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Research Article

Evaluation of Biological Activity of Crude Extracts from Plants used by Indigenous Communities of Pothohar Plateau, Pakistan

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Abstract: The present study aimed to evaluate the potential of eight plant extracts that are used by communities of the Pothohar Plateau. Selected plants were *Brassica campestris*, *Brassica oleracea* var. *italica*, *Allium sativum*, *Piper nigrum*, *Cinnamomum zeylanicum*, *Allium cepa*, *Olea europaea* and *Moringa oleifera*. The antimicrobial assessment was carried out by using the agar diffusion method and antioxidant activity was evaluated using 2,2-diphenyl-1-picrylhydrazyl free radical assay, phosphomolybdate assay, and reducing power assay, against the selected isolates. Antimicrobial and growth rate studies were carried out by using two Gram-negative, one Gram-positive, and two pathogenic fungal strains. Among all tested extracts *M. oleifera* appeared to have the highest bioactivity with a percentage inhibition of 89% against DPPH free radical, followed by *Allium sativum 81%, Allium cepa 75%, Olea europaea* 67%, *B. campestris 60%, B. oleracea 58%*. During the phosphomolybdate assay similar trends were obtained such as: *M. oleifera* 91% followed by *A. sativum* 85%, *A. cepa* 78%, *Brassica campestris* 72%, *Brassica oleracea* 70% and *Olea europaea* 65% in higher concentration (1000 µg/ml). In the case of antibacterial assay *Moringa oleifera* showed maximum zone of inhibition against *Staphylococcus aureus* (20 mm) followed by *Klebsiella pneumonia* (18 mm) and *Escherichia coli*, (17 mm) whereas, crude extract of *Allium sativum* showed maximum zone of inhibition 14.4 mm against *A. flavus;* whereas *M. oleifera* gave maximum zone of inhibition against *A. alternata*, followed by *A. sativum* and *A. cepa*. All the tested extracts showed bioactivities. This study indicates the antimicrobial and antioxidant potentials of *M. oleifera,* and *A. species*. Hence, it is recommended that the extracts of these plants should be further evaluated for their possible application as antimicrobial and antioxidant agents.

Keywords: Moringa, Pothohar, Plateau, Antimicrobial, Antioxidant.

1. INTRODUCTION

Since ancient times, plants have been used as medicine [1]. There are many different traditional systems of medicine, i.e., Ayurveda, Unani, and Chinese, which are still utilized by native people [2]. After the 17th century, modern synthetic medicine gained more trust of pharmacologist, due to their availability and rapid mode of action [3]. However, with time, due to the development of resistance in microbes, synthetic medicine is facing more challenges [4]. Therefore, these days there has been an increasingly growing interest in investigating plants for the development of new antimicrobial and antioxidant agents as well as an alternative for the utilization of chemically synthetic representatives [5]. Plants have different types of bioactive compounds, i.e., phenolic compounds, carotenoids, tocopherols, etc. These natural antioxidants are obtained from different sources and different plant parts. Sources such as fruits (grapes, pomegranate), vegetables, (broccoli, pumpkin), herbs, and spices decreased lipid oxidation and microbial activity [6]. *M. oleifera* is known as the miracle tree, people

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have used it for centuries due to its health benefits. *Olea europaea and Brassica campestris* are known for their strength to retard microbial growth, maintain the organoleptic characteristics of food and inhibit the pathogen's growth [7].

Pakistan occupies 80,943 km^2 and has a large biodiversity, with 6000 species of higher plants. But regrettably, only 10% of the plant species in Pakistan are reported for their medicinal potential. Pakistan is rich in native herbs and supplies a large scope for ethnobotanical studies. In Pakistan Traditional Unani medicine is largely used among the local communities. Pothohar plateau falls in a rainfed region of Pakistan [8]. It occupies the north of Punjab province of Pakistan with an elevation ranging from 1500-2000 feet.

