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Antimicrobial and Antioxidant Activities of Methanolic Extract and Fractions of *Epilobium roseum* (Schreb.) against Bacterial Strains

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Abstract

Present study aims to evaluate antimicrobial and antioxidant activities of the crude methanolic extract and different fractions of the *Epilobium roseum* (Schreb). The extract and fractions were used against pathogenic bacteria (*Bacillus subtilis*, *Bacillus atrophaeus, Klebsiella pneumonia* and *Pseudomonas aeruginosa*) and fungal strains (*Aspergillus niger* and *Aspergillus flavus*). The methanolic extract and their sub fraction n-hexane showed a prominent inhibition zone against all bacterial strains but inactive against fungal strains. The various extracts of *Epilobium roseum* (Schreb) from various parts were tested for their antioxidant activity by 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) assay. The IC_{50} of the stem and root of methanolic extract and their sub fraction n-hexane showed best activity ranged between 22.73 ± 6.92, 21.49 ± 6.26 and 14.94 ± 3.54, 13.92 ± 1.04 µg/ml compared to another fraction. The results support that *Epilobium roseum* can be used as antimicrobial and antioxidant agents. The results support the present study that *Epilobium roseum* (Schreb) has a potential source of natural antibacterial, antifungal and antioxidant potentials.

Keywords

Methanolic Extract, *Epilobium roseum*, *DPPH*, n-Hexan, Antioxidant Activity

1. Introduction

Plants are rich sources of modern medicines, chemical entities for synthetic

drugs and pharmaceutical intermediates [1]. Medicinal plants are widely used for therapeutic purposes; having a potential source of biological agents such as antioxidants and antimicrobial activity and their use is of greater demand nowadays [2] [3]. Thousands of medicinal species found in different parts of the world are being used from the ancient time, to improve flavor as well as antioxidant [4] and antimicrobial properties [5] [6] [7]. Recently the use of complementary and alternative medicines has been increased which lead to enhancing the market for herbal products worldwide [8]. According to the World Health Organization (WHO) 80% of developed countries use traditional medicine that is cheaper than synthetic medicine [9] [10]. It is important that the compounds obtained from medicinal plants have antioxidant and antimicrobial potential [10] [11] [12]. Scientific investigations showed that medicinal plants are used as an alternative healthcare treatment for several types of disease in many countries for their health contributions.

Previously, plant extracts were used against a number of bacterial species including *Bacillus cereus, Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* and were found effective at different concentrations [13]. Akinpelu *et al.* [14] studied the antibacterial potential of crude and butanolic extracts of *Persea americana* against *Bacillus cereus* and found the positive effects of the extracts at 25 and 10 mg/ml. Manandhar *et al.* [15] tested the antimicrobial activities of four different plant extracts against twelve pathogenic microorganisms and found that most of the extracts were able to reduce the growth of pathogenic microorganisms.

Epilobium roseum commonly known as Pale Willow herb, from Onagraceae family having worldwide distribution containing 160 - 120 flowering species, most important well-known species are *E. alpesterw, E. canum, E. billardierianum* and *E. parviflorum* growing on subarctic, subantarctic and temperate region [16]. This plant is also distributed in central Europe, Eastern Europe and North America. The flower extract is used for the treatment of prostate disorder (empty bladder and stronger urine flow and urgent need to pee), and abnormal growth (vinca alkaloid use against *in situ* malignant form) in central Europe [17] [18]. Epilobium roseum leaves are used for treatment of joint pain [16], increases sweating, central nervous system stimulant, dilates bronchioles (anti asthmatic use), diuretic and for muscle pain [19]. Moreover, *Epilobium roseum* is recently used as a: herbal medicine in Western countries for the treatment of acute onset of cold/flu, asthma, to raise blood pressure and hay fever.

The aim of this work was to evaluate the *in vitro* antimicrobial and antioxidant activities of the extracts of *Epilobium roseum* and to find out multiple resistance drugs such as the development of new synthetic antimicrobial and anti-oxidative drugs.

