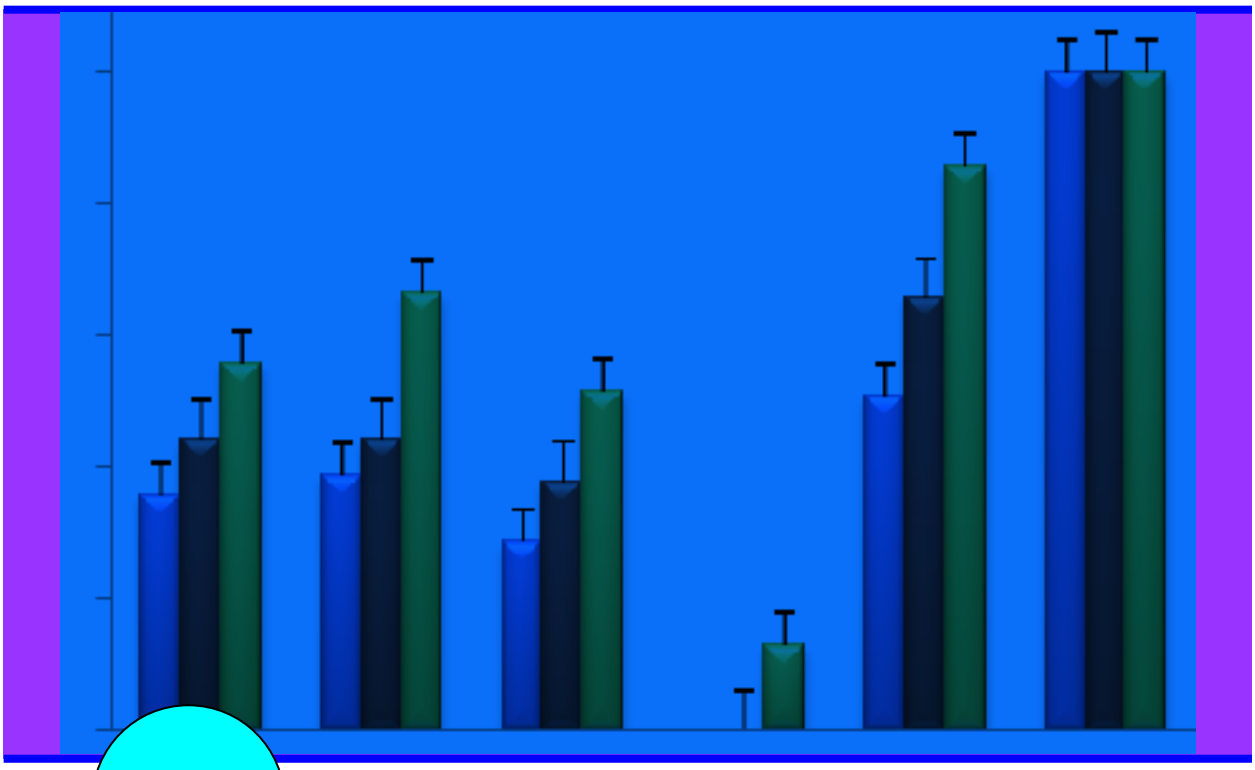

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Antimicrobial and antioxidant potential of *Periploca hydaspidis*

Rafi Ullah[1], Jehan Bakht[1] and Mohammad Shafi[2]

[1]Institute of Biotechnology and Genetic Engineering, The University of Agriculture Peshawar, Pakistan; [2]Department of Agronomy, The University of Agriculture Peshawar, Pakistan.

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Abstract

The present study investigates antimicrobial and anti-oxidant potential of different solvent extracted samples from *Periploca hydaspidis* through disc diffusion assay. *Pseudomonas aeruginosa* and *E. coli* were sensitive to crude extracts and all fractions measuring varying degree of growth inhibition. Similarly, the growth of *Citrobacter freundii* was not inhibited by crude methanolic extracts, n-hexane, ethyl acetate and aqueous extracted samples. Maximum growth inhibition was measured against *Klebsiella pneumoniae* exposed to aqueous fraction followed by *n*-hexane fraction. Our results also suggested that among various fungi, *Candida albicans* was sensitive to crude methanolic extracts, *n*-hexane and aqueous fractions compared with other fractions. The rest of the fungi under test were resistant to crude and all fractions showing no zone of inhibition. All the extracted samples of the stems and roots showed antioxidant activity, however, crude methanolic extract of stem tissues exhibited better antioxidant activity than the other extracts.

