ISSN 1330-9862
(FTB-2366)

original scientific paper

Comparative Antioxidant and Antimicrobial Activities of Phenolic Compounds Extracted from Five *Hypericum* Species

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Received: October 25, 2009
Accepted: April 6, 2010

Summary

Phenolic compounds were extracted from five *Hypericum* species (*H. perforatum*, *H. oblongifolium*, *H. monogynum*, *H. choisianum* and *H. dyeri* Redher) using ethanol. The crude extract (called fraction 1) was then fractionated using re-extraction to water (fraction 2), ethyl acetate (fraction 3), and acetone (fraction 4). The final residue was marked as fraction 5. The content of total phenolics in the fractions ranged from 21 mg of gallic acid equivalents per g (fraction 5 of *H. dyeri*) to 100 mg of gallic acid equivalents per g (fraction 5 of *H. choisianum*). Phenolic compounds present in the fractions showed antioxidant and antiradical properties investigated using DPPH radical scavenging activity, molybdate method, and reducing power. The strongest antiradical properties were noted for fraction 3 of *H. choisianum* (EC_{50}=11.2 μg/mL), whereas the weakest was for fraction 5 of *H. dyeri* (EC_{50}= 139.2 μg/mL). Fractions 1 and 5 of *H. dyeri* showed good antibacterial activity against *Escherichia coli*, while fractions 3–5 of *H. perforatum* were active against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Fractions 1–4 of *H. perforatum* were found most active against *Helminthosporium maydis* as determined by antifungal screening.

Key words: *Hypericum monogynum*, *H. oblongifolium*, *H. perforatum*, *H. choisianum*, *H. dyeri*, phenolic compounds, antioxidant activity, antimicrobial activities

Introduction

Hypericum (Guttiferae, syn. Clusiaceae) is a large genus of herbs or shrubs which grows widely in temperate regions. There are about 400 species of *Hypericum* genus and they are used as traditional medicinal plants in various parts of the world (*1*). Most of these species have been used for a long time for the treatment of external wounds and gastric ulcers, and also as sedative, antiseptic, and antispasmodic herbs in folk medicine (*1*).

In Pakistan the genus *Hypericum* is represented by nine species. Several studies have been published concerning *Hypericum perforatum* L., known as St. John's wort. This species has been reported to have antidepressant, anxiolytic, antiviral, wound-healing and antimicrobial activities (*1*).

Hypericum species have shown good antioxidant activity *in vitro* (*2*). Several studies have revealed antimicrobial activity of various extracts of *H. perforatum* (*3,4*). The components of *Hypericum perforatum* chiefly respons-

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ible for the antidepressant activity, hypericin and hyperforin, have been shown to increase serotonin levels. It has been reported that serotonin possesses a protective effect against oxidative damage in neuronal cells (*5*). An extract of *Hypericum perforatum* shoot exhibited an antilipid peroxidation property (*6*) and superoxide radical scavenging activity (*7*). *Hypericum hyssopifolium* exhibited significant DPPH radical scavenging activity (*1*) and free radical scavenging properties in cell-free and human vascular systems (*7*). Antioxidant activities of *Hypericum perforatum*, *H. androsaemum*, *H. triquetrifolium* Turra and *H. hyssopifolium* have also been evaluated (*8–10*). The antioxidant activities of methanol extracts of nine *Hypericum* species from the Balkans have been reported by Radulović *et al*. (*11*). Other species of the genus *Hypericum* also exhibited antimicrobial properties (*12–16*).

The aim of the present study is to evaluate comparative antioxidant and antimicrobial potential *in vitro* of five *Hypericum* species; *i.e. H. monogynum, H. oblongifolium, H. perforatum, H. choisianum* and *H. dyeri*.

Materials and Methods

Chemicals

All chemicals used were of analytical grade. Gallic acid was purchased from Acros (Geel, Belgium), 2,2'-diphenyl-1-picrylhydrazyl (DPPH) radical and trichloroacetic acid from Sigma-Aldrich (Buchs, Switzerland), sodium phosphate from Panreac (Castellar del Vallès, Barcelona, Spain), and ammonium molybdate from ABSCO Materials (Haverhill, Suffolk, UK). Folin–Ciocalteu phenol reagent, gallic acid, sodium carbonate, ascorbic acid, potassium ferricyanide, ferric chloride, sulphuric acid and other reagents were purchased from Merck (Darmstadt, Germany).

Plant material

Hypericum perforatum L., *H. oblongifolium* Wall, *H. monogynum* L., *H. choisianum* Wall and *H. dyeri* Redher were collected during the flowering period from different areas of Pakistan. All species were authenticated by Dr Habib Ahmad (Faculty of Science, Hazara University, Mansehra, Pakistan).