2. Materials and Methods

The plant sample *Epilobium roseum* (Schreb) was collected from the northern

area (Naran valley) of Khyber Pakhtunkhwa, Pakistan. The collected plant was identified with the help of Flora of Pakistan, taxonomist and various pictorial guides. The plant material was processed in Pakistan Council and Scientific industrial research (PCSIR) Peshawar, Pakistan.

The collected plant material (root and stem) of the *Epilobium roseum* (Schreb) was washed thoroughly with tap water to remove soil debris and surface sterilized with 70% ethanol and 1% perchloric acid for 30 sec under sterile condition. Further the collected plant material was dried in shade and grinded into powder. Approximately 200 g powdered plant materials were soaked in 600 ml methanol for 72 hrs three times and filtered through Whatman filter paper (No.1). The solvent was vaporized at 45˚C through rotary evaporator (RE801A-W), to obtain the crude extract [20]. The methanolic crude extract was further fractionated with n-Butanol), n-hexane, chloroform and ethyl acetate respectively and evaporated at 40˚C by Rotary Evaporator [17] [21].

The chemical used in present experiment are; 1,1-diphenyl-2-picrylhydrazyl (DPPH), Propyl gallate (PG), 3-Tert-Butyl-4-Hydroxyanisole (TBH), Sabourud dextrose agar (SDA), Dimethyl sulfoxide (DMSO), Nutrient broth and Nutrient agar.

The microorganisms used in the assay include two Gram positive and two Gram negative bacterial strains and two fungal strains as shown in the following **Table 1**.

Antifungal activities were performed by the method of Jagessar *et al.* [6]. Autoclaved prepared media (60 g of sabouraud dextrose agar/1000ml of distilled water) was poured in each Petri plate and was allowed to solidify. After solidification, circular discs (6 mm) of Whatman no 1 filter paper were placed in each petri plate. The spores of fungus strains (*A. niger* and *A. flavus*) were applied on the center of petri plates. Plant samples (1 mg/ml dimethyl sulfoxide and 1 mg/ml crude extract), were applied on the paper discs. Fluconazole was used as standard (positive control). The fungal culture was incubated at 26˚C for seven days. After seven days of incubation the zone of inhibition was measured [21].

Disc diffusion assay was performed for antibacterial activities [6]. The prepared media (28 g Nutrient agar/1000ml distil water), were poured into the Petri plates under sterile condition. The bacterial culture were homogenized with 8 ml nutrient broth (13 g Nutrient broth/1000ml dist water) in a test tube, and incubated in the shaking water bath (Model GLSC) for 16 hours (200 rpm) at 37˚C. After incubation bacterial cultures were diluted and standardized by comparing with 0.5 McFarland (turbidity) standards. The standardized bacterial culture (50 µl) was spread through a glass spreader on each nutrient agar plate and refrigerated for a few minutes. After refrigeration impregnated plates the filter paper disc (6 mm) and plant extract (6 µl) were applied on each paper disc. Azithromycin and tetracycline were applied on separate plates as standard. The plates were incubated at 37˚C for 24 hours. After 24 h of incubation inhibition zone was observed around each disc in mm.

Table 1. Details of the microorganism used in the assay.

Isolates Name	Types	Information of isolates
Bacillus atrophoeus	Gram positive	Clinical isolate obtained from PCSIR Lab. Complex Peshawar
Bacillus subtlilis	Gram positive	Clinical isolate obtained from PCSIR Lab. Complex Peshawar.
Kleibsiella pneumonia	Gram negative	ATCC# 9721
Pseudomonas aeruginosa	Gram negative	Clinical isolated obtained from PCSIR Lab. Complex Peshawar.
Aspergillus flavus	Fungus	Clinical isolate obtained from CIIT Abbottabad.
Aspergillus niger	Fungus	Clinical isolate obtained from CIIT Abbottabad.