Introduction

Herbal medicine is an important fields of traditional medicine in both rural and urban areas. For the proper utilization of herbal medicine and their potential as sources for new medicine it is of vital importance to investigate those medicinal plants, which have a good reputation in a more intensified way (Schopen, 1983; El-Faky et al., 1995; Awadh et al., 2001). A renewed curiosity has occurred in the last two decades to investigate the phytochemicals and antimicrobial activities of native and naturalized plants (Ho et al., 1992; Cragg et al., 1996; Oktay et al., 2003; Bakht et al., 2011a,b,c; 2012; 2013a,b,c; 2014a,b,c). Leaves, roots, flowers, whole plants and stems are used for as medicine for treatment of many diseases.

Periploca hydaspidis belongs to the Asclepiadaceae. It is a twining shrub, usually leafless; branches are smooth green and are 1.5 mm in diameter. Leaves (when present) are 40 x 1-3 mm, linear or lanceolate, nerves in leaves are obscure, petiole 1-2 mm long. Flowers are in lax axillary trichotomous cymes. Seeds are present in amount of 10-50 in a coating.

Materials and Methods

Crude extract preparation

The plant materials (stem) were washed thoroughly with distilled water to remove any dust particles, dried for seven days and grinded. One thousand gram of dried powder of stems were soaked in five liters of methanol in extraction flasks, kept at 24°C in dark for one week and shaken twice daily. The mixture was filtered through Whatman filter paper No. 1. The remaining solid residues were mixed with 2500 mL fresh methanol and the whole process was repeated thrice. The filtrate was dried at 45°C under vacuum pressure in a rotary evaporator. The semisolid extract was divided into two parts. One part (10 g) was used as crude methanolic extract and the second part (80 g) was used for further fractionation with different solvents.

Fractionation of crude extract

Eight gram of crude extract was dissolved in 500 mL sterile distilled water, mixed with *n*-hexane (300 mL), shaken gently and allowed to stand for 15 min. The upper *n*-hexane layer was obtained and the lower aqueous layer was re-extracted three times with fresh *n*-hexane. All fractions of *n*-hexane were pooled in a flask and dried at 45°C under vacuum pressure through rotary evaporator. The semisolid *n*-hexane fraction was dried as described earlier. Similar procedures were adopted for ethyl acetate and butanol fraction.

Preparation of media

Nutrient broth was used for shaking incubation and standardization and nutrient agar medium for the culturing and growth of all microorganisms. The required quantities of nutrient agar were poured into conical flasks and test tubes and sterilized. The nutrient agar medium was poured aseptically into sterilized petri plates in a Laminar flow hood and allowed to solidify for about an hour. After 24 hours, uncontaminated plates were used for further culturing of bacteria and fungi. The nutrient broth in flasks (approx. 20 mL/flask) was utilized for shaking incubation of microorganisms while nutrient broth in test tubes was used for standardization of microbial cultures.

Disc diffusion susceptibility assay

The antibacterial activity of different solvent extracted samples of *P. hydaspidis* was carried by disc diffusion assay as described in Bauer et al. (1996) and antifungal activity by Ramdas et al. (1998) against different bacterial and fungal strains (Table I). Nutrient agar media plates were inoculated with 18-24 hours cultures of microbial inoculums (a standardized inoculums 1-2 × 10^7 CFU mL^{-1} 0.5 McFarland Standard). Three discs of Whatman No. 1 filter paper (6 mm in diameter) were placed with the help of a sterile forceps on the media in petri plates. Plant extracts extracted in different solvents in concentration of 800 ppm in 12 µL volume were applied onto the discs. Antibiotics (6 µL per disc) as positive control and DMSO (6 µL $disc^{-1}$) as negative control were also applied on the discs in separate petri plates. Inoculated plates were kept at 37°C for 18-24 hours. The next day zones of inhibition were recorded in mm around the discs in each plate. The same procedure was repeated for 900 ppm and 1000 ppm concentrations of crude extracts and their fractions.

Positive controls

The following protocol was followed for positive control: a) Gram-positive bacteria: Ciprofloxacin 50 µg per 6 µL; b) Gram-negative bacteria: Ciprofloxacin 50 µg per 6 µL; c) Fungal strains: Fluconazole 50 µg per 6 µL.

