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Phytochemical Screening, *In vitro* Antioxidant and Antimicrobial Activities of the Crude Fractions of *Paeonia emodi* Wall. Ex Royle

[1]*Ghias Uddin,* [1]*Anwar Sadat and* [2]*Bina Shaheen Siddiqui*

[1]Phytopharmaceutical and Nutraceutical Research Laboratory, Institute of Chemical Sciences, University of Peshawar, Peshawar, KPK, Pakistan
[2]HEJ Research Institute of Chemistry, International Center of Chemical and Biological Sciences (IBCCS), University of Karachi, Karachi, Pakistan

Abstract: Accumulation of free radicals in body is harmful and can give rise to may stress related ailments. In the recent years, extensive research has been going on to find natural antioxidants from the plant sources. The ethanolic extract of the *P. emodi* was fractionated into *n*-hexane, dichloromethane, ethyl acetate and aqueous fraction was left behind which were then subjected to phytochemical screening. All the fractions were also assayed for their antioxidant potential using DPPH, hydrogen peroxide, superoxide anions, NO and ABTS. The aqueous and the EtOAc fractions were the most potent antioxidant showing the presence of various secondary plant metabolites *viz.,* flavonoids, steroids, tannins, saponins and alkaloids with remarkable scavenging activity against different active oxygen species such as DPPH, nitric oxide, hydrogen peroxide, ABTS and superoxide when compared to the standard butylated hydroxyl toluene (BTH). Antibacterial and antifungal activities of the crude fractions were also evaluated against selected strains of bacteria and fungus which showed significant antimicrobial activities.

Key words: Antioxidants · *Paeonia emodi* · Secondary metabolites · Total phenolic contents · Total flavonoids

INTRODUCTION

The human biochemistry is very diverse, a number of biochemical reactions have been taking place. One of intermediate product often formed is the active oxygen species that can give rise to oxidative damage to DNA, proteins and lipids, which can cause diseases like diabetes, cancer, ageing and many other degenerative conditions. Many secondary metabolites isolated from the plants possess remarkable antioxidant potentials such as flavonoids, tannins, terpenoids, vitamins, lignins and alkaloids etc. These compounds also proved to have anti-inflammatory, antibacterial, antifungal, antitumor, antimutagenic and antiviral activities. Intake of food rich in these antioxidants reduced the risk of cancer, diabetes and boost up the immune systems [1-4].

Paeonia is a genus of family Paeoneacae comprises of 40 species, which are mainly distributed in the North Temperate Zone especially in the Mediterranean region and Asia. Only one specie namely *Paeonia emodi* occurs in Pakistan. It grows well in the high altitude cool climate areas; flourish well in a deep, loamy and moist soil. In delicacy of the tincture and fragrance, they resemble to the rose. *P. emodi* is an erect leafy perennial herbs, 50 cm tall and glabrous. Leaves are biternate or ternate with lamina pale. Flowers are solitary, axillary and of red color. Bracts are leafy, petals are 8 and seeds vary from 3-5 [5].

P. emodi tubers are useful for the treatment of convulsions, hysteria, uterine diseases, colic and bile duct obstructions. It is given to children as a blood purifier, while seeds are cathartic and emetic. A dried flower infusion is useful in the treatment of diarrhea. Petals of various peony species are dried and crushed and tea made of it has been used as a cough remedy and as a treatment for hemorrhoids and varicose veins. *P. emodi* is a well known plant used medicinally to treat hypertension, palpitations and asthma. The literature survey revealed that *P. emodi* have shown significant biological activities

Corresponding Author: Dr. Ghias Uddin, Phytopharmaceutical and Nutraceutical Research Laboratory, Institute of Chemical Sciences, University of Peshawar, Peshawar, KPK, Pakistan.

such as lipoxygenase inhibiting activity, beta-glucuronidase inhibiting activity, cardiovascular and airway relaxant activities by peony roots extract, spasmolytic and spasmogenic activities, anticonvulsant, antianxiety and memory enhancing activities. Keeping such importance in view the present study is therefore investigated on the phytochemical composition, antioxidant potential, antifungal and antibacterial potential of *P. emodi* [6-8].

