special people called "Danyaln" (Shamans), inhale the smoke help Daylan to extract information about unforeseen things like diseases, evil deeds, and many other problems. The smoke of *Peganum harmala* L. and *Juniperus* species used for their cattle's sheds called "Dooban" reason behind this activity is actually myth to protect their cattle's and safe return to home. The inhabitants have great trust on some alpine plants as clean and effective for wealth and prosperity. These plants are *Betula utilis* D. Don, *Delphinium brunonianum* Royle, *Saussurea simpsoniana* Field & Garden, and *Primula macrophyla* D. Donare "Shujaa" (singular; Shujo) means sign of cleanliness. If anyone plucks them without any noble reason or either damage them they will suffer with any unknown disease of problem.

It is a common practice of villagers that they are not growing *Salix* species and *Juglans regia* in front of their resident/homes because they believe that the Salix species are symbol of sorrow while under the shed of the *Juglans regia*, the ghosts or evil spirits resides. While they believe that some Rosa species especially *Rosa webbiana*, and *Fragaria nubicola*, to cultivate in lawn and the fragrance of roses will bring happiness. *Peganum harmala* L. is common in used as an antiseptic and its smoke is called "Dooban" used to clean their houses and shops just prevent the evil deeds and diseases

4. DISCUSSION

It has been knowable that on earth there are about 0.3 million plants species, out of which 83% plants species have been studied [16,17]. There are about six thousand species of higher plants. It has reported that six hundred to seven hundred higher plants species are medicinally important [7]. Rural communities are mostly dependent on their natural resources, especially for the medication. Medicinal herbs are playing key role to control and treatment of many diseases [18, 19, 20, 21, 22].

The present study is also an effort to explore the hidden treasure floral wealth of an important area of Murruk nallah, Harmosh Valley, GB. Total 114 plants were reported from this area, belonging to 45 families. Maximum flora was reported included 85 herb species, 16 tree species trees, while 13 shrub species (Table 7). Floristic diversity of Pakistan's is due to its diverse climatic conditions, topography

Table 7. Detail List of the identified plant species from Murukh nallah, Haramosh valley, district Gilgit.

S. No	Botanical Name	Family	Habit
1	*Artemisia absinthium* L.	Asteraceae	Herb
2	*Artemisia brevifolium* Wall. ex DC.	Asteraceae	Herb
3	*Artemisia gmelini* Web.	Asteraceae	Herb
4	*Artemisia japonica* Thumb.	Asteraceae	Herb
5	*Artemisia santolinifolia* Turcz. Ex Krasch.	Asteraceae	Herb
6	*Artemisia scoparia* Waldst. & Kit.	Asteraceae	Herb

7	*Aster peduncularis* Wall.	Asteraceae	Herb
8	*Cirsium vulgaris* (Savi.)Ten.	Asteraceae	Herb
9	*Cremanthodium decaisnei* Clarke	Asteraceae	Herb
10	*Crepis flexuosa* (D.C.)Benth.	Asteraceae	Herb
11	*Erigeron alpinum* L.	Asteraceae	Herb
12	*Heteropappus altaicus* Willd.	Asteraceae	Herb
13	*Inula royleana* D.C	Asteraceae	Herb
14	*Leontopodium leontopodinum* (DC.) Hand. Mazz.	Asteraceae	Herb
15	*Ligularia thomsonii* (Clarke) Kitam	Asteraceae	Herb
16	*Saussurea condolleana* Clarke	Asteraceae	Herb
17	*Saussurea falconerii* Hook.f.	Asteraceae	Herb
18	*Saussurea simpsoniana* Field & Garden	Asteraceae	Herb
19	*Tanacetum falconeri* Hook.f.	Asteraceae	Herb
20	*Tanacetum falconeri* J.D. Hook,	Asteraceae	Herb
21	*Taraxacum officinale* F. H. Wiggers	Asteraceae	Herb
22	*Berberis orthobotrys* Bien. ex Aitch.	Berberidaceae	Shrub
23	*Eritichium canum* (Benth.)	Boraginaceae	Herb
24	*Heliotropum dasycarpum* Ledeb.	Boraginaceae	Herb
25	*Onosma hispida* Wall.ex G. Don	Boraginaceae	Herb
26	*Cardamine flexuosa* With.	Brassicaceae	Herb
27	*Sisymbrium orientale* L.	Brassicaceae	Herb
28	*Betula utilis* D. Don	Betulaceae	Tree
29	*Cannabis sativa* L.	Cannabaceae	Herb
30	*Silene moorcroftiana* Wall.	Caryophyllaceae	Herb
31	*Silene kanwarensis* Benth	Caryophyllaceae	Herb
32	*Silene vulgaris* (Moench) Garacke.	Caryophyllaceae	Herb
33	*Euonymus fimbriatus* Wall.	Celastraceae	Tree
34	*Euonymus hamiltonianus* Wall.	Celastraceae	Tree
35	*Chenopodium album* L.	Chenopodiaceae	Herb
36	*Chenopodium foliosum* L.	Chenopodiaceae	Herb
37	*Codonopsis clematidea* (Schrenk) C.B.	Campanulaceae	Herb
38	*Rhodiola heterodonta* Hook.f.thom	Crassulaceae	Herb
39	*Rhodiola wallichiana* (Hook.f) S.H.Fu	Crassulaceae	Herb
40	*Juniperus communis* L.	Cupressaceae	Shrub
41	*Juniperus excelsa* M. Bieb	Cupressaceae	Tree
42	*Juniperus macropoda*	Cupressaceae	Tree
43	*Juniperus turkestanica* Komarov	Cupressaceae	Tree
44	*Carex divisa* Hudson	Cypraceae	Herb
45	*Hippophae rhamnoides* L.	Elaeagnaceae	Shrub
46	*Ephedra gerardiana* Wall. ex stapf.	Ephedraceae	Shrub
47	*Ephedra intermedia* Schrenk& Meyer	Ephedraceae	Shrub
48	*Equisetum arvensis* L.	Equacetaceae	Herb
49	*Rhodondendron anthopogon* D.Don	Ericaceae	Shrub
50	*Euphorbia cornigera* Boiss.	Euphorbiaceae	Herb
51	*Corydalis govaniana* Wall.	Fumariaceae	Herb