Even though a great deal of work has been conducted on the ethno-medicinally used flora of this region. However, no significant scientific study was conducted on the bioactivities of important plants in this region. Therefore, the present study was planned to evaluate the bio-activities of traditionally utilized plants of the Pothohar plateau i.e., *Brassica campestris, Brassica oleracea* var. *italica*, *Allium sativum, Piper nigrum, Cinnamomum zeylanicum, Allium cepa, Olea europaea* and *Moringa oleifera.*

2. MATERIALS AND METHODS

2.1 Plant Selection

Species selected were *Brassica campestris*, *Brassica oleracea* var. *italica*, *Allium sativum, Piper nigrum, Cinnamomum zeylanicum, Allium cepa, Olea europaea* and *Moringa oleifera*.

2.2 Collection of Plant

Among selected plants, i.e., *Moringa oleifera* and *Olea europaea* leaves were collected from PMAS Arid Agriculture University, Rawalpindi on 3rd, March 2020. Leaves of *Brassica campestris* and *Brassica oleracea* var. *italica,* the bark of *Cinnamomum zeylanicum*, the seed of *Piper nigrum*, and bulbs of *Allium sativum* and *Allium cepa* were collected from the local market on 15th, April 2020. The specimens were identified with the help of taxonomists.

2.3 Processing and Drying

All the unwanted plant parts were removed, shade dried, at room temperature, ground to powder, and stored in an airtight container with labels, a schematic diagram for crude extract is shown in Figure 1.

2.4 Extract Preparation

Plant extracts were prepared by following the methodology of Qin *et al.* [9]. The ratio between the plant powder material and the solvent was 1:6. The mixture was allowed to stand for seven days and filtered. The filtrates were again treated with the same procedure and filtrates were evaporated. The extract obtained was stored at 4^{o}C. The solvent we used is methanol. Our goal is the isolation of polar natural products such as polyphenols, flavonoids, etc. For the extraction of such polar natural products, a highly polar solvent such as methanol is particularly suited.

2.5 Bioactivities of Crude Methanolic Extracts

2.5.1. Antioxidant activities of crude extracts

For antioxidant activity, the plant samples were mixed in methanol solvent. It was then diluted by making different concentrations (62.5, 125, 250, 500, and 1000 µg/mL).

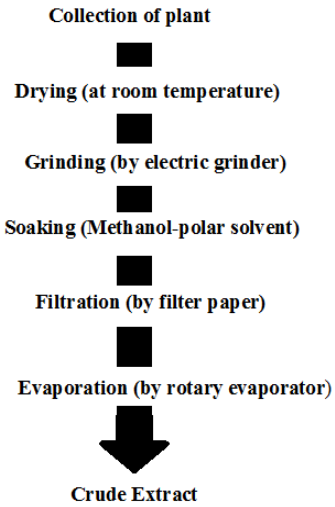

Fig. 1. Scheme for crude extract preparation.

2.5.1.1. DPPH radical scavenging activity assay

Antioxidant potential for crude extracts was evaluated by using the DPPH standard. Free radical scavenging bioassay was carried out by Hara *et al.* [10] with few modifications. To prepare a stock solution, DPPH was dissolved in methanol solvent. The stock solution was then stored in darkness at 20 °C for future use. Then DPPH solution was diluted by methanol, to obtain a particular absorbance at 517 nm. The samples were then mixed with a small quantity of obtained solution. The solution was incubated for 15 minutes in darkness. Positive control was Ascorbic acid while negative control was DMSO.

2.5.1.2. Phosphomolybdate assay

The antioxidant activities were examined by the method phosphomolybdate given by Gupta *et al.* [11]. The samples were mixed with our prepared reagents. These test tubes were then covered using aluminum foil. These covered tubes were incubated in a water bath at 95 °C. The covered tubes were cooled down at room temperature. Absorbance was calculated at 765 nm in comparison with a blank by using a spectrophotometer. Ascorbic acid was used as a positive control. The percentage increase in absorbance was measured.

2.5.1.3. Reducing power assay

The reducing power assay was done by the methodology of Gupta *et al.* [11]. The plant extract solutions, phosphate buffer, potassium ferri-cyanide, and tri-chloroacetic acid were mixed and incubated. These mixtures were then diluted then $FeCl_3$ was mixed. Then absorbance was observed at 700 nm by using a spectrophotometer. The increased absorbance value was the indication of high reducing power activity.