The free radical scavenging activity of the plant extract was measured in vitro by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) assay [22] [23]. Fresh solutions of 1-diphenyl-2-picrylhydrazyl (DPPH) were prepared (3.2 µl DPPH dissolved in 25 ml methanol), and kept at 4˚C. From the solution (methanolic solution of DPPH) approximately 90 µl were added to 10 µl of plant extracts in different concentrations, incubated at 37˚C for 30 minutes. The absorbance was measured at 490 nm using a multiplate reader (Bio-Tek Elx800 USA). Propyl gallate (PG) and 3-tert-butyl-4-hydroxyanisole (TBH) were used as standard. Inhibition percentage of the radical scavenging activity of the test sample was compared with the standard and calculated according to the equation of Veeru *et al.* [24].

$$\text{DPPH inhibition } (\%) = \left[(Ab - Aa) / Ab \right] \times 100$$

where *Aa*, absorbance values of the test sample and *Ab*, absorbance value of the blank sample.

Extracts concentration at 50% inhibitions (IC_{50}) is calculated from the graph plotted of inhibition percentage against extracts concentration.

Statistical Analysis

All the experimental data of antimicrobial and antioxidants are statistically analyzed by mean ± standard deviation (SD) triplicated. Antioxidants values IC_{50} are analyzed from linear regression analysis using Graph pad prism 5 software.

3. Results and Discussion

It has long been recognized that naturally occurring substances in higher plants have antimicrobial and antioxidant activity [25] [26]. Among the available methods, disc diffusion method for antimicrobial assay and on the other hand, the compounds distributed in plants have the ability to scavenge free radicals by single-electron transfer [27]. The development of microbial resistance to presently avail-able antibiotics led to the search for new antimicrobial agents [28]. Due to the problem of microbial resistance to antibiotics, attention is given toward biologically active components isolated from plant species commonly used as herbal medicine, as they may offer a new source of antimicrobial activities [29]. In the present finding the extracts of crude methanolic extract and various fractions of the *Epilobium roseum* (Schreb) tested for antimicrobial activity against bacterial

strains *i.e. Bacillus atrophaeus, Bacillus subtilis*, *Klebsiella pneumonia, Pseudomonas aeruginosa* and two fungal strains *i.e. Aspergillus niger* and *Aspergillus flavus.* Chloroform and n-butanol fraction of root *Epilobium roseum* (Schreb) have the highest zone of inhibition against tested bacteria. Ethyl acetate and Methanol extract have moderate activity, while no activity was observed by aqueous fraction against *Pseudomonas aeruginosa* (Table 2, Figure 1). The methanolic crude extract of root and their fraction showed no activity against tested strains of fungi (*A. niger* and *A. flavus*) (Figure 1). Ethyl Acetate fraction and n-hexane fraction of stem showed best activity against both Gram positive and Gram-negative bacteria while water and n-butanol fraction mostly inactive (Table 3, Figure 1). The first remarkable aspect of the results obtained was that no one of the extracts and their fraction inhibited the growth of tested strains of fungi (*A. niger* and *A. flavus*) (Table 4).

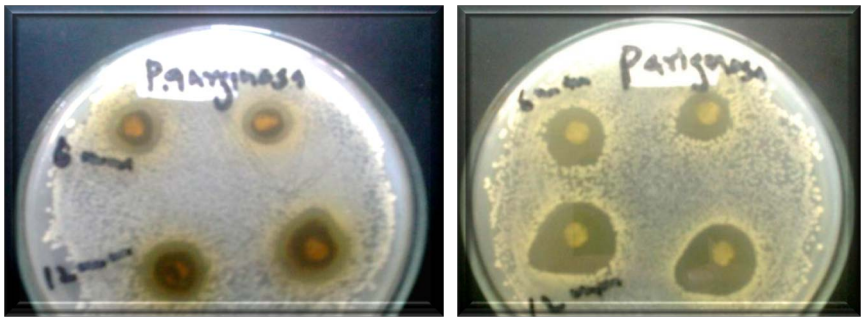

Figure 1. Zone of inhibition (mm) of methanolic extract and fractions of "*Epilobium roseum* (Schreb)" Root stem.