Test organisms for bioassays

Microorganisms used in the determination of antimicrobial activities of different plant extracts were as follows: Gram-positive bacteria *Staphylococcus aureus*; Gram-negative bacteria *Pseudomonas aeruginosa*, *Salmonella* Typhi, *Escherichia coli*, *Proteus vulgaris*, *Enterobacter aerogenes*; and fungal strains *Aspergillus niger, Aspergillus flavus, Alternaria solani* and *Helminthosporium maydis.* Pure bacterial and fungal cultures (clinical isolates) were obtained from the Centre of Biotechnology and Microbiology, University of Peshawar, Peshawar, Pakistan. Different bacterial and fugal strains were maintained on nutrient agar (NA; Oxoid, Basingstoke, Hampshire, UK) and Sabouraud dextrose agar (SDA; Oxoid), respectively. Bacterial cultures were prepared by transferring two to three colonies into a tube containing 20 mL of nutrient broth (Oxoid) and grown overnight at 37 °C, while fungal cultures were prepared in SDA. Fresh culture suspensions equivalent to a 0.5 McFarland standard (10^8 CFU/mL) were used for inoculation.

Preparation of extracts and fractions

The air-dried and powdered aerial parts of five species were extracted with ethanol using a Soxhlet apparatus. The respective extracts were filtered and dried under reduced pressure at a temperature below 50 °C. Various fractions were obtained (Fig. 1) by solvent extraction.

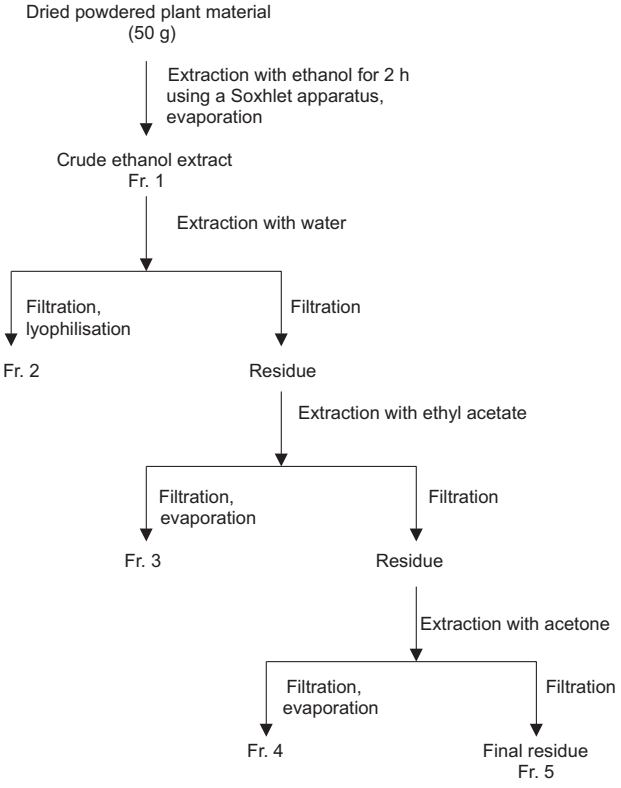

Fig. 1. Scheme of the extract fractionation

Total phenolic content

The content of total phenolic compounds in each fraction was estimated using the Folin-Ciocalteu phenol reagent (*17*). The results were reported as mg of gallic acid equivalents (GAE) per g of extract.

Antioxidant activity

The antioxidant activity of the fractions was evaluated by the method of Prieto *et al*. (*18*) with slight modifications. Briefly, an aliquot of 0.3 mL of sample solution in methanol was combined in an Eppendorf tube with 2.7 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The effective concentration of the sample was 50 μg/mL in the reaction mixture. For the blank, 0.3 mL of ethanol were mixed with 2.7 mL of the reagent. The tubes were capped and incubated in water bath at 95 °C for 90 min. After the samples had cooled to room temperature, the absorbance of the aqueous solutions was measured at

695 nm against a blank. The results were reported as μmol of ascorbic acid equivalents (AAE) per g of extract.

DPPH radical scavenging activity

DPPH radical scavenging activity of the phenolic fractions of *Hyperium* species was evaluated according to the method described by Blois (*19*) with slight modifications. Briefly, a 1-mM solution of DPPH radical solution in methanol was prepared and 1 mL of this solution was mixed with 3 mL of sample solutions in ethanol (containing 20–100 μg of phenolic fraction) and control (without sample). After 30 min, the absorbance was measured at 517 nm.