52	*Gentianoides tianschanica* Ruper ex Kusn.	Gentianaceae	Herb
53	*Swertia petiolata* D. Don	Gentianaceae	Herb
54	*Geranium pratense* L.	Geraniaceae	Herb
55	*Ribes alpestre* Decne.	Grossulariaceae	Shrub
56	*Juncus compressus* Jacq.	Juncaceae	Herb
57	*Juglans regia* L.	Juglandaceae	Tree
58	*Isodon regusus* (Wall.ex Benth) Codd.	Labiatae	Herb
59	*Mentha longifolia* Benth.	Labiatae	Herb
60	*Mentha royleana* Benth	Labiatae	Herb
61	*Nepeta discolor* Royle ex Benth.	Labiatae	Herb
62	*Salvia nubicola* Wall ex Sweet	Labiatae	Herb
63	*Stachys tibetica* Vatke.	Labiatae	herb
64	*Thymus linearis* Benth.	Labiatae	Herb
65	*Morus alba* L.	Moraceae	Tree
66	*Morus nigra* L.	Moraceae	Tree
67	*Fraxinus hookeri* Wenzing	Oleaceae	Tree
68	*Olea ferruginea* Royle	Oleaceae	Tree
69	*Epilobium angustifolium* L.	Onagraceae	Herb
70	*Colutea nepalensis* Sims	Papilionaceae	Shrub
71	*Medicago sativa* L.	Papilionaceae	Herb
72	*Trifolium repens* L.	Papilionaceae	Herb
73	*Picea smithiana* Wall.	Pinaceae	Tree
74	*Pinus wallichiana* A.B. Jacksn	Pinaceae	Tree
75	*Plantago depressa* Willd.	Plantaginaceae	Herb
76	*Plantago lanceolata* L.	Plantaginaceae	Herb
77	*Plantago major* L.	Plantaginaceae	Herb
78	*Aconogonon alpinum* var. Stewartii S.P.Hong	Polygonaceae	Herb
79	*Bistorta affinis* (D.Don) Green	Polygonaceae	Herb
80	*Rheum spiciforme* Royle.	Polygonaceae	Herb
81	*Rheum webbianum* Royle	Polygonaceae	Herb
82	*Rumex nepalensis* Spreng.	Polygonaceae	Herb
83	*Primula denticulata* Smith.	Primulaceae	Herb
84	*Primula macrophylla* D.Don	Primulaceae	Herb
85	*Pyrola rotundifolia* L.	Pyrolaceae	Herb
86	*Aconitum violaceum* Jack. Ex stapf	Ranunculaceae	Herb
87	*Delphinium brunonianum* Royle.	Ranunculaceae	Herb
88	*Pulsatilla wallichiana* (Royle) Ulbr.	Ranunculaceae	Herb
89	*Cotoneaster integerrima* Medik	Rosaceae	Shrub
90	*Fragaria nubicola* Lind.ex Land.ex Lancaita	Rosacacae	Herb
91	*Patentilla anserina* L.	Rosaceae	Herb
92	*Prunus armeniaca* L.	Rosaceae	Tree
93	*Rosa webbiana* Wall.ex Royle	Rosaceae	Shrub
94	*Sorbus tianshanica* Rupr.	Rosaceae	Shrub
95	*Spiraea canesens* D.Don.	Rosaceae	Shrub
96	*Gallium verum* L.	Rubiaceae	Herb

97	*Haplophyllum gilesii* Hemsl.	Rutaceae	shrub
98	*Salix iliensis* Regel	Salicaceae	Tree
99	*Bergenia stracheyi* Hook. & Thoms.	Saxifragaceae	Herb
100	*Eupharasia platyphlla* Penn.	Scrophulariaceae	Herb
101	*Pedicularis bicornuta* Klotzsch.	Scrophulariaceae	Herb
102	*Scrophularia nudata* Penn.	Scrophulariaceae	Herb
103	*Verbascum thapsus* L.	Scrophulariaceae	Herb
104	*Physochlaina praealta* Decne.	Solanaceae	Herb
105	*Solanum nigrum* L.	Solanaceae	Herb
106	*Daphne mucronata* Royle	Thymelaeaceae	Tree
107	*Carum Carvi* L.	Umbelliferae	Herb
108	*Ferula nathrax* Boiss.	Umbelliferae	Herb
109	*Haracleum candicans* Wall. Ex. DC	Umbelliferae	Herb
110	*Playtitiana lasiocarpa* (Boiss.) Rech.f. & Riedl	Umbelliferae	Herb
111	*Pleurospermum candollei* (DG.) Clarke	Umbelliferae	Herb
112	*Pleurospermum hookeri* Clarke var. thomsani	Umbelliferae	Herb
113	*Urtica dioica* L.	Utricaceae	Herb
114	*Pegnum harmala* L.	Zygophyllaceae	Herb

and variation of altitudinal variations. It is observed that more than 5700 plants species are exist in Pakistan, out of these 400 plant species are endemic [23, 28].

It is fact that, rural communities are most dependent on their natural vegetation for medication after food and fodder [4]. Some most important plants of study area used for traditional medicines are; *Saussurea simpsoniana* Field & Garden, *Cicerbita gilgitensis*, *Berberis orthobotrys* Bien. ex Aitch, *Onosma hispida* Wall. ex G. Don, *Betula utilis* D. Don, *Chenopodium foliasum* L, *Geranium pratense* L, *Thymus linearis* Benth, *Primula macrophyla* D.Don, *Rheum spiciforme* Royle, *Pulsatilla wallichiana (*Royle) Ulbr, *Delphinium brononianum* Royle, *Aconitum violaceum* Jack. Ex stapf, *Spiraea canesens* D.Don, *Sorbus tianchanica* Rupr, *Bergenia stracheyti* Hook & Thoms, *Rhodendron anthopogon*, *Pleurospermum candollei* (DG.) Clarke, *Carum Carvi* L, *Ferula nathrax* Boiss, *Urtica dioica* L. and *Pegnum harmala* L.

All natural vegetation's either timber forests and non-timber flora are under pressure due to fast urbanization, over grazing, and deforestation need to address them as early as possible [24, 25, 26, 27]. The present study has given a pavement for the young researcher to conserve these species for future generations. Phytochemical compounds can be isolated from these herbal plants to synthesize herbal drugs which can create great improvement in herbal industries and can lead to new innovative herbal drugs.

5. CONCLUSION

Northern Pakistan is full of natural treasure especially in the sense of natural vegetation. Reported 114 plants, belong to rare and precious species. Communities have no proper acknowledgment about them. Even they have no any idea for their sustainable utilization, and proper harvesting methods. This will cause the eradication of some important species very soon.

6. RECOMMENDATIONS

On the basis of our research, the few recommendations are:

- ✓ Deforestation, random collection and over exploitation of medicinal plants are great threat should aware the communities about importance the natural resources of the valley.
- ✓ The participatory action, and proper training is needed for local communities how to support conservation practices and sustainable

utilization.

- ✓ Overgrazing can be controlled by rotational grazing methods, which is major threat for the endangered flora.
- ✓ Special training is required for the sustainable utilization of these plants for medications, and even for commercial utilization.

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Proceedings of the Pakistan Academy of Sciences:
B. Life and Environmental Sciences 56 (3): 113–120 (Special Issue, 2019)

ISSN: 2518-4261 (print), ISSN 2518-427X (online)

Pakistan Academy of Sciences

Research Article

Antimicrobial Effect of *Psidium guajava* L. Leave Extract in Correlation with Biofilm Formation and Metallo-β-Lactamase Production in Multidrug Resistant *Pseudomonas aeruginosa*

Rabina Thapa[1], Bijendra Raj Raghubanshi[2], and Anjana Singh[*1]

[1]Central Department of Microbiology, Tribhuvan University, Kathmandu, Nepal
[2]KIST Medical College and Teaching Hospital, Imadol, Lalitpur, Nepal

Abstract: This study was aimed to determine antibacterial effect of *P. guajava* leave extracts and correlation of metallo-β-lactamase (MBL) production and biofilm formation with MDR *P. aeruginosa* isolated from different clinical samples. The study was carried out in the Kathmandu Institute of Science and Technology (KIST) medical college and teaching hospital and Central Department of Microbiology, Tribhuvan University, Kathmandu, Nepal. A total of 45 isolates of *P. aeruginosa*, isolated from different clinical samples were identified by standard microbiological techniques and antimicrobial susceptibility of the isolates was tested by Kirby-Bauer disk diffusion method on Muller Hinton agar as per CLSI guidelines. The ability to form biofilm was detected using the microtiter plate assay. MBL production was screened by Imipenem disk diffusion method and confirmed by Imipenem-EDTA combined disk method. P. guajava leave extracts were prepared using absolute methanol and hydroethanol solvent at different ratios. The antimicrobial activity of *P. guajava* leave extract against the pseudomonal isolates was determined by agar well diffusion method. Out of 45 isolates of *P. aeruginosa*, 30 (67%) were multidrug resistant (MDR) isolates, 30 (67%) were biofilm producers and 6 (13%) were metallo β lactamase (MBL) producers respectively. The methanol extract of fresh P. guajava leave (13mm) showed higher activity and least activity by 7:3 hydroethanol extract of dried *P. guajava* leave (6mm) toward the *P. aeruginosa* isolates. The methanol extract may be an alternative source for Pseudomonal infection treatment as antimicrobial resistance to available drugs which is increasing day by day. However, it should be standardized and tested in animal models before its application.