DPPH radical scavenging activity

DPPH radical scavenging activity of different solvent extracted samples was determined according to the

Table I

Bacterial strains used during the experiment

Microbial species	Gram	Details of the microbial strains used
Bacteria		
Klebsiella pneumoniae	Negative	Clinical isolate obtained from the Department of Microbiology, Quaid-I-Azam University Islamabad, Pakistan
Pseudomonas aeruginosa	Negative	ATCC # 9721
Staphylococcus aureus	Positive	ATCC # 6538
Bacillus subtilis	Positive	Clinical isolate obtained from the Department of Microbiology, Quaid-I-Azam University Islamabad, Pakistan
Escherichia coli	Negative	ATCC # 25922
Xanthomonas campestris	Negative	ATCC # 33913
Citrobacter freundii	Negative	ATCC # 8090
Fungus		
Candida albicans		ATCC # 10231. Plant Pathology Department, The University of Agriculture Peshawar KPK Pakistan
Trichoderma reesei		ATCC # 26921. Plant Pathology Department, The University of Agriculture Peshawar KPK Pakistan
Acremonium alternatum		ATCC # 60645. Plant Pathology Department, The University of Agriculture Peshawar KPK Pakistan
Penicillium hrysogenum		ATCC # 11709. Plant Pathology Department, The University of Agriculture Peshawar KPK Pakistan
Rhizopus oryzae		ATCC # 20344. Plant Pathology Department, The University of Agriculture Peshawar KPK Pakistan

method described by Mensor et al. (2001). The stock solutions of the samples were diluted to final concentrations of 250, 125, 50, 25, 10 and 5 µg/mL in methanol. 1 mL of a 0.3 mM DPPH methanol solution was added to 2.5 mL solution of the extract and was allowed to react at room temperature for 30 min under complete dark. The absorbance of the resulting mixture after the reaction was taken at 518 nm using UV visible spectrophotometer. The readings were converted to percentage antioxidant activity (% AA) following the methods of Subathraa and Poonguzhali (2012).

$Q= 100(A_o-A_s)/A_o$

Where; Q = % antioxidant activity, A_o =Absorbance of pure DPPH and A_s = Absorbance of the sample.

Statistical analysis

Data are presented as mean values of three replications. MSTATC computer software was used for statistical analysis (Russel and Eisensmith, 1983). Least significant difference (LSD) test was employed to compare significant difference among means (Steel et al., 1997).

Results

Figure 1 represents the antimicrobial activity of samples extracted from the stem of *P. hydaspidis*. The data indicated that *Klebsiella pneumoniae* was more susceptible to crude, *n*-hexane, ethyl acetate and aqueous extracts at all concentrations (800, 900 and 1000 ppm disc^{-1}) and was also sensitive to butanol fraction at highest concentration (1000 ppm disc^{-1}) only. Significant reduction in the growth of *Klebsiella pneumoniae* was observed due to the exposure of the microbe to all extracted samples except butanol fraction. Among these samples, aqueous extracted fraction showed the maximum zone of inhibition (85.7% at 1000 ppm disc^{-1}, 65.7% at 900 ppm disc^{-1} and 50.7% at 800 ppm disc^{-1}) as compared to other fractions. Crude methonolic extract, *n*-hexane and ethyl acetate inhibited the growth of bacteria at all the three concentrations used, while the butanol fraction inhibited the growth of *K. pneumoniae* at 1000 ppm only. *Pseudomonas aeruginosa* was sensitive to crude methanolic and aqueous fractions at all three concentrations used, however, *n*-hexane, ethyl acetate and butanol fractions showed no inhibitory effect on *P. aeruginosa* measuring no zone of inhibition. Among the tested samples, aqueous extracted fraction showed highest maximum zone of inhibition i.e. 54.5% at 1000 ppm disc^{-1}. Crude methanolic extract at 800 ppm disc^{-1} concentration measured 31.8%. The data also revealed that *Staphylococcus aureus* was sensitive to crude methanol, ethyl acetate and butanol fractions showing highest maximum zone of inhibition with ethyl acetate at 1000 ppm disc^{-1} concentration followed by crude methanolic extract (35.7%) and butanol fractions (33.9%) at the same concentration. Our results further suggested that *S. aureus* was completely resistant to n-hexane and water extracted fractions showing 0% zone of inhibition.