MARERIALS AND METHODS

Plant Materials: *P. emodi* plant was collected in July 2009 from Hazara Division of Khyber Pakhtunkhwa, Pakistan and identified by Taxonomist Mr. Abdul Majid, Department of Botany, Hazara University where the voucher specimen was preserved at the herbarium. The whole plant was shade dried for about 27 days at room temperature and grained into fine pieces (12.5 kg).

Extraction: The plant material (12.5 kg) was extracted thrice with ethanol of commercial grade (20 liters) at room temperature in a steel tank. The combined extract was concentrated in the rotary evaporator at 40°C under vacuum to get crude extract (600 g), which was subjected to solvent-solvent separation with *n*-hexane, dichloromethane and ethyl acetate while aqueous fraction was left behind.

Chemicals: Ethanol used for the crude extraction was of commercial grade (95%) purchased from local supplier. Dragendroff's reagent, methanol, gallic acid, commercial saponins, ferric chloride, hydrochloric acid, magnesium metal strips, were purchased from BDH, England and blood agar from Biolab, South Arica. Chloroform, H_2SO_4, Folin-Ciocalteu reagent, Na_2CO_3, vanillin, aluminium chloride, potassium acetate, phosphate buffer, $K_3Fe(CN)_6$, trichloroacetic acid (TCA), 2-thiobarbituric acid (TBA), thiocyanate (FTC), butylated hydroxyl toluene (BHT), 2, 2-diphenyl-1-picrylhydrazyl (DPPH), 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid (ABTS), potassium persulphate, sodium nitroprusside, hydrogen peroxide, sulfanilic acid, glacial acetic acid, naphthylethylenediamine dichloride, potassium metabisulphite (PMS), NADH were purchased from Merck, USA.

Phytochemical Screening: A small portion of each fraction was used for the phytochemical tests to identify secondary metabolites such as tannins, flavonoids, alkaloids, saponins and steroids in accordance with the methods described below [9-12].

Tannins: 1 g of each fraction was dissolved in 10 ml of distilled water and filtered. A blue coloration resulting from the addition of ferric chloride reagent to the filtrate indicated the presence of tannins in the extract.

Alkaloids: 0.5 g of the each fraction was dissolved in 5 ml of HCl (1%) on steam bath. A milliliter of the filtrate was treated with few drops of Dragendroff's reagent. Turbidity or precipitation was taken as indicative of the presence of alkaloid.

Flavonoids: 0.2 g of each fraction was dissolved in 2 ml of methanol and heated for a while. A chip of magnesium metal was added to the mixture followed by the addition of a few drops of concentrated HCl. The occurrence of a red or orange coloration was indicative of the flavonoids.

Saponins: Freshly prepared 7% blood agar plate was used and wells were made in it. The crude extract dissolved in 10% methanol, which was used to fill the wells bored in the blood agar plates. 10% methanol was used as a negative control while commercial saponins solution was used as a positive control. The plates were incubated at 35°C for 6 hours. Complete haemolysis of the blood around the extract was evidentiary of presence of saponins.

Steroids: 0.5 g of each fraction was dissolved in 3 ml of chloroform and filtered. Few drops of concentrated H_2SO_4 were carefully added to the filtrate to form lower layer. A reddish brown color at the interface was considered as positive test for steroid ring.

Total Phenolic Contents: The amount of phenol in each extract of *P. emodi* was determined with Folin-Ciocalteu reagent using the method of Spanos *et al*. [12] as modified by the Crop Research Institute Report [13]. 2.5 ml of 10% Folin-Ciocalteu reagent and 2 ml of Na_2CO_3 (2% w/v) was added to 0.5 ml of each sample (3 replicates) of the plant extract solution (1 mg/ml). The resulting mixture was incubated at 45°C with shaking for 15 min. The absorbance of the samples was measured at 765 nm using UV/visible light. Results were expressed in milligrams of gallic acid (0-0.5mg/ml) dissolved in distilled water.