Keywords: Disk diffusion method, alternative source, treatment, antimicrobial resistance, standardized

1. INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative ubiquitous bacterium that can be isolated from sources such as soil, plants, animals and humans [1]. It is an important pathogen which causes various infections: Urinary Tract Infection (UTI), Respiratory Tract Infection (RTI), otitis media, skin and soft tissue infections, bacteremia and serious systemic infections particularly in people with compromised immune systems including burn sufferers, cystic fibrosis, cancer and AIDS [2, 3]. The increasing use of antibiotics and growing numbers of invasive procedures, together with the development of intrinsic and acquired resistance mechanisms of *P. aeruginosa*, cause the evolution of numerous multi drug resistant (MDR) *P. aeruginosa* outbreaks in clinical settings [4]. Although Carbapenems are the antibiotics of choice for several Pseudomonal infection, resistance is evolving due to production of MBLs which are broad spectrum enzymes that hydrolyses most beta-lactam antibiotics except Monobactams [5]. *P. aeruginosa* can form biofilms which exponentially increase antibiotic resistance [6]. Biofilms are the aggregation of cells which protect bacteria from environmental stresses as well as from the host immune system and antimicrobials [7]. The biofilms are composed of one or more extracellular polymers such as polysaccharide that holds the cell community together [8]. Metallo-**β**-lactamases are a diverse set of enzymes that catalyze the hydrolysis of a broad range of **β**-lactam drugs

Received: February 2019; Accepted: September 2019
* Corresponding Author: Anjana Singh; anjanas67@gmail.com

including carbapenems. The dissemination of the genes encoding these enzymes among Gram-negative bacteria has made them an important cause of resistance.

Synthetic antibiotics are widely used to cure infections; however indiscriminate use of such antibiotics causes antimicrobial drug resistance, necessitating the use of medicinal plants as the alternative therapeutic agents [9]. *Psidium guajava* L leave are important and commonly used to treat diseases like diabetes mellitus II, gastroenteritis, blood coagulation, gastric mucosal injury, etc. *P. guajava* leave contain active chemical compounds such as saponins, flavonoids, tannins, eugenol and triterpenoids. Poly phenolic compounds that dominate *P. guajava* leave are flavonoids (>1.4%) and tannins [10]. The activities possessed by *P. guajava* leave are antiviral, anti-inflammatory, anti-plaque and antimutagenic and thus, it can be helpful for prevention and treatment of diseases [11]. The main aim of this research is to find out the effect of *P. guajava* leave extract on multidrug resistant biofilm and metallo β lactamase producing pseudomonas respectively.

2. MATERIALS AND METHODS

2.1 Method

The method was quantitative carried out at the Microbiology laboratory of KIST Medical College and Teaching hospital, Gwarko, Lalitpur, a tertiary care hospital and Central Department of Microbiology, Tribhuvan University, Kirtipur, from April to October, 2018.

2.1.1 Specimen

Samples included pus, wound swab, blood, sputum and urine from in and out patients visiting the hospital.

1.1.2 Identification and Antibiotic Susceptibility Testing of Pseudomonas aeruginosa

The identification of *P. aeruginosa* isolates were carried out on the basis of standard microbiological procedures and antibiotic susceptibility testing was done by modified Kirby Bauer disc diffusion method following CLSI guidelines, 2014.

2.2 Detection of Metallo β Lactamase (MBL)

2.2.1 Screening of MBL Production by Imipenem Disk Diffusion Method

According to the CLSI recommendation, MBL production was screened using Imipenem disk same as antibiotic susceptibility test on MHA agar plate and resistant zone was noted. Resistant zone indicated a probable MBL producing strain which was further confirmed by phenotypic confirmatory test.

2.2.2. Confirmation of MBL production by Imipenem- EDTA Combined Disk Method

EDTA of 0.5M was prepared with distilled water and sterilized by autoclaving. Imipenem disk was supplemented with EDTA by dispensing 10µl of the solution to each Imipenem disk.

Imipenem-EDTA combined disk method (CDT) was performed. A lawn culture of test isolates was prepared. After allowing it to dry for five minutes, two Imipenem discs, one with 0.5 M EDTA and the other a plain Imipenem disc, was placed on the surface of agar plates approximately 30mm apart. The plates were incubated overnight at 37. An increase in zone diameter of ≥7mm around Imipenem + EDTA disk in comparison to Imipenem disk alone indicated production of MBL [12].

2.3 Detection of biofilm production

Biofilm detection by microtitre plate culture method (MPC):

The overnight grown cultures of *P. aeruginosa* from agar plates were inoculated in trypticase soy broth (TSB) with 1% glucose. Stationary-phase 18-hr culture of *P. aeruginosa* was diluted 1:100 with fresh TSB. Individual well of sterile polystyrene 96 wells flat bottom tissue culture plates were filled with 200µl of diluted culture broth. Uninoculated broth was considered as negative control. The plates were incubated at 37˚C for overnight. After 24 hours of incubation, content of each well was gently discarded by tapping the plates downwards. The wells were washed three times with 200µl of PBS (pH 7.2) in order to remove planktonic bacteria. Adherent bacteria were fixed with 99% methanol for 10-15 min. After drying the plates, stained for 10 min with 0.1% crystal violet (CV). Excess stain

was removed by washing the wells with distilled water and plate was kept for drying at an inverted position. After the plate was air dried, the dye bound to the adherent cells was re-solubilized with 160 µl of 95% ethanol. The OD of each well was measured at 570 nm using ELISA reader. These OD values were taken as index of bacteria that adhere to the surface and formed biofilm. Experiments were carried in triplicate and their mean was taken for the analysis. Interpretation of biofilm production was done according to the standard criteria [13].

2.4 Preparation of methanol and hydro ethanol extracts of *P. guajava* leave

Methanol extract was prepared by taking absolute methanol as solvent, hydro ethanol extracts by taking ethanol and water in different ratios (7:3, 1:1 and 3:7). The leave pieces were added to different solvents in sterile flasks and wrapped with aluminum foil to avoid evaporation. The mixtures were kept for 3 to 4 days at room temperature. The flasks were placed on a platform shaker at 70 rpm. After 3-4 days of soaking in solvent, the mixtures were transferred to tubes and centrifuged for 10 min at 4,000 rpm at 25°C. The supernatant was collected and stored at 4°C until use (Figures 3, 4).