Table 2. Antibacterial activity of crude methanolic extract and fractions of "*Epilobium roseum* (Schreb)" Root.

Zone of Inhibition in Mean ± STD µg/ml								
Isolates	Extract-Fractions						Standard	
Bacterial strains	Methanol	n-Hexane	Chloroform	Ethylacetate	n-butanol	Water	Az	Tetra
B. atrophoeus	14.0 ± 0.5	10.7 ± 1.0	22.1 ± 0.1	17.7 ± 1.4	20.2 ± 0.2	16 ± 1.0	24 ± 0	25 ± 0
Bacillus subtlilis	15.3 ± 0.5	09.0 ± 1.8	17.3 ± 1.5	12.7 ± 1.3	18 ± 1.0	16 ± 1.0	21 ± 1	23 ± 0
K. pneumonia	13.3 ± 1.3	12.2 ± 1.0	14.2 ± 1.0	12.7 ± 1.5	16 ± 1.0	0 9 ± 1.0	21 ± 1	23 ± 0
P. aeruginosa	07.9 ± 0.1	12.7 ± 0.0	10.0 ± 1.0	14.7 ± 1.5	15 ± 1.5	00 ± 00	20 ± .0	19 ± 0

Table 3. Antibacterial activity of crude methanolic extract and fractions of "*Epilobium roseum* (Schreb)" Stem.

(Zone of Inhibition in Mean ± STD µg/ml)								
Isolates	Extract-Fractions						Standard	
Bacterial strains	Methanol	n-Hexane	Chloroform	Ethyl acetate	n-butanol	Water	Az	Tetra
B. atrophoeus	19.1 ± 0.5	17.1 ± 1.0	12.0 ± 1.0	10.3 ± 1.0	00.0 ± 0.0	9.3 ± 0.5	23 ± 0.0	22 ± 00
B. subtlilis	15.0 ± 1.0	15.0 ± 1.0	11.0 ± 1.0	13.7 ± 1.5	00.0 ± 0.0	0.0 ± 0.0	20 ± 1.0	21 ± 05
K. pneumonia	17.3 ± 0.5	14.3 ± 0.5	120 ± 0.58	13.0 ± 1.0	00.0 ± 0.0	0.0 ± 0.0	25 ± 1.0	24 ± 00
P. aeruginosa	15.3 ± 1.0	16.7 ± 1.0	13.0 ± 1.00	10.0 ± 1.0	09.7 ± 1.0	0.0 ± 0.0	20.0.0	20 ± 01

Table 4. Antifungal activity of crude methanolic extract and fractions of "*Epilobium roseum* (Schreb)" Stem.

Isolates	Extract-Fractions (Zone of Inhibition in Mean ± STD µg/ml)						Standard
Fungal Strains	Methanole	n-Hexane	Chlorofom	Ethylacetate	n-butanol	Water	Fluco
A. niger	0.00 ± 0.0	0.00 ± 0.0	0.0 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.0 ± 0.0	20.0 ± 0
A. flavus	0.00 ± 0.0	0.00 ± 0.0	0.0 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.0 ± 0.0	23.0 ± 2

Az-Azithromycine, Tetra-Tetramycine, (standard used for antibacterial activity) Fluco-Fluconazole (Standard used for antifungal activity).