The data revealed that *Bacillus subtilis* was completely resistant to *n*-hexane, ethyl acetate and butanol fractions showing no zone of inhibition. However, the same bacterium was sensitive to crude methanolic extract and water fractions. Highest zone of inhibition was given by aqueous fraction at 1000 ppm disc^{-1} concentration (55% ZI) followed by 900 ppm disc^{-1} (48.8% ZI) and 800 ppm disc^{-1} (25% ZI) of the same fraction. Lowest ZI was noted for crude methanolic extract at 800 ppm disc^{-1} concentration (9.6% ZI). *Escherichia coli* were sensitive to all concentrations of different extracts. Maximum ZI was shown crude methanolic extract at 1000 ppm disc^{-1} concentration (70.3% ZI) while lowest ZI was noted for ethyl acetate fraction (25.2% ZI). Crude, *n*-hexane and butanol fractions did not inhibit the growth of *Xanthomonas campestris* whereas ethyl acetate and water extracted fractions reduced its growth. Maximum ZI was revealed by ethyl acetate at 1000 ppm disc^{-1} concentration and minimum by aqueous fraction at 800 ppm disc^{-1} concentration. The data also indicated that only butanol fraction was effective to inhibit the growth of *Citrobacter freundii* at all concentrations used (37.7%, 30.4% and 23.2% ZI at 1000 ppm disc^{-1}, 900 ppm disc^{-1} and 800 ppm disc^{-1} respectively). *C. freundii* was completely resistant to crude methanolic, *n*-hexane, ethyl acetate and water extracted fractions. *C. albicans* was sensitive to crude methanolic extract, *n*-hexane and aqueous extracted samples and completely resistant to ethyl acetate and butanol fractions. The data revealed that highest ZI was shown by crude methanolic extract at 1000 ppm disc^{-1} (57.6% ZI) followed by aqueous extracted sample (54.5% ZI) at the same concentration. Our results also indicated that all other strains used during the present study were completely resistant to different extracts at all concentrations.

DPPH free radical scavenging assay was carried out for the determination of antioxidant activity of with five different solvents extracted from the stem tissues. The data on the antioxidant activity of the stem crude methanolic, *n*-hexane, ethyl acetate, butanol and water extracted samples at different concentration (5, 10, 25, 50, 100, 125 and 250 µg/mL) is demonstrated in Figure 2. The data confirmed that all the extracted samples exhibited antioxidant activity at both the lowest and highest concentration when calculated in comparison to positive control (gallic acid). The data revealed that highest antioxidant potential (96.3%) was demonstrated by crude methanolic extracted sample at 250 µg/mL followed by 95.6% of the same extract at 125 µg/mL and 90.9% by butanol extracted sample at 250 µg/ml. The data further indicated the lowest antioxidant