2.5 Determination of antimicrobial activity of *P. guajava* leave extractions against *P. aeruginosa*

Antimicrobial activity of *P. guajava* leave extract was tested by well-diffusion method according to the standard of the National Committee for Clinical Laboratory Standards. Diameter of 5 mm wells were punched into the MHA medium using a sterile cork borer and inoculated with the test bacterium. Exactly 0.1ml aliquots of each test extract were dispensed into each well. Methanol and hydroethanol solvents alone were used as control. Disk of Tobramycin was placed at the centre. After 24 hours of incubation at 37°C, each plate was examined for inhibition zones (mm).

2.6 Data analysis

The data obtained were analyzed by using SPSS software for Windows (version 21). A value of $\alpha \leq 0.05$ was assumed wherever applicable and 95% confidence intervals along with the exact p-values were presented.

Fig. 3. Antimicrobial activity of fresh *P. guajava* leave extract

MFLE: methanol fresh leave extract, EFLE1: hydroethanol fresh leave extract (5:5), EFLE2: hydroethanol fresh leave extract (3:7), EFLE3: hydroethanol fresh leave extract (7:3) and NS: normal saline

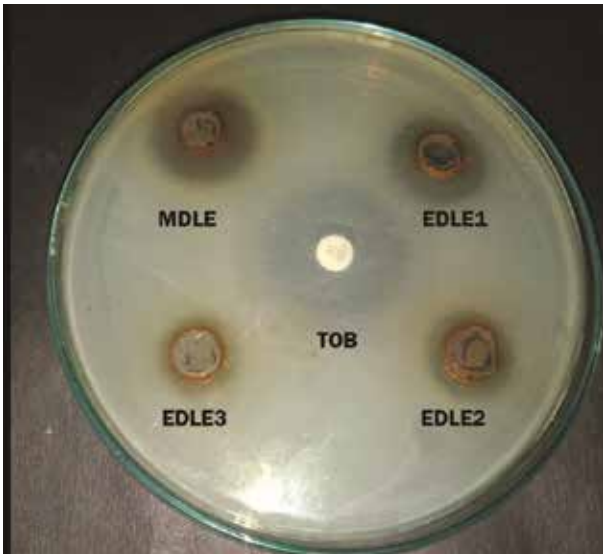

Fig. 4. Antimicrobial activity of dried *P. guajava* leave extract

MDLE: methanol dried leave extract, EDLE1: hydroethanol dried leave extract (5:5), EDLE2: hydroethanol dried leave extract (3:7), EDLE3: hydroethanol dried leave extract (7:3) and TOB: Tobramycin

3 RESULTS

A total of 3500 specimens were processed during the six months of duration. Bacterial growth was observed in 900 (25.71%) samples. Out of the total specimens, *P. aeruginosa* isolates were 45 (5%).

3.1 MBL and biofilm production in MDR *P. aeruginosa*

Out of 45 isolates of *P. aeruginosa,* 30 (66.67%) were MDR isolates. The total MBL producing *P. aeruginosa* was found to be 6 (13.33%) by Imipenem-EDTA combined disk method and all of them were found to be MDR strains while 24 (80%) were negative for MBL production. Out of 30 MDR isolates, 29 (97%) were biofilm producers of which 15 (50%) were strong and 14 (47%) were moderate biofilm producers and out of 15 non MDR isolates only 1 (7%) was found to be biofilm producer (Figure 1).

3.2 Antimicrobial activity of *P. guajava* leave

P. guajava leave extract (fresh and dried) showed antimicrobial activity against all types of *P. aeruginosa* isolate, both drug resistant and drug sensitive. However, the activity was quite lower than Tobramycin (16mm), the antibiotic standard used, in both ATCC culture and clinical culture of *P. aeruginosa.* Methanol extract of fresh leave extract showed higher activity (13mm) than that of dried leave extract (12mm). Among different concentration hydroethanol extract, 5:5 hydroethanol extract showed greater inhibition followed by 3:7 hydroethanol extract and least by 7:3 hydroethanol extract. However, the activity was comparatively high in fresh extracts than in dried extracts (Figure 2).

4 DISCUSSION

Resistance to antimicrobial agents is an increasing clinical problem and is a recognized public health threat. *P. aeruginosa* showed a particular propensity for the development of resistance. The emergence of resistance in *P. aeruginosa* also limits future therapeutic choices and is associated with increased rate of mortality and morbidity [14, 15]. This study was carried out to assess MDR, MBL production, biofilm production and antimicrobial activity of *P. guajava* leave extract in *P. aeruginosa* isolated from different clinical specimens of in patients and out patients at Medical College and Teaching Hospital. The total of 45 (5%) isolates of *P. aeruginosa* were isolated and followed by antibiotic susceptibility testing. MDR was shown by 30 (66.67%) of *P. aeruginosa* isolates tested. Similar study made by Fatima et al. (1999) showed 73.9% MDRPA isolates [16]. Drug resistance in *P. aeruginosa* is multifactorial either through membrane permeability and efflux system or through its virulence factors or acquired genetically by plasmid which may lead to a super bugs, that are difficult to treat [17]. Emergence of MDR is related