Radical scavenging activity was calculated with the following equation:

$$\text{Radical scavenging activity} = (1 - \frac{A_{\text{sample}}}{A_{\text{control}}}) \times 100 \qquad /1/$$

Reducing power

Reducing power of phenolics present in the fractions was determined as described by Oyaizu (*20*). The suspension of the fractions (5–25 μg/mL) in 1 mL of distilled water was mixed with 2.5 mL of 0.2 M phosphate buffer (pH=6.6) and 2.5 mL of 1 % (by mass per volume) potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. Following this, 2.5 mL of 10 % (by mass per volume) trichloroacetic acid were added and the mixture was then centrifuged at 1750×*g* for 10 min. An aliquot of 2.5 mL of the upper layer was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1 % (by mass per volume) $FeCl_3$, and the absorbance of the mixture was read at 700 nm. The results were reported as mg of ascorbic acid equivalents (AAE) per g of extract.

Antibacterial screening

The antibacterial tests were performed using the agar well diffusion assay (*21–23*). Agar plates were prepared using sterile nutrient agar (Oxoid). Bacterial strains of standardized cultures were evenly spread onto the surface of the agar plates using sterile swab sticks. Five wells (6 mm diameter) were made in each plate with a sterile borer. A volume of 100 μL of ethanolic, aqueous, ethyl acetate, acetone and final extracts (10 mg/mL) of five *Hypericum* species were added to each well. A volume of 100 μL of absolute alcohol per well was used as a negative control. For a positive control, 100 μL of streptomycin (2 mg/mL) were used. Diffusion of the extracts and controls was allowed at room temperature for 1 h in a laminar flow cabinet. The agar plates were then covered with lids and incubated at 37 °C for 24 h. The plates were observed for the presence of inhibition of bacterial growth, which was indicated by a clear zone around the wells. The size of the zones of inhibition was measured and the antibacterial activity expressed in terms of the average diameter of the inhibition zone in millimeters. The absence of a zone of inhibition was interpreted as the absence of activity.

Antifungal activity assay

For antifungal activity, the tube dilution test was employed (*23,24*). A volume of 5 mL of medium (SDA) was added to each screw-capped test tube and they were autoclaved at 121 °C for 15 min. To the tubes containing 5 mL of sterile SDA, ethanolic, aqueous, ethyl acetate, acetone and final extracts (400 μg/mL) of five *Hypericum* species and fuconazole (200 μg/mL) in absolute alcohol were added. The tubes were kept in the upright position overnight to check the sterility. On the next day, the tubes were inoculated with the fungal culture in the upright position, and then incubated for 10 days at 27–30 °C. Each compound or extract was tested against four fungal cultures. The negative control tubes contained 5 mL of SDA, 1.0 mL of absolute alcohol and 0.1 mL of fungal culture. The positive control tube contained 5 mL of SDA, 200 μg/mL of fuconazole in 1 mL of absolute alcohol and fungal cultures. After 10 days, the results were recorded according to the formula:

$$\text{Growth inhibition} = \frac{\text{Linear growth of negative control} - \text{Linear growth of sample}}{\text{Linear growth of negative control}} \times 100 \qquad /2/$$

The degree of activity was recorded in five grades according to the percentage inhibition of growth: inactive (0), low (0–30 %), moderate (30–50 %), good (50–70 %) and significant (70 % and above).

Statistical analysis

Statistical analysis was performed with the analysis of variance (ANOVA) followed by Tukey's *post hoc* test. Statistical calculations were done using Minitab v. 11.0 software (Minitab Inc, State College, PA, USA).

Results and Discussion

Total phenolic content

The content of total phenolics in fractions 1–5 of the five *Hypericum* species is reported in Fig. 2. The crude extracts (marked as fraction 1) obtained from *H. perforatum*, *H. oblongifoilum*, *H. monogynum*, *H. choisianum* and *H. dyeri* were characterized by their contents of total phenolics expressed as GAE: 71.6, 76.8, 72.7, 96.7, and 58.1 mg/g, respectively. Fraction 2, obtained from the crude extracts using water re-extraction, showed a content of total phenolics expressed as GAE ranging from 46.0 (*H. monogynum*) to 92.7 mg/g (*H. choisianum*). The following re-extractions using ethyl acetate and acetone resulted in the content of total phenolics expressed as GAE in the range of 46.3 to 73.1 (fraction 3) and 46.0 to 87.8 mg/g (fraction 4). The content of total phenolics in the final residue (fraction 5) ranged from 21.1 (*H. dyeri*) to 100 mg/g (*H. choisianum*).