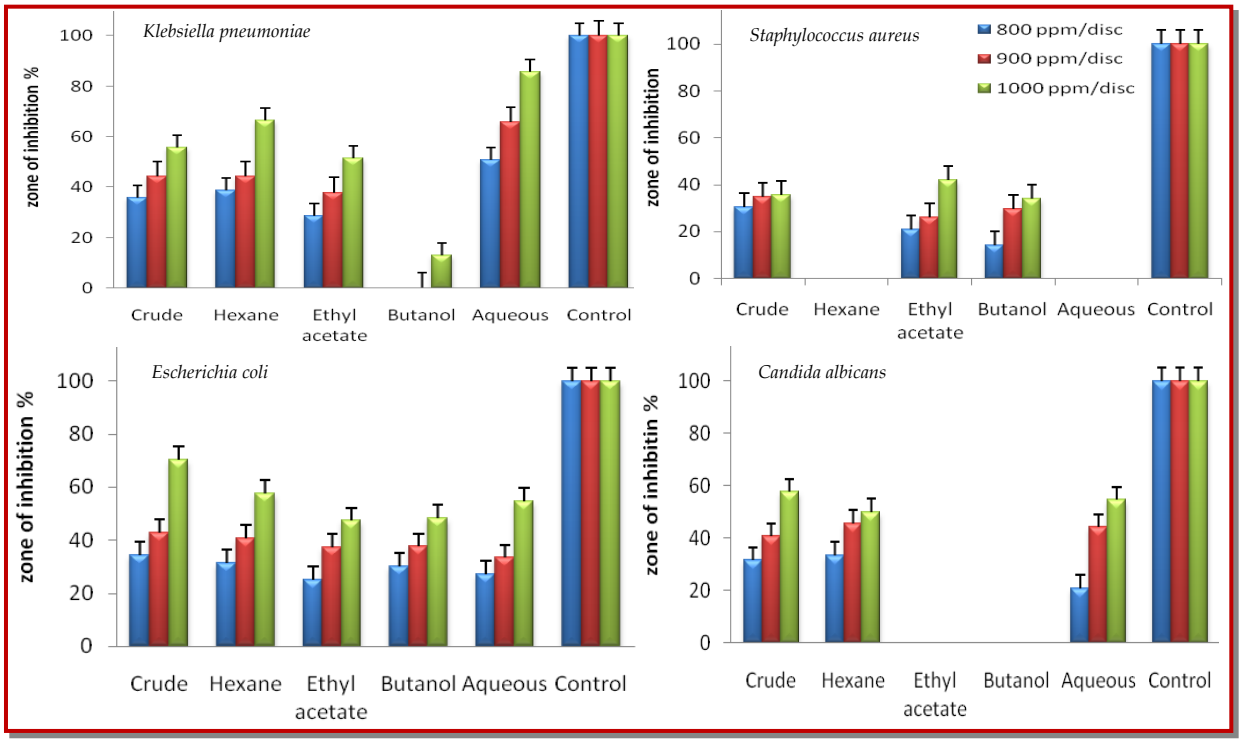

Figure 1: Antimicrobial activity of crude methanol, n-hexane, ethyl acetate, butanol and water extracted samples from the stems of *Periploca hydaspidis* by disc diffusion assay (Bar shows LSD at $p<0.05$)

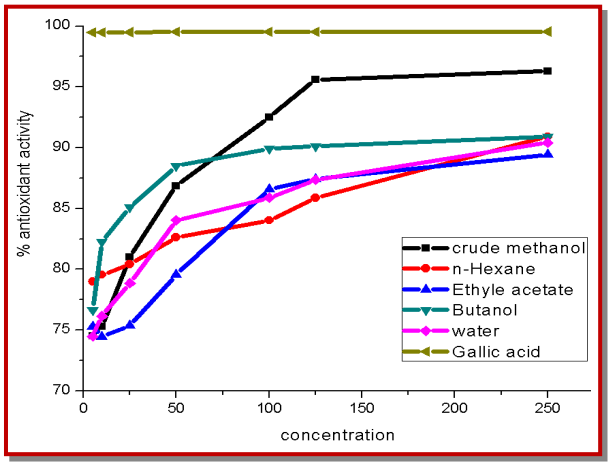

Figure 2: Antioxidant potential (%) of different solvent extracted samples from the stems of *Periploca hydaspidis* extracted with different solvents

activity (74.5%) was noted for water extracted sample at 5 µg/mL concentration. The order of antiradical scavenging potential of different extracted samples of the stem was crude methanolic extract>butanol> ethylacetate> water>*n*-hexane at different concentrations. The results suggested that radical scavenging activity of different extracts decreased with the decrease in its concentration. The data indicated that all the extracts of the root showed free radical scavenging activity at both minimum and maximum concentrations compared with the positive control (gallic acid). The results showed that maximum antioxidant potential of 97.5% was measured by crude methanolic extracts at 250 µg/mL followed by 97.2% and 96.1% by the same extract at 125 and 100 µg/mL respectively. The data also suggested that minimum antioxidant activity (66.5%) was noted by butanol extracted sample at 5 µg/mL. The order of the antiradical scavenging potential of the different extracted samples was crude methanolic extract>*n*-hexane>ethyle acetate>water>butanol extracted samples at different concentrations. Free radical scavenging activity of different extracts decreased with the decrease in its concentration.

Discussion

The current research investigates the antimicrobial activities of different solvent extracted samples from the stem of *P. hydaspidis* using disc diffusion assay. Among twelve different microbial species used, seven were bacteria (Gram positive and Gram negative) and five were fungi. Our results revealed that *K. pneumoniae* was susceptible to crude methanolic extract, *n*-hexane, ethyl acetate and aqueous extracts at all concentrations showing highest zone of inhibition when compared with other fractions. These results are in agreement with Chathradhyunthi et al. (2009) and Rauf et al. (2012). *P. aeruginosa* was sensitive to crude methanolic and water extracts at all the three concentrations used, where *n*-hexane, ethyl acetate and butanol fractions did

not inhibit the growth of *P. aeruginosa*. Among different fractions, aqueous was more effective to control the growth of *P. aeruginosa* at highest concentration. Our results agree with Rauf et al. (2012). The data also suggested that *S. aureus* was sensitive to crude methanolic extract, ethyl acetate and butanol fraction inhibiting the activity of *S. aureus*. *S. aureus* on the other hand was resistant to n-hexane and water extracted fractions showing no ZI. Our results are in agreement with Sivasankaridevi et al. (2013).