Quantitative Determination of Flavonoids: Aluminum chloride colorimetric method was used for flavonoids determination. 1 ml of each sample (1 mg/ml) was mixed with 3 ml of methanol, 0.2 ml of 10% aluminum chloride, 0.2 ml of 1 M potassium acetate and 5.6 ml of distilled water and remained at room temperature for 30 min. The absorbance of the reaction mixture was measured at 420 nm with UV spectrophotometer (Shimadzu UV-Vis 1700). The content was determined from extrapolation of calibration curve, which was made by preparing gallic acid solution (0-0.8 mg/ml) in distilled water. The concentration of flavonoids was expressed in terms of mg/ml.

Quantitative Determination of Proanthocyanidin: Total proanthocyanidin was determined based on the procedure of Sun *et al*. [14]. About 3 ml of vanillin-methanol (4% v/v) and 1.5 ml of HCl was added to 0.5 ml (1 mg/ml) of aqueous extract and vortexed. The resulting mixture was allowed to stand for 15 min at room temperature followed by the measurement of the absorbance at 500 nm. Total proanthocyanidin content was expressed as gallic acid equivalent (mg/ml) from the standard curve.

DPPH Antioxidant Assay: The method of Liyana-Pathiana and Shahidi [15] was used for the determination of scavenging activity of DPPH free radical. 1 ml of 0.135 mM DPPH prepared in methanol was mixed with 1 ml of aqueous extract ranging from 0.2-0.8 mg/ml. The reaction mixture was vortexed thoroughly and left in dark at room temperature for 30 min. The absorbance was measured at 517 nm. The scavenging ability of the plant extract was calculated using the following equation;

$$\text{DPPH Scavenging activity (\%)} = \frac{[(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})]}{\text{Abs}_{\text{control}}} \times 100$$

where $\text{Abs}_{\text{control}}$ is the absorbance of DPPH + methanol, $\text{Abs}_{\text{sample}}$ is the absorbance of DPPH radical + sample i.e. plant extract.

2, 2'-Azino-bis (3-ethylbenzthiazoline-6-sulphonic acid (ABTS) Scavenging Activity: The method of Re *et al*. [16] was adopted for the determination of ABTS activity of the plant extract. The working solution was prepared by mixing two stock solutions of 7 mM $ABTS^+$ solution and 2.4 mM potassium persulphate solution in equal amount and allowed to react for 12 hours at room temperature in dark. The resulting solution was later diluted by mixing 1 ml of freshly prepared $ABTS^+$ solution followed by the measurement of absorbance at 734 nm. The percentage of scavenging inhibition capacity of $ABTS^+$ of the extract was calculated and compared with Butylated hydroxyl toluene (BHT). The percent of scavenging inhibition capacity of $ABTS^+$ of the extract was calculated from the following equation;

$$\% \text{ inhibition} = \frac{[(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})]}{\text{Abs}_{\text{control}}} \times 100$$

Nitric Oxide Scavenging Activity: The method of Garratt *et al.* [17] was adopted to determine the nitric oxide radical scavenging activity of different extracts of *P. emodi*. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generate nitric oxide, which interacts with oxygen to produce nitrite ions determined by the use of Griess reagents. 2 ml of 10 mM sodium nitroprusside dissolved in 0.5 ml phosphate buffer saline (pH 7.4) was mixed with 0.5 ml of plant extract at various concentrations (0.2-0.8 mg/ml). The mixture was incubated at 25°C. After 150 min, 0.5 ml of incubation solution was withdrawn and mixed with 0.5 ml of Griess reagent [(1.0 ml sulfanilic acid reagent (0.33% in 20% glacial acetic acid at room temperature for 5 min with 1 ml of naphthylethylenediamine dichloride (0.1% w/v)]. The mixture was incubated at room temperature for 30 min. The absorbance was measured at 540 nm. The amount of nitric oxide radical was calculated following the following equation.

$$\% \text{ inhibition of NO} = \frac{[A_0 - A_1]}{A_0} \times 100$$

where A_0 is the absorbance before reaction and A_1 is the absorbance after reaction has taken place.