The yield of ethanol extract (content of fraction 1) ranged from 8.0 (*H. oblongifolium*) to 18.0 % (*H. dyeri*) (Fig. 2). In the study by Radulović *et al.* (*11*), the methanol extracts from *Hypericum species* from the Balkans ranged from 0.8 to 35.3 %. The content of crude aqueous extract of *H. perforatum* was 28.6 % (*4*).

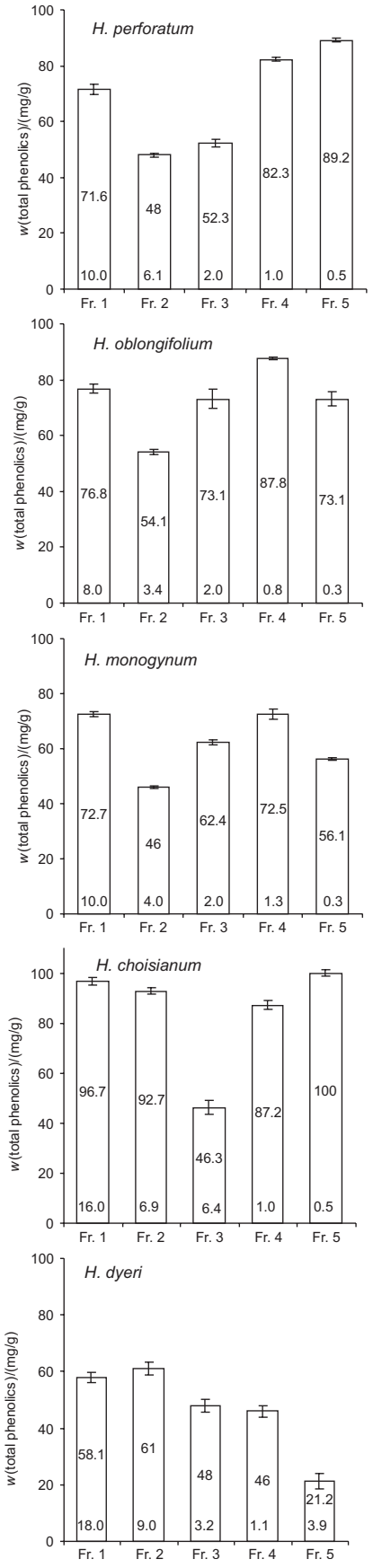

Fig. 2. Content of total phenolics (in mg/g) in fractions separated from *Hypericum* species. Values at the bottom of the bars express the content of individual fractions (in %) in the plant material

Antioxidant activity

The antioxidant activity of the fractions was measured spectrophotometrically using phosphomolybdenum method, which is based on the reduction of Mo(VI) to Mo(V) by the sample analyte and the subsequent formation of green phosphate/Mo(V) compounds with a maximum absorption at 695 nm. The antioxidant capacity of various fractions of the five *Hypericum* species was compared (Table 1). All fractions showed remarkable activities. The same trend in activity was observed in most cases as in the DPPH radical scavenging activity and reducing power. The highest antioxidant activities as AAE were noted for fraction 2 of *H. dyeri* (1385 μmol/g), and fractions 1 (1345 μmol/g) and 4 (1287 μmol/g) of *H. monogynum*. The lowest value (96 μmol/g) was noted in the final residue (fraction 5) of *H. dyeri.*

Table 1. Antiradical activity of five *Hypericum* species extracts against DPPH radical

Plant species and fraction number	RSA at 100 μg/mL / %	EC_{50} / μg/mL
H. perforatum		
Fr. 1	(90.3±0.8)^{ab}	32.8
Fr. 2	(88.3±0.7)^{b}	36.4
Fr. 3	(66.9±1.1)^{c}	63.2
Fr. 4	(90.9±0.4)^{a}	33.2
Fr. 5	(91.9±0.4)^{a}	15.5
H. oblongifolium		
Fr. 1	(91.8±0.5)^{a}	29.2
Fr. 2	(92.7±0.9)^{a}	28.9
Fr. 3	(85.2±0.3)^{b}	38.8
Fr. 4	(92.7±0.5)^{a}	19.8
Fr. 5	(91.0±0.2)^{a}	31.1
H. monogynum		
Fr. 1	(92.3±1.3)^{a}	28.5
Fr. 2	(81.1±0.6)^{b}	52.2
Fr. 3	(67.4±0.8)^{ad}	61.8
Fr. 4	(89.7±0.3)^{d}	33.5
Fr. 5	(89.2±0.3)^{6}	34.8
H. choisianum		
Fr. 1	(92.1±0.5)^{ab}	39.8
Fr. 2	(91.1±0.2)^{b}	38.8
Fr. 3	(45.5±0.2)^{c}	110.2
Fr. 4	(84.6±0.3)^{d}	39.8
Fr. 5	(93.1±0.3)^{a}	19.9
H. dyeri		
Fr. 1	(86.0±0.3)^{a}	49.2
Fr. 2	(90.4±0.2)^{b}	41.8
Fr. 3	(80.3±0.4)^{c}	55.2
Fr. 4	(70.4±0.5)^{d}	69.4
Fr. 5	(35.1±0.8)^{e}	139.2
Gallic acid	98.2±0.5	11.2