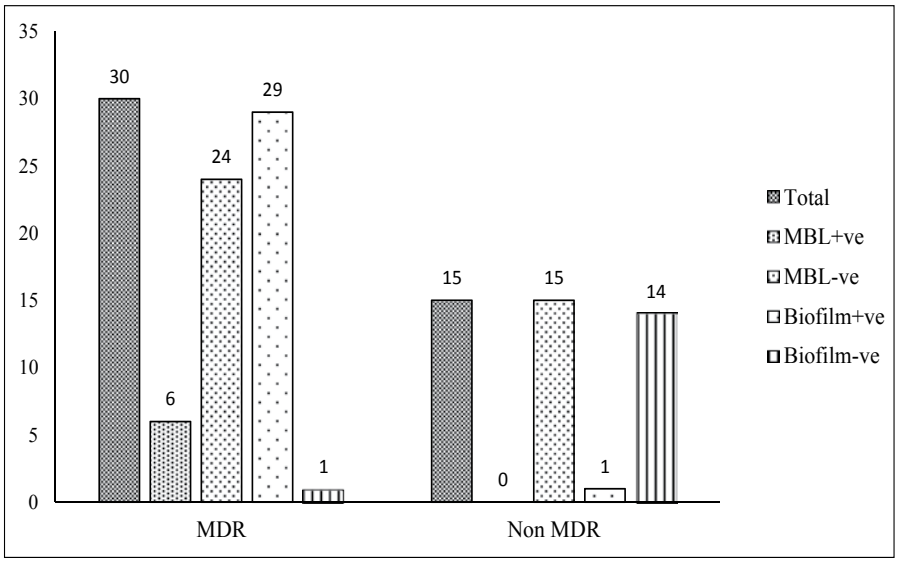

Fig. 1. MBL and biofilm production in MDR and non MDR *P. aeruginosa*

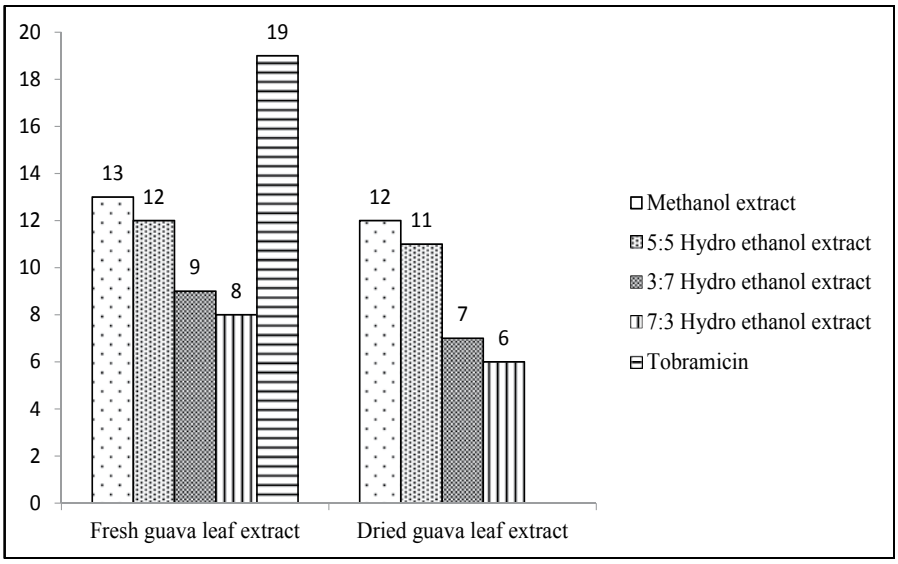

Fig. 2. Antimicrobial activity of *P. guajava* leave extract on *P. aeruginosa* isolates

to the empirical use of antibiotics rather than their rational use as majority of patients undergo broad spectrum antibiotics before sample collection. Drug resistance should be prevented by encouraging judicial use of antibiotics.

In this study, out of 45 isolates, 6 (13.33%) *P. aeruginosa* isolates were found to be positive for MBL production with MDR. Similarly, Thapa et al (2017) reported 14.2% of MBL producing *P. aeruginosa* isolates during the study [18]. Our result varied much more compared to Acharya et al (2017) who reported 68.6% positive isolates for MBL production by the Imipenem-EDTA disk diffusion test [19]. MDRPA has appeared as an issue of great concern with emergence of MBLPA [20]. Carbapenem resistance in *P. aeruginosa* is most commonly due to production of MBL [21]. Carbapenem resistant *P. aeruginosa* has become prevalent globally [22, 23]. MBL production in *P. aeruginosa* is associated with treatment failure, longer hospital stay and significant morbidity and mortality [24]. Routine detection of MBL may ensure optimal patient care and timely introduction of appropriate infection control procedures [25].

In this study, 30 (66.67%) biofilm producing *P. aeruginosa* were isolated. Out of total biofilm producer, 29 were MDR and their association was statistically significant (<0.05). Similar study made by Maita et al (2014) showed biofilm producers statistically significant while all non-biofilm producers were non MDR. The *P. aeruginosa* isolates producing biofilm were reported high about 79.4% [27]. Antimicrobial resistance is an innate feature of bacterial biofilms and many studies have shown that biofilm formation is higher in MDR strains [28]. One of the most medical important biofilm forming bacteria is *P. aeruginosa* which is usually associated with human nosocomial infections [29, 30 and 31]. Extracellular matrix of biofilm acts as barrier for any antibiotics and increase resistance to these antibiotics [8]. Biofilm producing bacteria are 10 to 1,000 times more resistant to antimicrobial agents than the planktonic cell. There is high level of antibiotic resistance among biofilm-forming *P. aeruginosa* strains. The differences in the various reports about the prevalence of biofilm formation may be attributed to the variation in the sites of infection, multiple subcultures of bacteria, method of biofilm detection, species-specific and bacterial strain [32]. Regular screening of biofilm formation and monitoring antimicrobial resistance profile of *P. aeruginosa* is very important as it may help to formulate an effective antimicrobial strategy in a clinical setting while dealing with infections caused by this organism [26].

In the study, *P. guajava* leave extract (fresh and dried) showed antimicrobial activity against all types of *P. aeruginosa* isolates both drug resistant and drug sensitive. However, the activity was quite lower than the antibiotic standard used Tobramycin (16mm), in both ATCC culture and

clinical culture of *P. aeruginosa.* Methanol extract of fresh leave extract showed higher activity (13mm) than that of dried leave extract (12mm). Among different concentration hydro ethanol extract, 5:5 hydroethanol extract showed greater inhibition followed by 3:7 hydroethanol extract and 7:3 hydroethanol extract respectively. However, the activity was comparatively high in fresh extracts than in dried extracts. Similar study made by Gitika et al (2016) reported maximum zone of inhibition for the methanol extracts of *P. guajava* leave [33]. The antibacterial activity of different *P. guajava* extract was found to be significant against both *E. coli* and *P. aeruginosa* but less significant than the standard antibiotics [34]. In another study, the methanol and ethyl acetate extracts were found to exhibit broader spectrum activity and the methanol extract was more active comparatively [35]. Ethanol was reported as the best solvent compared to acetone for tannin extraction from *P. guava* leave [36]. Khadka 2018 made a similar study where 83.67% isolates of *P. aeruginosa* were biofilm producer and *P. guajava* leave tea was able to kill *P. aeruginosa* [37]. It was analyzed that higher levels of tannin content was equivalent to higher antibacterial activity. The leave contain many fungistatic and bacteriostatic agents and important oxidants [11]. Synthetic antibiotics are widely used to cure infections, but necessitating the use of medicinal plants as the alternative therapeutic agents. Medicinal plants and plant-derived products are cost-effective and easily obtainable and have promising efficacy to treat infectious diseases, and thus they may be useful in eradicating new emerging microbial strains [9]. Recently, scientists have found evidence that specific combinations of phytochemicals are more effective in protecting against diseases than the isolated compounds, pointing to a need to study the synergy among active compounds in plants, for example, by experimenting with plant extracts [38].

5 CONCLUSION

Multidrug resistance *P. aeruginosa* create great challenges in the therapy. Effective means need to be developed to control this problem. Regular antimicrobial susceptibility surveillance may assist in monitoring of the resistance patterns antibiotic policy and introduction of effective antibiotics for better patient management. Biofilm production and MBL production in *P. aeruginosa* was found to be linked with MDR property of the organisms. Thus, early detection of biofilm production and MBL production may be helpful in controlling the infection by resistant strains. Many researches have been demonstrating the presence of a wide variety of bioactive compounds in *P. guajava* leave capable of showing beneficial effects on human health. In the present study, *P. guajava* leave extracts with methanol and various concentrations of ethanol showed significant inhibitory activity against *P. aeruginosa* isolates with fresh leave showing more activity than dried one. On the basis of this study, it can be said that *P. guajava* leave extracts can be effective antibacterial agent that can be a good source to treat and control many diseases. However, extensive research in clinical trials needed so that it can be used for prevention.

6 ACKNOWLEDGEMENTS

Authors are grateful to University Grants Commission for providing financial support. Further like to thank Central Department of Microbiology, Tribhuvan University and KIST Medical College and Teaching Hospital for providing laboratory facilities.