Scavenging Activity of Superoxide Anion: Measurement of superoxide anion radicals scavenging activity of different fractions was based on the method described by Liu *et al*. [18]. Superoxide radicals are generated in PMS–NADH systems by oxidation of NADH and assayed by the reduction of NBT. In these experiments, the superoxide radicals were generated in 3 ml of Tris-HCl buffer (16 mM, pH 8.0) containing 1 ml of NBT (50 μM), 1 ml NADH (78 μM) and crude fraction (25–50 μg). The reaction was started by adding 1 ml of PMS solution (10 μM) to the mixture. The reaction mixture was incubated at 25°C for 5 min; the absorbance was read at 560 nm against blank samples using gallic acid as a control. Decreased absorbance of the reaction mixture indicated increasing superoxide anion scavenging activity. The percentage inhibition of superoxide anion generation was calculated using the following formula;

$$\% \text{ inhibition} = \frac{[A_0 - A_1]}{A_0} \times 100$$

where A_0 was the absorbance of the control (gallic acid) and A_1 was the absorbance in the presence of extract.

Hydrogen Peroxide Scavenging Activity: Scavenging activity of hydrogen peroxide by the plant extract was determined by the method of Ruch *et al*. [19]. Plant extract (4 ml) prepared in distilled water at various concentration was mixed with 0.6 ml of 4 mM H_2O_2 solution prepared in phosphate buffer (0.1 M pH 7.4) and incubated for 10 min. The absorbance of the solution was taken at 230 nm against blank solution containing the plant extract without H_2O_2. The percentage inhibition was calculated using the following formula;

$$\% \text{ inhibition} = \frac{[A_0 - A_1]}{A_0} \times 100$$

where A_0 was the absorbance of the control (gallic acid) and A_1 was the absorbance in the presence of extract

Antibacterial Activity: The antibacterial activity was evaluated by the agar well diffusion method [20, 21] in which one loop full of 24 hours old culture containing approximately 104-106 CFU was spread on the surface of Mueller-Hinton Agar plates. Wells were dug in the medium with the help of sterile metallic cork borer. Stock solutions of the test samples in the concentration of 3 mg/ml were prepared in dimethyl sulfoxide (DMSO) and 100 μl dilutions were added in their respective wells. The antibacterial activity of each extract was compared with standard drug imepinem (positive control) and DMSO (negative control). The antibacterial activity was determined by measuring zones of inhibition usually of each sample wells Antifungal Activities: The antifungal activity was determined by the Agar tube dilution Method [22]. The crude extract was dissolved in DMSO (24 mg/1ml). Sterile Sabouraud's dextrose gar medium (5 ml) was placed in a test tube and inoculated with the sample solution (400 μg/ml) kept in slanting position at room temperature for overnight. The fungal culture was then inoculated on the slant. The samples were incubated for 7 days at 29°C and growth inhibition was observed and percentage growth inhibition was calculated with reference to the negative control by applying the equation;

$$\%\text{Inhibition of fungal growth} = \frac{\text{100-linear growth in test (mm)}}{\text{Linear growth in control (mm)}} \text{X } 100$$