Data are expressed as mean values±standard deviations (N=3); values marked by the same letter in the same column of the same species are not significantly different ($p>0.05$)

Antiradical activity

The DPPH radical is a stable organic free-radical with an absorption band between 515 and 528 nm. It loses this absorption when accepting an electron or a free radical species, which results in a visually noticeable discolouration from purple to yellow. Because the DPPH radical can accommodate many samples in a short period and is sensitive enough to detect active ingredients at low concentrations, it has been extensively used for screening antiradical activities of plant extracts (*8*). The DPPH radical scavenging activity of various fractions of five *Hypericum* species was studied.

All fractions separated from the five *Hypericum* species showed scavenging activity against the DPPH radical, but their antiradical properties were different (Fig. 3, Table 2). For *H. perforatum*, the highest activity was noted for fraction 5. For *H. monogynum*, the activities were shown by all fractions even at the lowest concentration (20 μg/mL). Among the crude extracts of the five plant species, the highest activity (EC_{50}=28.5 μg/mL) was observed in *H. monogynum*, while the aqueous extract of *H. dyeri* exhibited the lowest activity (EC_{50}=49.2 μg/mL). The ethyl acetate fraction of all species had lower activity, except that of *H. choisianum* (EC_{50}=11.2 μg/mL). A very strong activity was shown by the acetone fraction of *H. oblongifolium*, to a maximum (EC_{50}=19.8 μg/mL). Among the residual fractions, the highest antiradical activity (EC_{50}=15.5 μg/mL) was observed for *H. oblongifolium*, while the weakest (EC_{50}=139.2 μg/mL) for *H. dyeri*. Gallic acid in the same assay exhibited EC_{50}=11.2 μg/mL. The significant DPPH radical scavenging activities of the fractions were due to their corresponding phenolic contents; fractions with higher phenolic contents showed remarkable scavenging activities (Table 1). In the studies of Zou *et al*. (*8*) and Silva *et al*. (*25*), the antiradical activity of the ethanol extract obtained from *H. perforatum* was characterized by EC_{50} of 10.6 and 49.0 μg/mL, respectively.

The reducing power serves as a strong indicator of the antioxidant activity and was determined using a modified iron(III) to iron(II) reduction assay. In this assay, the yellow colour of the test solution changes to various shades of green and blue depending on the reducing power of the extracts or compounds. The presence of reductants in the solution causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. Therefore, Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm (*6*). Comparative reducing power of various extracts/fractions (crude ethanolic, aqueous, ethyl acetate, acetone, and final residue) of five *Hypericum* species was studied (Fig. 4, Table 2). All samples showed some degree of reducing power; however, as anticipated, their reducing power was inferior to that of the standard. Like the scavenging activity, the reducing power of the samples increased with the increase of concentration. There were statistically significant differences observed among the same fractions ($p<0.05$) of different plants except for the final fractions of *H. dyeri*, where no significant difference ($p>0.05$) was observed in various fractions of the same plant. The crude extract of *H. oblongifolium* showed the highest reducing power expressed as AAE (449 mg/g) for all five species. For the aqueous and acetone fractions, the highest activity expressed as AAE (449 and 498 mg/g, respectively) was observed in *H. oblongifolium*. The ethyl acetate fractions of all species possessed lower activity expressed as AAE except for *H. dyeri* (402 mg/g). The greatest reducing power expressed as AAE (528 mg/g) was observed in the final fraction of *H. choisianum*, while the lowest (145 mg/g) was in the final fraction of *H. dyeri.* The reducing power might be due to either the presence of phenolics or some other reducing agents present in the plant; correlation in phenolic content, reducing power, and DPPH radical scavenging activity was observed in most cases (Table 2). The fraction with higher phenolic content showed remarkable scavenging activity and reducing power.