Free radical-scavenging are well-known mechanisms widely used to determine the free radical-scavenging activity, inhibit lipid oxidation [30]. The screening and characterization of antioxidants derived from natural sources has gained much attention and efforts have been put into identifying compounds as suitable antioxidants to replace synthetic ones [31]. Antioxidants are widely distributed in most of the medicinal plants and show a range of biological activities [32]. They also scavenge the free radicals and help in prevention of different diseases [33]. Most of the natural antioxidants are responsible for the conversion of lipid radicals into stable products and prevent the oxidation of lipoproteins [34]. DPPH radical scavenging activity can be reduced through hydrogen donating ability [1] [35]. Oxidative stress increases superoxide radical concentration in all cells, hence inducing several pathophysiological Processes [36] [37]. Antioxidant activities of the *Epilobium roseum* (Schreb) extract and their fraction are measured by DPPH assay shown in (**Table 5**). The IC50 values for radical scavenging activities of PG, TBH and different extract fractions of the *Epilobium roseum* using the DPPH colorimetric method. In the DPPH assay conducted on the Ethyl acetate extracts, root and stem both had the lowest IC50 value among the other fractions (03.96 ± 0.39 and 02.90 ± 0.12 µg/mL), followed by chloroform fraction (07.98 ± 2.09 and 04.13 ± 0.41 µg/mL), n-butanol fraction (12.21 ± 5.71 and 03.44 ± 0.69 µg/mL), methanolic extract (14.94 ± 3.54 and 13.92 ± 1.04 µg/mL) and n-Hexane fraction (22.94 ± 2.06 and 24.12 ± 2.12 µg/mL). It was evident that the extracts showed hydrogen donating ability and therefore the extracts could serve as free radical scavengers, acting possibly as primary antioxidants [38]. The positive control PG-Propyl gallate, TBH-3-tert-butyl-4-hydroxyanisole had an IC50 value 50.01 ± 0.23, 52.50 ± 0.57 and 60.03 ± 0.03, 55.10 ± 1.21 µg/mL. The above IC50 values showed that n-Hexane fraction and methanolic extract demonstrated even higher radical scavenging activities than the other fractions in the DPPH assay. The DPPH activity of the plant was observed in the following order: n-hexane fraction of stem > n-hexane fraction of Root > methanolic extract of root > methanolic extract of stem. IC50 values were 24.12 ± 2.12 > 22.94 ± 2.06 > 17.04 ± 0.54 > 13.12 ± 1.01 > 12.21 ± 1.51 µg/ml for n-hexane fraction of stem > n-hexane fraction of Root > methanolic extract of root > methanolic extract of stem respectively. Although antioxidant effects of various extracts were low as compared to standard. Havesi *et al.* [39] reported that the aqueous acetone extract of *E. parviflorum* exhibited the higher antioxidant effect in the DPPH assay than other antioxidants and inhibited the lipid peroxidation determined by the TBA assay.

Table 5. Free radical scavenging activity (DPPH) of "*Epilobium roseum* (Schreb)" Root and Stem crude methanolic extract and fractions.

DDPH assay IC_{50} ± STD µg/ml		
Extract/Fractions	*E. roseum root*	*E. roseum* stem
Methanolic extract	17.04 ± 0.54	13.12 ± 1.01
N-Hexane fraction	22.94 ± 2.06	24.12 ± 2.12
Chloroform fraction	06.38 ± 4.59	02.73 ± 0.84
Ethyl acetate fraction	02.96 ± 2.39	02.69 ± 0.26
n-butanol fraction	12.21 ± 1.51	03.44 ± 0.69
PG (Control)	50.01 ± 0.23	52.50 ± 0.57
TBH (Control)	60.03 ± 0.03	55.10 ± 1.21

Values are given as mean ± standard deviation of triplicate experiments. PG-Propyl gallate, TBH-3-tert-butyl-4-hydroxyanisol; DPPH, 1,1-diphenyl-2-picrylhydrazyl.

4. Conclusion

The present study demonstrated that the methanolic extract and different fractions of the *Epilobium roseum* (Schreb) possess strong antimicrobial and antioxidant properties which confirm the ethnopharmacological uses of the native medicinal plant *E. roseum*, and are the first ever report in Pakistan.

Availability of Data

All the data is available in the submitted manuscript. There are no additional files associated with this MS.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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