2.5.2. Antibacterial assays

2.5.2.1. Sample preparation

The antibacterial potential of selected plant extracts was tested at different concentrations (62.5 µg/ml – 1000 µg/ml). The extracts of selected species were weighed. Samples were dissolved in DMSO to prepare a stock solution. The stock solution was used to prepare five different concentrations, i.e., 62.5, 125, 250, 500, and 1000 µg/ml. Positive control Ciprofloxacin was prepared with the same concentrations.

2.5.2.2. Test microorganisms

The three different bacterial strains were used in this study, two Gram-negative (*Escherichia coli*, and *Klebsiella pneumonia*) and one Gram-positive (*Staphylococcus aureus).* Muller-Hinton Broth (MHB) was used to maintain microorganisms at 4°C until further use. For the re-culturing of microorganisms, the nutrient broth medium was dissolved in distilled water. Sealed the flask with a cotton plug before allowing for autoclave. The selected bacterial cultures were inoculated under aseptic conditions. These cultures were grown at 37 °C for 24 hours in a shaker incubator at 150 rpm (rotation per minute).

2.5.2.3. Agar well diffusion method

The antibacterial activity was examined by the methodology given by Dilshad *et al.* [12] with few modifications. Nutrient medium was prepared and poured in pre-labeled petri plates up to 1/3 of its volume. The media was inoculated with 10 mL of inoculum. The petri plates were left till solidification. Sterilized cork borer was used to make wells. Wells were made in every plate, for extracts, negative and positive control. Then nutrient media (20 µl) was added in each well. Tested Extracts were poured into respective wells. These plates were incubated at 37 °C for a day and a zone of inhibition was calculated. The experiment was done thrice, and then the mean value was calculated.

Positive control = Diameter of inhibition zone by standard drug (Ciprofloxacin)

2.5.3. Antifungal activity

2.5.3.1. Fungal strains

Aspergillus flavus and *Alternaria alternata* were used in the study. These fungal cultures were preserved on SDA media (Sabouraud dextrose agar) in a slanting position at 27 °C. After 7 days, the fungal spores were ready for determination of antifungal activity.

2.5.3.2. Agar tube dilution method

Antifungal activity was determined by the tube dilution method of Dilshad *et al.* [12]. Sabouraud dextrose agar (SDA, Merck) was dissolved in distilled water (32.5 g in 500 ml). Placed the mixture on a hot plate, a magnetic stirrer was used for proper mixing. Then 5 ml of media were poured and then autoclaved at 120 °C for 15 minutes and allowed to cool. After cooling just before solidification, plant extract was added in several concentrations, i.e., 10, 25, and 50 µg/ml. Then these tubes were shaken well and allowed to solidify. After that, a 4 mm inoculum was placed in each tube and incubated for 7 days at 28 °C.

3. RESULTS AND DISCUSSION

To evaluate the antioxidant properties of plants, it is suggested to use multiple tests with a variety of conditions [13]. In the present study, three different assays were performed, i.e., DPPH free radical, reducing power assay, and phosphomolybdate assay. The eight plant species were examined (Table 1).

Upon evaluation, it was observed that crude extract of *Moringa oleifera* showed maximum activity against DPPH free radical with percentage inhibition of 89% (1000 µg/ml), 81% (500 µg/ml), 77% (250 µg/ml), 65% (125 µg/ml), 56% (62.5 µg/ml). Results of *M. oleifera* were followed by *Allium sativum* 81% (1000 µg/ml) and *Allium cepa* 75% (1000µg/ml). The percentage inhibition was found dependent on concentrations. The minimum antioxidant activity was shown by plants belonging to the Brassicaceae family. *B. campestris* and *B. oleracea* displayed percentage inhibition of 60% and 58% in higher concentration (1000 µg/ml) and the lowest value was 23% and 20% at 62.5 µg/ml, respectively (Figure 2). The percentage inhibition declared by *Olea europaea* is better than the plants belonging to the Brassicaceae family 67% (1000 µg/ml), 56% (500 µg/ml), 40% (250 µg/ml), 35% (125 µg/ml), 26% (62.5 µg/ml). A similar percentage inhibition (40%) was displayed by *B. campestris* and *O. europaea* at 250 µg/ml.

Table 1. Selected plants and their documented uses.