Table 2. Antioxidant activity and reducing power of phenolic fractions of *Hypericum* species

Plant species and fraction number	Antioxidant activity expressed as AAE	Reducing power expressed as AAE
	μmol/g	mg/g
H. perforatum		
Fr. 1	(762±52)[a]	381
Fr. 2	(613±50)[b]	274
Fr. 3	(955±82)[c]	179
Fr. 4	(962±51)[c]	315
Fr. 5	(1256±128)[d]	528
H. oblongifolium		
Fr. 1	(1286±152)[a]	324
Fr. 2	(1009±48)[b]	494
Fr. 3	(1079±83)[b]	285
Fr. 4	(1307±69)[a]	498
Fr. 5	(888±181)[c]	394
H. monogynum		
Fr. 1	(1345±61)[a]	449
Fr. 2	(987±77)[b]	293
Fr. 3	(987±77)[b]	190
Fr. 4	(1287±89)[a]	324
Fr. 5	(652±43)[c]	298
H. coisianum		
Fr. 1	(1051±159)[a]	366
Fr. 2	(912±79)[a]	337
Fr. 3	(738±92)[a]	149
Fr. 4	(942±60)[a]	283
Fr. 5	(1410±60)[b]	536
H. dyeri		
Fr. 1	(994±109)[a]	327
Fr. 2	(1385±141)[b]	402
Fr. 3	(758±56)[ac]	306
Fr. 4	(610±45)[c]	259
Fr. 5	(96±17)[d]	145

Data are expressed as mean values±standard deviations (*N*=3); values marked by the same letter in the same column of the same species are not significantly different ($p>0.05$)

Antimicrobial activity

As shown in Table 3, the phenolic fractions from the five studied *Hypericum* species showed some antibacterial activity against all of the tested microorganisms, with

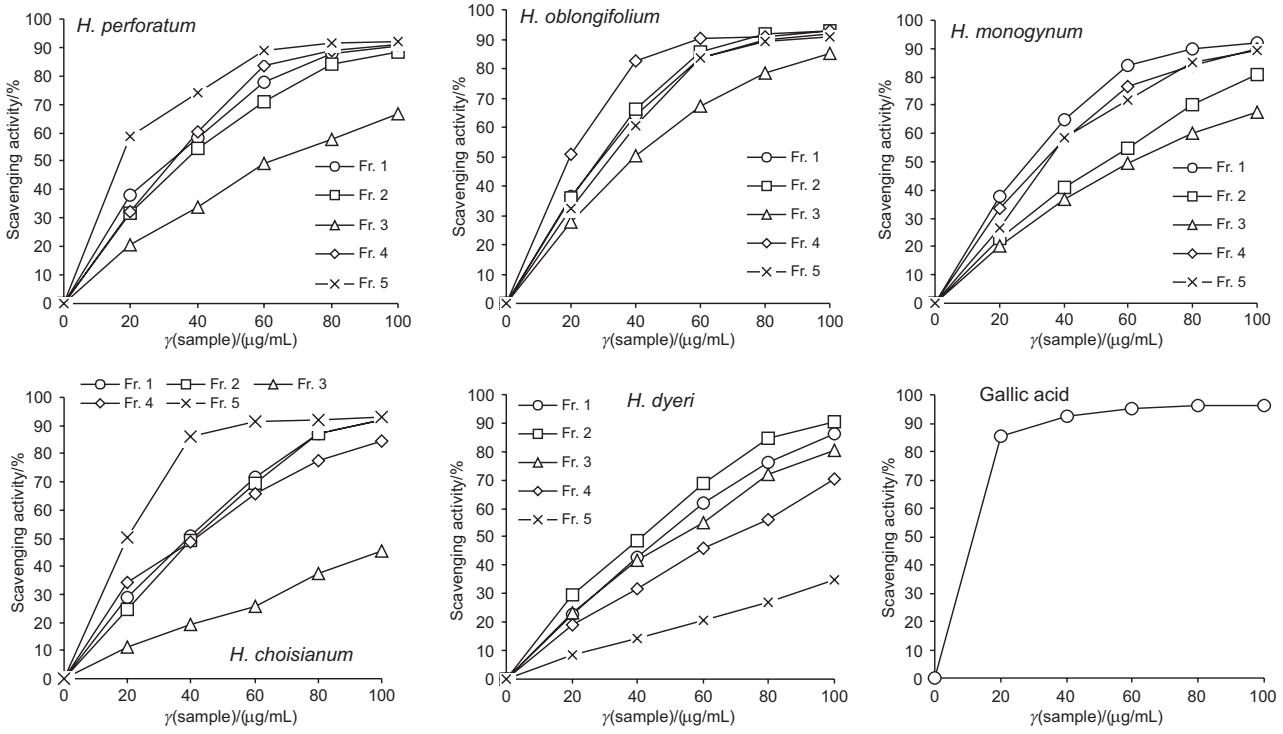

Fig. 3. Antiradical activity of the phenolic compounds present in fractions against DPPH radical