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Proceedings of the Pakistan Academy of Sciences:
B. Life and Environmental Sciences 56 (3): 121–125 (Special Issue, 2019)

ISSN: 2518-4261 (print), ISSN 2518-427X (online)

Pakistan Academy of Sciences

Research Article

Taxonomical and Phytochemical Characterization of Two Highly Traded Medicinal Species of Genus *Berberis*

Sidra Nisar Ahmed[1,2*], Mushtaq Ahmad[2], Muhammad Zafar[2], Sofia Rashid[2,3], Ghulam Yaseen[2], Shomaila Ashfaq[2], and Shazia Sultana[2]

[1]Department of Botany, The Women University Multan, Pakistan
[2]Department of Plant Sciences, Quaid-i-Azam University, Islamabad 45320, Pakistan
[3]Department of Biosciences, COMSATS University, Islamabad, Pakistan

Abstract: TThe medicinal plants serve as an important natural source and potentially safe medicinal drugs that can play a significant role in moderating human health by contributing towards herbal medicines. The genus *Berberis* exhibits evergreen and deciduous shrubs like *Berberis aristata* DC and *Berberis lyceum* Royal. These medicinal plants are used as a remedy for swollen and sore eyes, skin diseases, healing of broken bones, curative piles, jaundice and diarrhea. The morphological and organoleptic studies concluded that fruit of *Berberis aristata* encloses two seeds while *Berberis lyceum* encloses three seeds. The total flavonoid content (134.33mg GAE/g) and phenolic content (39.23 mg QE/g) in *Berberis aristata* is high as compared to flavonoid content (115.01 mg GAE/g) and phenolic content (33.03 mg QE/g) of *Berberis lyceum*. The results achieved provide us with valuable information for botanical quality control and species identification and assist us to detect adulterations in commercial as well as in laboratory samples.

Keywords: Berberis, medicinal uses, flavonoids, phenols

1. INTRODUCTION

The medicinal plants are the valuable natural resource and offers raw material for pharmaceutical industry, modern and traditional practices as well as preservation of traditional knowledge. The increased demand of plant extracts used in cosmetic industries, pharmaceutical and food industries recommends that systematic studies of plants is essential for trace of their chemical compounds and their role in medicine [1]. There are almost 8000 species of known medicinal values in South Asia that are considered as cheap and effective traditional source against many diseases. A survey of natural plant wealth of Pakistan displayed that there is abundant growth of valuable medicinal species in Azad Kashmir, Kotli Sattian, Murree Hills, Hazara, Kurrum Agency, Northern areas and Baluchistan [2]. The evergreen and deciduous shrubs of *Berberis aristata* and *Berberis lyceum* belongs to family Berberidaceae and are a part of traditional medicines long time ago. They are semi deciduous shrub, 2 to 4 meter high, leaves are lanceolate or narrowly obovate-oblong, entire or with a few large spinous teeth, arranged alternately on stem [3]. Inflorescence a racemes, flowers yellow born in auxiliary clusters longer than the leaves. The fruit of *Berberis aristata* is edible with medicinal and nutritional values both in fresh and dried form [4]. It is an ovoid, bluish black berry almost 10 mm long [5, 6] and exhibits a central position in Georgian and Persian cuisines [7]. It is rich in vitamins, minerals and fibers that are vital for good health. Its fruit is used to prepare prickles, jams, syrups, candies and acts as a food additive. Medicinally, it is laxative, useful in sores, piles, and eye infections, and effective against kidney dilemmas [8].

Berberis lyceum is considered important medicinal plant in practice of herbal medicine. Its roots are used as remedy for swollen and sore

Received: February 2019; Accepted: September 2019
* Corresponding Author: Sidra Nisar Ahmed; nisar.sidra3@gmail.com

eyes, healing of broken bones internal injuries, gonorrhea, curative piles, unhealthy ulcers, acute conjunctive and in chronic ophthalmic [9]. It is also used as bitter tonic and possesses antifungal, antibacterial, diaphoretic, anti-inflammatory, anti-diabetic and hypoglycemic properties [10-12]. The fruit is rich in vitamin A, calcium, copper, potassium and phosphorus [13, 14]. The increasing trend in plant based natural medicines has directed the phytochemical evaluation of numerous plant species [15]. Phytochemicals are chemical compounds occurred in plants and implies different characters to them. Spectrophotometric and chromatographic techniques are applied to measure quantity of bioactive compounds like flavonoids, alkaloids and polyphenolic compounds [16]. The flavonoids and phenolics are one of the important antioxidants which are used either to enhance nutrition or treated as antioxidant additives [17]. Among flavonols, Quercetin is very important and exhibits good antioxidant activity. Plants are compared with reference to presence of quercetin level as they supposed to show antioxidant properties [18]. Their antioxidant potential serves as a leading role in advancement of modern medicines for hepatitis and cancer [19]. It helps in protection of human body from various diseases by termination of free radicals. This Study is aimed to carry out microscopy of taxonomical characters and chemical evaluation of species within genus to show variation in qualitative and quantitative characters as well as serves as an innovation for pharmaceutical industries to extract valuable compounds for drug preparation.

2. MATERIALS AND METHODS

2.1. Plant samples

The medicinal plants were collected in the year 2016-2017 according to their flowering time for current study. The precise and correct botanical name authorization is achieved through the International Plant Name Index (IPNI). The voucher specimens were submitted in the Herbarium of Pakistan (ISL), Quaid-i- Azam University, Islamabad. The collected plants were washed, shade dried and some part was ground for phytochemical analysis.

2.2. Extraction procedure

The dried plant powder was mixed in methanol in a ratio 1:10 w/v, centrifuged at 6000 for 15 minutes. Then mixture was filtered through what man filter paper no. 1 and concentrated the filtrate using a rotary evaporator.

2.3. Examination of Taxonomic Characters using Light and Scanning Electron Microscopy (SEM)

2.3.1. Morphological Examination

The morphological characterization constitutes analysis of macroscopic and microscopic characters. The binocular dissecting microscope (SZF model Kyowa, Japan) of 5X, 10X and 20X magnifying power was used. The plant specimens were studied both qualitatively and quantitatively along with aid of different floras [20].

2.4. Phytochemical analysis

2.4.1. Quantification of Total Phenolic Content (TPC)

The total phenolic content was estimated by method given by [21]. Each plant extract was taken in methanol solution in concentration of 1mg/1mL. Take 1 mL of this extract and mixed with 5 mL of 10% diluted Folin –Ciocalteu's reagent and 4 mL $NAHCO_3$ (7.5%). Place this mixture at room temperature for 90 min and then absorbance was measured at 760 nm. Gallic acid was taken as a standard and results were expressed as mg Gallic acid equivalents per gram dry weight (mg GAE/g).

2.4.2. Quantification of Total Flavonoid Content (TFC)

The flavonoid content was measured by Aluminium Chloride calorimetric method described by [22] with some modifications. Add 2 mL methanol extract of each plant sample, 0.1 mL 10 % diluted $AlCl_3$, 0.1 mL 1 molar KCH_3COOH and 2.8 mL ionized water. Place this mixture for 40 min at room temperature and then measured absorbance at 415 nm. Quercetin was used as a standard and results were expressed as mg Quercetin equivalents per gram dry weight (mg QE/g).