Plants	Phytochemical	Antimicrobial activity	References
Allium sativum	Sulfoxides	Antibacterial	[24]
Piper nigrum	Alkaloid	Antimicrobial	[24]
Olea europaea	Secoiridoids	Anifungal. antibacterial	[25]
Cinnamomum zeylanicum		Antibacterial, antifungal	[26]
Allium cepa		Antibacterial	[27]
Brassica campestris	Flavonoid	Antibacterial	[28]
Brassica oleraceae var. italica		Antibacterial	[29]
Moringa oleifera		Antibacterial	[30]

Minimum inhibition was seen in the lowest concentration (62.5 µg/ml) in all selected species. Against phosphomolybdate ion, the percentage inhibition in the case of *M. oleifera* was 91% followed by *A. sativum* (85%), *A. cepa* (78%) *Brassica campestris* (72%), *Brassica oleracea* (70%) and *Olea europaea* (65%) in higher concentration (1000 µg/ml). *P. nigrum* and *C. zeylanicum* showed the same percentage of inhibition 53% (250 µg/ml). Similar *P. nigrum* and *B. oleracea* showed the same percentage inhibition 70% (1000 µg/ml). Similar findings were obtained during the reducing power assay. The high antioxidant potential of *M. oleifera* has previously been reported [14]. Khunchalee and Surapat [15] also tested different extracts of *M. oleifera* and got a very high activity against DPPH free radical, i.e., leaves extract in ethanol (89.33%), leaves extract in methanol (84.05%), leaves extract in water (74.85%), seeds extract in methanol (89.09%) and pod extract in methanol (86.36%), respectively.

In our study, the other two species that showed antioxidant activity were *A. cepa and A. sativum*. Alliums. Mainly, *A. cepa, A. sativum, A. ursinum,* and *A. ampeloprasum*, are a rich source of bioactive compounds, which have a major role in the pharmacological properties of plants. The extracts of *A. cepa* bulb peel and *A. sativum* bulb have previously been studied and expressed notable antioxidant activity in *in vitro* antioxidant assays, such as DPPH, ABTS, TAC, and FRAP [16]. Allium species have been recognized in the treatment of infective diseases as an effective antimicrobial agent. Many fungi, bacteria, and viruses have been