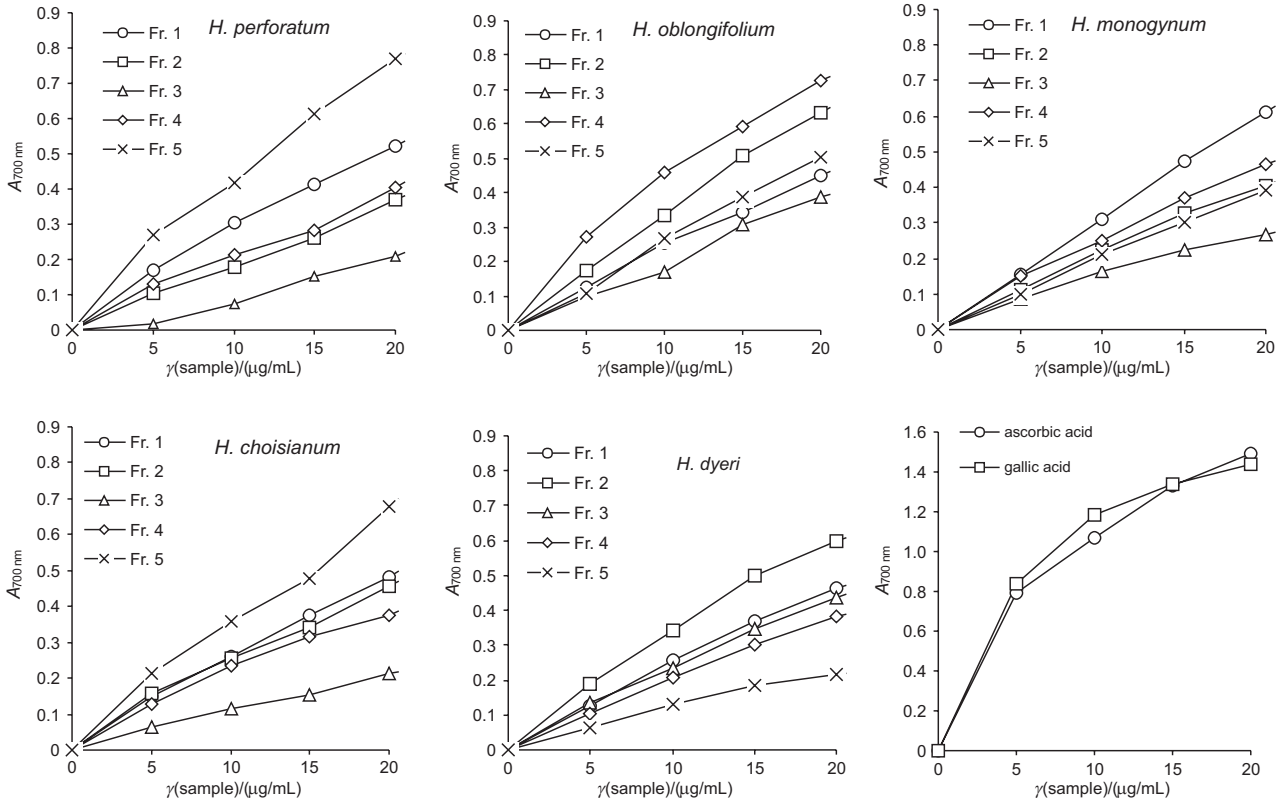

Fig. 4. Reducing power of the phenolic compounds present in fractions

Table 3. Antibacterial activities of phenolic fractions of *Hypericum* species as diameter of growth inhibition zone (in mm)