3. RESULTS AND DISCUSSION

The detailed taxonomical and phytochemical characterization of *Berberis aristata* and *Berberis lyceum* is documented in Table 1. The *Berberis*

Table 1. Botanical description of Medicinally important plants species of B*erberis aristata* and *Berberis lyceum*

S. No	Features	*Berberis aristata* DC.	*Berberis lyceum* Royle
1	**Family name**	Berberidaceae	Berberidaceae
2	**Common names**	English name: Indian berberry, Tree turmeric. Local name: Zereshk, Sumlu, Kashmal Trade name: Tursh, Darhald	English name; Indian berberry Local name:Sumbloo, Ishkeen,Kala
3	**Distributional range**	Plant is native to Nepal and distributed in India, Bhutan, sub- tropical & temperate Asia, Europe and America. In Pakistan, plant is found in Kashmir and Himalayas.	In world, it is found in temperate and tropical Asia, Africa and Europe. In Pakistan it grows in Kashmir, Baagh, Muree, Swat, Gilgit ,Hunza, Nagar, Chilas, Mansehra.
4	**Habit and Habitat**	Plant is spiny evergreen Shrub.	Plant is deciduous spiny shrub of 2 to 3 m height.
5	**Phenological status (Flowering / Fruiting time)**	Early April till end of May / June -July.	March-April/June-July
6	**Medicinal Values**	Fruit is used in vaginal, stomach and uterine disorders, wound healing, diarrhea, dermal and optical ailment.	Used to cure diabetes, piles and spleen ailments. Plant extract is good for cough, Ear, Nose and Throat troubles, and eye diseases. It is a good source for healing of broken bones
7	**Botanical description**	An erect, glabrous, spiny shrub, 2-3.5 m high with yellow fragile bark having deep furrowing. Internodes 3- 4.5 cm long. Leaves glossy, verticillate, reticulate venation, spinous toothed, sub-acute to obtuse, obovate to elliptic lamina, 4.2cm×1.3cm, dark green from upper and light green from lower side. Inflorescence has 16-20 flowers in drooping clustered position, golden yellow color, complete, hermaphrodite, actinomorphic, Sepals 6.5 mm long, obovate , smaller than inner 10 mm long; Petals elliptic, 8.5 mm long ;Stamens 6.6 mm long; ovules 5. Fruit is oblong-ovoid, 6.2 mm × 3.4 mm bluish purple berries with 3seeds .	A deciduous shrub, 3 - 4.5 m high, erect, pale yellow branches. Internodes 2 cm long. Leaf sub sessile, lamina oblanceolate to obovate, acuminate tip, having larger spines arranged alternately on stem, 1.7-2.8 cm × 0.8-1.2 cm. Inflorescence raceme, flower pale yellow , 4-7cm long, born in axillary clusters longer than leaves, pedicels 6-12, tubular sepals obovate, 5-5.5 cm ×3.5mm, central and inner sepals are larger than outer ones, petals obovate with notch at tip, petals shorter than sepals and stamens are shorter as compared to petals; ovules 4, Fruit shiny black ovoid berries 0.9-1cm × 0.6-0.7cm.

species show variation in morphology and may shows complications in taxonomic identification [23]. The detailed morphology and organoleptic analysis depicted that fruit of *Berberis aristata* encloses two seeds (Fig. 1 A & B) and *B. lyceum* has three seeds (Fig. 2, C & D). Fruit of *Berberis lyceum* and its allied species are purplish black with seeds and appears in form of bunches [24].

It is necessary to evaluate chemical composition of medicinal plants with the help of standards to check their nutritional value as well as to authenticate genuine source. In present project, total phenolic and flavonoid content in *Berberis aristata* fruit is high in comparison with *Berberis lyceum* (Fig. 3). Earlier studies highlighted that different researchers developed various markers to differentiate *Berberis aristata* from its allied

Fig. 1. *Berberis aristata* (A:field photo B: Fruit)

Fig. 2. *Berberis lyceum* (C: field photo D: Fruit)

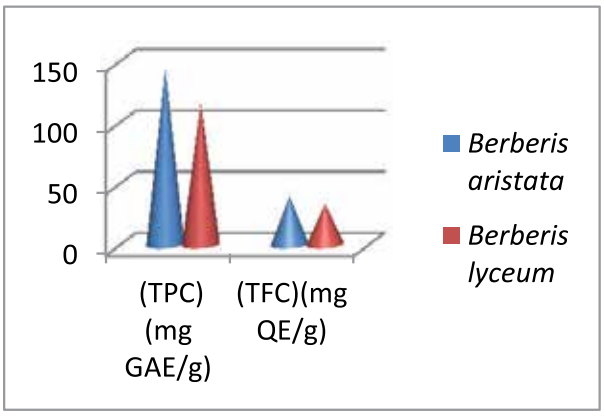

Fig. 3. Quantification of Total Phenol Content and Total Flavonoid Content

species [25].

4. CONCLUSION

The work will contribute towards correct identification and authentication of medicinal plants and a milestone at industrial and pharmaceutical level. The microscopy and chemical evaluation of species shows a comparison within genera and provides data for antioxidant evaluation as well as preparation of antioxidant drugs from such remarkable plant sources.

5. ACKNOWLEDGMENT

Authors are grateful to HEC Pakistan for Indigenous Fellowship for a Ph.Ddegree in Pakistan.

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Proceedings of the Pakistan Academy of Sciences:
B. Life and Environmental Sciences 56 (3): 127–129 (Special Issue, 2019)

ISSN: 2518-4261 (print), ISSN 2518-427X (online)

Pakistan Academy of Sciences

Workshop Recommendations

Strengthening the Role of Complementary Medicine to Address Health Workforce Shortages in Primary Health Care in Asia

Tasleem Akhtar[1*], and Zabta K. Shinwari[2*]

[1]Professor Emeritus Khyber Medical University, Peshawar
[2]Pakistan Academy of Sciences, Islamabad, Pakistan

Background: According to the World Health Organization (WHO), "Traditional medicine refers to health practices, approaches, knowledge and beliefs incorporating plant, animal and mineral based medicines, spiritual therapies, manual techniques and exercises, applied singularly or in combination to treat, diagnose and prevent illnesses or maintain well-being"[1]. Globally there is a resurgence of interest in traditional medicine and other non-conventional health care systems. Such systems include Chinese medicine, Ayurveda, Herbal medicine, Tibb Unani, Homeopathy, Acupuncture, Chiropractic, Osteopathy, bone-setting and many others. Variously named as Indigenous, folk, Traditional and Complementary Medicine (T&CM) and/or Complementary and Alternate Medicine (CAM), these systems of health care were until recently predominantly used by the poor and rural communities. They are however currently finding favor with the more educated and affluent communities of the developed world, and their use is rapidly expanding. The positive features of T&CM responsible for the rapid expansion of their use include diversity, flexibility, easy accessibility, relative low cost, low levels of technological input, relative low side effects and growing economic importance (WHO)[5]. Along with their affordability and accessibility these systems are firmly embedded in the belief systems of communities and are culturally compatible. Taking cognizance of the growing demand for and use of T&CM WHO developed guidelines and strategies for T&CM integration into conventional health care systems by member countries for the period 2002-2005 and recently 2014-2023. The 2014-2023 strategy has two key goals: to support Member States in harnessing the potential contribution of T&CM to health, wellness and people centered health care and to promote the safe and effective use of T&CM through the regulation of products, practices and practitioners. These goals will be reached by implementing three strategic objectives: 1) building the knowledge base and formulating national policies; 2) strengthening safety, quality and effectiveness through regulation; and, 3) promoting universal health coverage by integrating T&CM services and self-health care into national health systems[1].