RESULT AND DISCUSSION

Phytochemical Screening: Various fractions of *P. emodi* were subjected to phytochemical analysis, which revealed the presence of various plant secondary metabolites like steroids, flavonoids, alkaloids, saponins and tannins. Results are summarized in the Table 1. These results showed that *P. emodi* contains important classes of compounds i.e. flavonoids, proanthocyanidin, steroids, alkaloids and saponins. Total phenolic contents of the aqueous extract was found to be 0.385 mg gallic acid equivalent/g of the extract power and ethyl acetate fraction showed total phenolic contents 0.421 mg of gallic acid equivalent/g of the extract power while dichloromethane and *n*-hexane fraction showed no significant phenolic contents. The total flavonoids contents were estimated to be 0.680 mg of gallic acid equivalent/g for aqueous extract, 0.589 mg gallic acid equivalent/g for ethyl acetate extract and 0.314 mg gallic acid equivalent/g of dichloromethane extract. In the same way proanthocyanidin contents was found to be 0.004 mg gallic acid equivalent/g for aqueous extract and 0.005 mg gallic acid equivalent/g for ethyl acetate fraction (Table 2). These compounds are known to possess immense biological activities and thus responsible for various biological activities of the plant extracts.

Free Radical Scavenging Activities: The percentage scavenging activities of the various subtractions of the ethanolic plant extract for DPPH, ABTS, hydrogen peroxide, nitric oxide and superoxide anion radical were summarized in the tables. Both aqueous (Table 3) and ethyl acetate (Table 4) sub-fractions showed significant radial scavenging activities while dichloromethane (Table 5) and *n*-hexane (Table 6) fractions showed no significant scavenging activities. Table 1 indicated that *P. emodi* contains considerable quantity of flavonoids. Flavonoids are oxidized by radicals, resulting in a more stable, less-reactive radical. In other words, flavonoids stabilize the reactive oxygen species by reacting with the reactive compound of the radical. Because of the high reactivity of the hydroxyl group of the flavonoids, radicals are made inactive, according to the following equation [11].

$$\text{Flavonoid (OH)} + R^{\bullet} \rightarrow \text{Flavonoid (O}^{\bullet}\text{)} + RH$$

Antimicrobial Activities: Bioactivities are an essential part of drug discovery from the plant origin and screening of various fractions against different strains of bacteria

Table 1: Estimation of phytochemical components of various fractions of *P. emodi*

Fractions	Tannins	Flavonoids	Steroids	Alkaloids	Saponins
n-Hexane	-	-	+	-	-
Dichloromethane	-	+	+++	++	-
Ethyl acetate	+	+++	++	+	+
Aqueous	+++	+++	+	-	+++

+++ indicated appreciable amount (positive within 5 mins.), ++ indicated moderate amount (positive after 5 mins. but within 10 mins), + indicated trace amount (positive after 10 mins. but within 15 mins) and – indicated complete absence.

Table 2: Total phenol, flavonoids and proanthocyanidin contents

Fractions	Total phenol	Total flavonoids	Total proanthocyanidin
n-Hexane	-	-	-
Dichloromethane	0.043	0.314	0.002
Ethyl acetate	0.421	0.589	0.005
Aqueous	0.385	0.680	0.004

Table 3: Percent radical scavenging activities of aqueous fraction of *P. emodi* and BTH as a standard at different concentration

Extract/BTH (mg/ml)	Superoxide anion	Nitric oxide	DPPH	Hydrogen peroxide	ABTS
0.2	59.50 (60.50)	30.50 (40.50)	40.50 (42.50)	54.00 (68.00)	48.50 (51.50)
0.4	64.00 (72.50)	38.50 (45.50)	45.00 (53.00)	60.00 (73.50)	54.00 (62.50)
0.6	68.50 (76.50)	54.50 (62.00)	53.50 (74.00)	64.50 (76.50)	61.00 (77.00)
0.8	72.50 (79.50)	61.50 (80.50)	68.50 (82.00)	71.00 (80.00)	68.50 (78.00)

BTH values in parenthesis.

Table 4: Percent radical scavenging activities of ethyl acetate fraction of *P. emodi* and BTH as a standard at different concentration

Extract/BTH (mg/ml)	Superoxide anion	Nitric oxide	DPPH	Hydrogen peroxide	ABTS
0.2	40.50 (60.50)	23.50 (40.50)	43.50 (42.50)	55.00 (68.00)	43.00 (51.50)
0.4	46.00 (72.50)	29.50 (45.50)	48.00 (53.00)	63.00 (73.50)	48.50 (62.50)
0.6	51.50 (76.50)	36.50 (62.00)	52.50 (74.00)	69.50 (76.50)	56.00 (77.00)
0.8	58.00 (79.50)	42.50 (80.50)	68.50 (82.00)	73.00 (80.00)	61.00 (78.00)

BTH values in parenthesis.