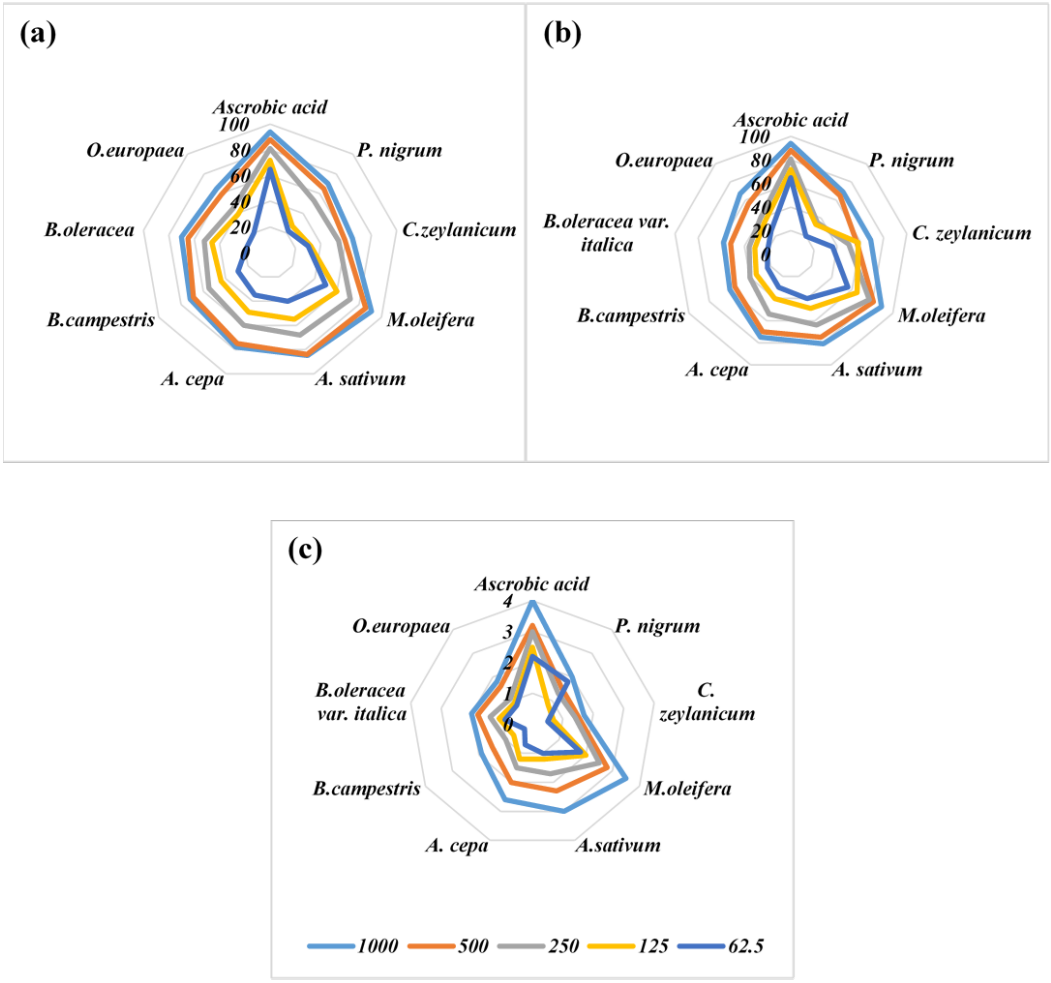

Fig. 2. Antioxidant activity of crude methanolic extract of selected species by (a) Phosphomolybdate assay, (b) DPPH Assay, and (c) Reducing power assay.

found vulnerable to various Allium solvent extracts [17]. They are known to have antioxidant potential due to the presence of high amounts of organosulfur compounds, polyphenols, and flavonoids [18].

The antimicrobial screening of the plants is considered an important parameter for the assessment of any plants to be used as preservatives for food and food products [19]. To study the antibacterial activity of crude methanolic extract of selected species both Gram-positive and Gram-negative bacterial strains were used (Figure 3). The

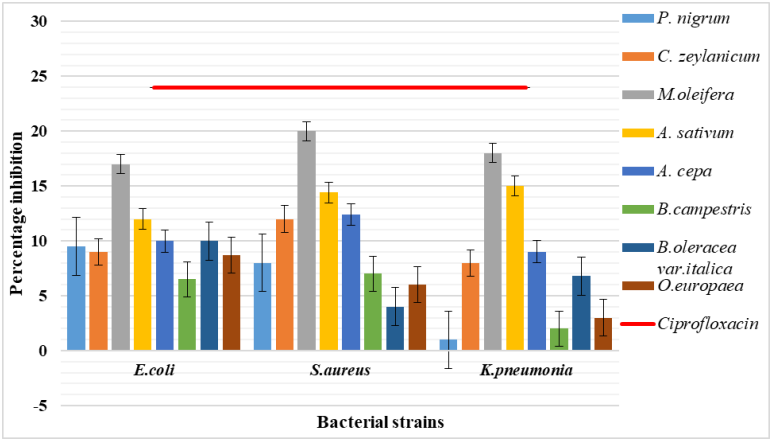

Fig. 3. Antibacterial activity of crude methanolic extract of selected species.

bacteria species were chosen as they are common pathogenic bacteria infecting humans and animals. Similarly, two fungal strains (*Aspergillus flavus* and *Alternaria alternata*) were used in the present study to evaluate the fungal activity of selected species (Figure 4). The crude methanolic extract of selected species represented the antibacterial activity against three bacterial strains. The crude extract of *Moringa oleifera* showed a maximum zone of inhibition against *Staphylococcus aureus* (20 mm) followed by *Klebsiella pneumonia* (18 mm) and *Escherichia coli* (17 mm).