An immediate need for T&CM integration into national health systems is in the area of Human Resources for Health (HRH). The critical shortage of trained health workers in low and middle income countries was documented in 2004 and reported in the World Health Organization (WHO) 2006 World Health Report[2,3]. The estimated shortage

[1]WHO Traditional Medicine Strategy 2014-2023. https://apps.who.int/iris/bitstream/handle/10665/92455/9789241506090_eng.pdf
[2]Human Resources for Health-Overcoming the Crisis .Joint Learning Initiative. 2004
[3]WHO. The World Health report 2006 – working together for health. Geneva: World Health Organization; 2006
[4]A Universal Truth: No Health Without a Workforce. Third Global Forum on Human Resources for Health Report, November 2013. Global Health Workforce Alliance and World Health Organization.http://www.who.int/workforcealliance/knowledge/resources/hrhreport2013/en/

* Corresponding Authors: Tasleem Akhtar; tasleem.akhtar@gmail.com
Zabta K. Shinwari; shinwari2008@gmail.com

of 4.3 million in the two reports had increased to 7.2 million by 2013 and is predicted to reach 12.9 million by 2035[4]. This shortage has impeded the achievement of past national and international health goals and will be a serious barrier to achieving current and future goals[5]. Rapid strengthening of HRH therefore is considered a vital and urgent part of policies and strategies to achieve health in countries like Pakistan who have crisis level HRH, shortages. A task shifting approach for the deployment of Community Health Workers (CHWs) and traditional medicine practitioners is recommended to achieve rapid expansion of the health workforce. A study by Celluti et al found that where there is necessary support and certain conditions are observed, CHWS and TM workers can make significant contribution to health services delivery and achievement of universal health coverage.[6]

Conference Recommendations

The following recommendations are based on the presentations and discussion during the conference and are in line with the strategies recommended in the WHO 2014 – 2023 Strategic Frame Work.

1. Establish Higher Education and Research to Enhance Quality and Expand the Knowledge base of Traditional and Complementary Medicine

In the developed countries, Integrative Medicine has achieved recognition as a specialty field and Academic Centers/ Institutes and Universities have been established to provide higher education and undertake quality research in the field. In the United States, the American Board of Integrative Medicine (ABIM) awards Certification in the field like in all other medical specialties. Asian countries and more especially Pakistan need to establish such institutions. The institutions however, need to be established within the existing universities and academic centers in order to ensure that the field of integrative medicine doesn't develop in isolation from other such institutions in the domain of medical and health care field.

2. Integrate Traditional Medicine with Primary Health Care (PHC)

WHO's traditional medicine strategy 2014-2023's Strategic objective 3 aims to promote universal health coverage by integrating T&CM services into health care service delivery and self-health care. The following recommendations are given to achieve the objective 3 of the strategy:

2.1. Recognize HRH crisis as a health policy priority and consider T&CM integration in PHC as one of the strategic objectives for addressing the crisis;
2.2. Build T&CM knowledge base to inform policies and strategies;
2.3. Review and revise existing regulations to strengthen safety, quality and effectiveness of T&CM therapies and practices;
2.4. Review and revise T&CM training curricula to include some essentials of modern health care, such as the following:

2.4.1. Professionalism

Healing the sick is a profession and Healers are professionals. Professionals have a legal and ethical relationship of trust with those they serve. Health Professionalism is a three-part promise:

a) To acquire and maintain value system which emphasizes that the interests of the patients and the public will supersede the self-interests of practitioners;
b) Acquire and upgrade knowledge and technical skills necessary for providing good health care; and
c) Develop interpersonal skills necessary to communicate and work together with patients.

2.4.2. Medical Ethics

The teaching of "medical ethics" must become a part of T&CM practitioners pre-service and continuing education curricula. Health professionals are required to develop 'respect' for individuals, 'do no harm' to their patient, work for 'doing good' for their patients and exercise 'fairness' in the distribution of goods and benefits to their patients.

[5]O'Brien P and Gostin LO. Health Worker Shortages and Global Justice. October 2011. Milbank Memorial Fund. http://www.milbank.org/uploads/documents/HealthWorkerShortages_Mech/HealthWorkerShortages_Mech.html
[6]Deployment of community health workers in response to health workforce shortages. AIDS 2010, 24 (suppl 1):S45–S57. http://hsrc.himmelfarb.gwu.edu/cgi/viewcontent.cgi?article=1357&context=sphhs_policy_facpubs

2.4.3. Avoidance of mixing single active ingredient modern medicines with herbal and traditional medicines

They must be made to understand that modern single ingredient drug's use requires a good understanding of their pharmacology. They should stick to their whole herb therapies and avoid mixing modern medicines like antibiotics, steroids and psycho-active drugs in their herbal medicines formulations.

2.4.4. Recognition of Drug interactions

The widespread belief that whole herbs formulations are harmless is not correct. Concurrent use of herbs with modern medicine may mimic, magnify, or oppose the effect of drugs. The apparently harmless garlic can interact with some modern drugs and cause serious interaction like bleeding when taken with low dose aspirin and Warfarin etc.

2.4.5. Timely referral of patients for appropriate therapy and management

As emphasized by Hippocrates 2400 years back, don't attempt to treat conditions for which you have no knowledge and skill- refer them. Timely referral is an essential function of PHC. T&CM practitioners need to be linked with modern medicine practitioner and health care facilities for the purpose of timely referral of patients who need modern medical management.

2.4.6. Adoption and transmission of essential health promotion and disease prevention messages which are a function of PHC workers

These include Antenatal Care and Delivery by Trained Workers; Child vaccination; Mother and Child Nutrition; Drinking of Clean Water and Personal and Environmental Hygiene. Teaching of health promotion and disease prevention maybe made a compulsory part of T&CM curricula.

3. Additional recommendations relevant to Pakistan and some other Asian countries:

These include the following:

3.1. Suppliers of medicines based on natural products must ensure that harvesting of the products from the animal/organisms species providing the source compounds is sustainable or the animals are domesticated in order to meet demands.

3.2. Many species are endangered because of their (real or perceived) health benefits. Efforts must be made to eliminate illegal trade in such endangered species. Such efforts should include an awareness campaign about the scientific basis for 'no medical effects' of such produces, e.g. donkeys, rhino horn, tigers, rhinoceroses, sea horses and pangolins.

3.3. Due attention be given to implication of success models on T&CM approach from neighboring countries.

3.4. Cultivation of medicinal plants at national level (for example, in Billion Tree Tsunami Programme in Pakistan) is encouraged.

3.5. There should be red listing of indigenous medicinal plants at country level as well as documentation of traditional knowledge at national level.

3.6. Periodic training workshops (mainly for collectors of medicinal plants, etc.), symposiums, conferences should be held for capacity building at government level.

3.7. Knowledge about medicinal plants be provided at school level.

3.8. Efforts be made to bridge the gap between academia and industry for integrated / coordinated use of three systems of medicines namely, allopathic, homeopathic and unani.

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ACKNOWLEDGEMENTS (font size 10)**:** In a brief statement, acknowledge financial support and other assistance.

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2. Bialek, W. & S. Setayeshgar. Cooperative sensitivity and noise in biochemical signaling. *Physical Review Letters* 100**:** 258–263 (2008).
3. Kay, R.R. & C.R.L. Thompson. Forming patterns in development without morphogen gradients: differentiation and sorting. *Cold Spring Harbor Perspectives in Biology* 1: doi: 10.1101/cshperspect.a001503 (2009).

b. **Books**

4. Luellen, W.R. *Fine-Tuning Your Writing*. Wise Owl Publishing Company, Madison, WI, USA (2001).
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c. **Book Chapters**

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