The data also revealed that *Bacillus subtilis* was completely resistant to *n*-hexane, ethyl acetate and butanol fractions showing no effect on its activity. Maximum growth inhibition was noted by aqueous fraction at highest concentration. Our results are agreement with Hughes and Lawson (1991), Bekenblia (2004) and Santas et al. (2010). Our results also indicated that *E. coli* were sensitive to all extracts of the plant at all concentrations used. The highest ZI was measured by crude extract when applied at highest concentrations. These results agree with Dimayuga and Garcia (1991) and Udayasankar et al. (2012). The results demonstrated crude methanolic extract, *n*-hexane and butanol fractions did not reduce the growth of *Xanthomonas campestris*, however, ethyl acetate and water extracted fractions inhibit its growth. Highest reduction was noted for ethyl acetate at 1000 ppm $disc^{-1}$ concentration. Similar results are also reported by Dimayuga and Garcia (1991) and Samy and Ignacimuthu (2000). The data also suggested that butanol was more effective against *C. freundii* at all concentrations used However, *C. freundii* was completely resistant to crude methanolic extract, n-hexane, ethyl acetate and water extracted fractions. Similar results were shown by Chathradhyunthi et al. (2009), Dimayuga and Garcia (1991), Rauf et al. (2012), and Sivasankaridevi et al. (2013).

Our results suggested that growth of *C. albicans* was reduced by crude methanolic extract, *n*-hexane and aqueous extracted samples. Ethyl acetate and butanol fractions showed no activity against *C. albicans*. The data indicated that maximum inhibition was measured by crude methanolic extract at highest concentration. The other fungal strains tested during the present study were completely resistant to different extracts at all concentrations showing no zone of inhibition. The results agree with Bergeron et al. (1996), and Jones et al. (2000), Prince and Prabakaran (2011), Bakht et al., (2013 a,b).

Compounds having the ability to scavenge free radicals are produced by the natural machinery of the plants. These compounds include phenolic acids, lignins, tannins, alkaloids, terpenoids, flavonoids, stilbenes, coumarins, amines and vitamins etc. (Cait et al., 2003). Some of these compounds have anti-inflammatory, antimutagenic, antibacterial, antiviral, antiatherosclerotic, antitumor and anticarcinogenic capabilities (Sala et al., 2002). Ingestion of natural antioxidants through diet or through medicine prepared from plants can reduce the risk of happening of cancer, heart disease, diabetes, and other complications connected with the presence of free radicals (Veerapur et al., 2009). Besides, the projected life of food and food goods can be improved by accretion of antioxidants (Cook and Samman, 1996).

DPPH free radical scavenging assay was carried out for the determination of antioxidant activity of different solvent extracted samples at different concentration from the stem tissues. Interestingly we found that the subject plant in our current study possesses high antioxidant potential. The data indicated all the samples of the stem extracted with different solvents exhibited antioxidant activity at both the low and high concentration when calculated in comparison to the positive control. The highest antioxidant potential was noted in crude methanolic extracts at 250 µg/mL followed by the same extract at 125 µg/mL and butanol extracted samples at 250 µg/mL. These results suggested that compounds which having radical scavenging potential were accumulated at high level in these two extracts (Jamshed et al., 2012). The lowest antioxidant activity was demonstrated by water extracted sample at 5 µg/mL. The overall order of radical scavenging potential of the different extracted samples of the stem was crude methanol>butanol> ethyl acetate>water>*n*-hexane extracted sample at different levels of concentration (Hajji et al., 2009). Closer examination of our data further revealed that radical scavenging activity of the extracts decreased with the decrease in concentration of the extracts indicating its dose dependent pattern. Our data for the roots samples revealed that maximum antioxidant potential was measured by crude methanolic extracts at 250 µg/mL followed by the same extract at 125 µg/mL. Similarly, minimum antioxidant activity was established by butanol extracted sample at 5 µg/mL. The order the antiradical scavenging potential of the different extracted samples of the stem was crude methanol>*n*-hexane>ethyl acetate>water>butanol extracted samples at different levels of concentration (Hajji et al., 2010). The data further revealed that the antiradical scavenging activity of the extracts decreased with the decrease in concentration of the extracts. Our data showed that all the extracts of the roots showed antioxidant activity at lowest concentration of 5 µg/mL (Jamshed et al., 2012).

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Conflict of Interest

Authors declare no conflict of interest

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Author Info
Jehan Bakht (Principal contact)
e-mail: jehanbakht@yahoo.co.uk