It was noticed that after *Moringa oleifera* the antibacterial activity of crude extract of *Allium sativum* was highest which was further followed by *A. cepa. A. sativum* showed 14.4 mm against *S. aureus*, followed by 12 mm against *E. coli.* The minimum antibacterial activity was shown by *Piper nigrum* (1 mm) against *Klebsiella pneumonia*. Similarly, *Brassica campestris* (2 mm) and *Olea europaea* (3 mm) showed a minimum zone of inhibition against *Klebsiella pneumonia*. *Piper nigrum* and *Cinnamomum zeylanicum* showed the same results against *E. coli.* Among all bacterial strains, *Staphylococcus aureus* showed maximum resistance. *Moringa oleifera* has previously been reported by many studies to have antibacterial activity. Awasthi *et al.* [20] conducted a comparative assessment of ethnobotany and antibacterial activity of *Moringa oleifera* Lam. in Nepal. They concluded that *M. oleifera* is a promising medicinal plant and More research is needed on its ethnomedicinal and biochemical capabilities. Miladiarsi *et al.* [21] prepared an ointment from Moringa leafs and claimed that it has antibacterial activity against *Staphylococcus aureus* with an inhibition zone of 15% (16.1 mm).

Allium species also appear to have high antibacterial activity. According to Oyawoye *et al.* [22] the high antibacterial activity of allium species is due to the large quantity of phenolic compounds in them. Benkeblia [23] assessed the antibacterial activity of different phenolic compounds against *Staphylococcus aureus a*nd *Salmonella enteritidis* and obtained similar results. The crude methanolic extract of selected species was investigated against *Aspergillus flavus* and *Alternaria alternata.* According to the findings, the crude extract of Allium sativum showed a maximum zone of inhibition14.4mm against Aspergillus flavus whereas *Moringa oleifera* gave a maximum zone of inhibition against *Alternaria alternata,* followed by *Allium sativum* and *Allium cepa*. The minimum antioxidant activity was shown by *Olea europeae*. During our evaluation, *M. oleifera* appeared to be the most potent plant followed by *Allium sativum* and *Allium cepa*. Previous studies also showed that extracts obtained from leaves of *M. oleifera* contain bioactive properties such as antioxidant, anti-inflammatory, antilipidemic, antimicrobial, anti-hyperglycemic, among others [12]. The high antioxidant activity of plant extracts is due to the presence of phenolic compounds such as tannins, flavonoids, and steroids. Some of the bioactive activities of plants such as antimicrobial, anti-inflammatory, anticarcinogenic, and antiatherosclerotic are also due to the antioxidant capacity of plants.

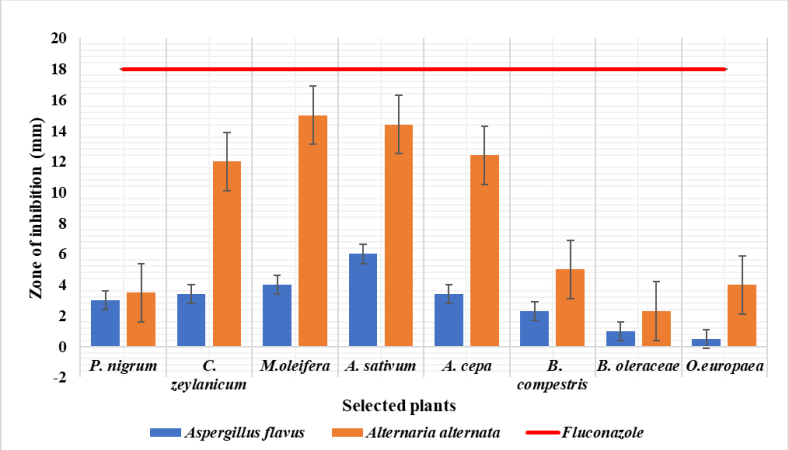

Fig. 4. Antifungal activity of crude methanolic extract of selected species.

4. CONCLUSIONS

Plants used by local communities of Pothohar Plateau, Pakistan appeared to have notable bioactivities. Particularly *M. oleifera* is the most active plant with strong activity against free radicals (89% DPPH, 91% phosphomolybdate) and infectious microorganisms *(Staphylococcus aureus,* 20 mm; *Klebsiella pneumonia,* 18 mm; *Escherichia coli* 17 mm). Other plants that showed activity were *A. sativum* and *A. cepa*. Therefore, it is recommended that these plants should be further explored and characterization of active compounds should be performed with the aim of novel drug development against drug-resistant microbes.

5. CONFLICT OF INTEREST

The authors declare no conflict of interest.

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