Table 5: Percent radical scavenging activities of Dichloromethane fraction of *P. emodi* and BTH as a standard at different concentration

Extract/BTH (mg/ml)	Superoxide anion	Nitric oxide	DPPH	Hydrogen peroxide	ABTS
0.2	19.50 (60.50)	8.50 (40.50)	12.50 (42.50)	14.00 (68.00)	07.00 (51.50)
0.4	23.00 (72.50)	12.00 (45.50)	14.00 (53.00)	16.50 (73.50)	11.50 (62.50)
0.6	27.00 (76.50)	15.20 (62.00)	18.50 (74.00)	19.00 (76.50)	14.00 (77.00)
0.8	30.00 (79.50)	18.50 (80.50)	21.50 (82.00)	20.50 (80.00)	16.00 (78.00)

BTH values in parenthesis

Table 6: Percent radical scavenging activities of *n*-hexane sub-fractions of *P. emodi* and BTH as a standard at different concentration

Extract/BTH (mg/ml)	Superoxide anion	Nitric oxide	DPPH	Hydrogen peroxide	ABTS
0.2	00.00 (60.50)	00.00 (40.50)	00.00 (42.50)	00.00 (68.00)	00.00 (51.50)
0.4	00.00 (72.50)	00.00(45.50)	00.00 (53.00)	00.00 (73.50)	00.00 (62.50)
0.6	05.00 (76.50)	04.00 (62.00)	07.00(74.00)	03.00 (76.50)	00.00 (77.00)
0.8	06.00 (79.50)	05.50 (80.50)	10.00 (82.00)	04.50 (80.00)	00.00 (78.00)

BTH values in parenthesis

and fungi is the first step. Gram *+ve* and gram *–ve* strains were used to evaluate the antibacterial activities of the various fractions and the results are summarized in the Table 6. The result revealed that ethyl acetate fraction is the most active showing moderate to significant activity against various strain of the selected bacteria. The ethyl acetate fraction showed significant activity against *Bacillus subtilis* with zone of inhibition of 27 mm as compare to the standard with zone of inhibition of 35 mm.

The antifungal activities were summarized in Table 7 showing some moderate antifungal activities. The ethyl acetate fraction showed moderate activity, the EtOAc

Table 7: Antibacterial activities of various fractions of *P. emodi*

Bacterial species	Zone inhibition of Std. drug (mm)	Zone inhibition (mm)			
		n-Hexane	Chloroform	Ethyl acetate	Aqueous
Escherichia coli	35	-	14	24	18
Bacillus subtilis	36	-	10	27	16
Shigella flexaneri	36	-	7	23	15
Staphylococcus aureus	43	-	4	9	6
Pseudomonas Aeruginosa	32	-	8	22	12
Salmonella typhi	40	-	10	16	11

Table 8: Antifungal activities of various fractions of *P. emodi*

Fungal species	Std. Drug Mic μg/ml	% inhibition			
		n-Hexane	Chloroform	Ethyl acetate	Aqueous
Trichophyton longifusis	Miconazole 70	-	-	16	-
Candida albicans	Miconazole 110.8	-	18	31	22
Aspergilus flavus	Amphotericin 20	-	-	28	15
Microsporum canis	Miconazole 98.4	-	-	-	-
Fusarium solani	Miconazole 73	-	-	35	16
Candida glaberata	Miconazole 110.8	-	-	21	-

fraction showed highest activity against *Fusarium solani* with zone of inhibition of 35 mm indicating that there are some compounds present especially in the ethyl acetate fraction, which possess good antibacterial and antifungal activities that may contribute to the successful application of such natural compounds for the treatment of infection disorder caused by fungus and bacteria.

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