Plant species and fraction number	*Escherichia coli*	*Staphylococcus aureus*	*Enterobacter aerogenes*	*Salmonella* Typhi	*Proteus vulgaris*	*Pseudomonas aeruginosa*
H. perforatum						
Fr. 1	(11.3±1.9)^{a}	(13.2±1.9)^{ab}	(15.5±2.3)^{a}	(10.6±2.0)^{a}	(10.3±1.8)^{a}	(11.2±2.5)^{a}
Fr. 2	(11.2±2.1)^{a}	(14.5±1.9)^{ab}	(11.4±2.2)^{a}	(11.2±2.2)^{a}	(11.1±2.1)^{a}	(12.1±2.1)^{a}
Fr. 3	(10.6±1.7)^{a}	(16.4±2.4)^{ab}	(11.2±2.1)^{a}	(10.1±2.1)^{a}	(11.4±2.3)^{a}	(18.3±2.1)^{b}
Fr. 4	(11.2±1.5)^{a}	(19.1±2.3)^{c}	(11.1±2.0)^{a}	(10.5±2.0)^{a}	(11.3±2.2)^{a}	(15.5±2.2)^{ab}
Fr. 5	(11.2±2.2)^{a}	(18.2±2.1)^{cb}	(10.9±2.8)^{a}	(11.6±2.2)^{a}	(10.5±2.3)^{a}	(15.4±2.0)^{ab}
H. oblongifolium						
Fr. 1	(10.2±1.7)^{a}	(18.8±2.0)^{a}	(10.6±2.2)^{a}	(11.4±2.5)^{a}	(12.2±2.2)^{a}	(16.6±1.6)^{ab}
Fr. 2	(10.1±1.5)^{a}	(11.5±1.9)^{b}	(10.3±1.8)^{a}	(11.2±2.1)^{a}	(12.1±2.0)^{a}	(14.3±1.9)^{ab}
Fr. 3	(11.2±1.9)^{a}	(19.2±2.3)^{a}	(11.1±1.8)^{a}	(10.8±2.3)^{a}	(11.2±1.8)^{a}	(17.2±2.0)^{ab}
Fr. 4	(11.5±1.5)^{a}	(19.3±2.1)^{a}	(10.4±2.1)^{a}	(11.6±2.2)^{a}	(12.5±2.1)^{a}	(18.1±2.4)^{a}
Fr. 5	(12.8±1.9)^{a}	(12.2±2.0)^{b}	(10.8±2.2)^{a}	(12.2±2.3)^{a}	(10.1±2.1)^{a}	(12.3±2.1)^{b}
H. monogynum						
Fr. 1	(12.8±2.0)^{a}	(21.1±1.8)^{a}	(11.7±2.0)^{a}	(10.1±2.0)^{a}	(13.4±2.3)^{a}	(20.3±2.2)^{a}
Fr. 2	(13.1±1.9)^{a}	(14.2±1.8)^{b}	(11.2±1.9)^{a}	(15.4±2.0)^{a}	(10.8±2.5)^{a}	(13.6±2.1)^{b}
Fr. 3	(11.4±1.6)^{a}	(14.3±1.9)^{b}	(11.5±1.9)^{a}	(15.5±1.9)^{a}	(11.5±2.5)^{a}	(13.4±2.3)^{b}
Fr. 4	(11.7±1.9)^{a}	(14.6±2.1)^{b}	(11.4±2.3)^{a}	(13.6±1.6)^{a}	(11.4±2.7)^{a}	(14.1±2.8)^{b}
Fr. 5	(14.6±2.0)^{a}	(11.6±2.7)^{b}	(13.2±2.0)^{a}	(12.2±1.9)^{a}	(14.2±2.1)^{a}	(13.2±2.0)^{b}
H. coisianum						
Fr. 1	(10.3±2.3)^{a}	(15.2±2.1)^{a}	(11.2±2.2)^{a}	(12.2±1.5)^{a}	(11.3±2.4)^{a}	(16.5±2.5)^{a}
Fr. 2	(10.2±2.9)^{a}	(10.3±2.3)^{a}	(11.2±2.6)^{a}	(11.1±2.0)^{a}	(12.6±2.9)^{a}	(13.7±2.1)^{a}
Fr. 3	(11.1±1.8)^{a}	(12.4±1.9)^{a}	(11.1±2.1)^{a}	(11.3±2.4)^{a}	(12.8±2.5)^{a}	(16.6±2.1)^{a}
Fr. 4	(11.4±1.9)^{a}	(10.2±1.9)^{a}	(10.1±2.3)^{a}	(12.4±2.2)^{a}	(13.5±2.5)^{a}	(14.3±2.3)^{a}
Fr. 5	(10.1±1.8)^{a}	(11.1±1.7)^{a}	(11.2±1.9)^{a}	(12.2±2.4)^{a}	(13.6±2.0)^{a}	(17.1±2.3)^{a}
H. dyeri						
Fr. 1	(19.7±2.5)^{a}	(11.3±2.0)^{a}	(10.3±1.8)^{a}	(12.6±2.1)^{a}	(12.3±1.9)^{a}	(12.5±2.5)^{a}
Fr. 2	(23.1±2.7)^{a}	(12.6±1.3)^{a}	(11.5±1.9)^{a}	(12.3±2.0)^{a}	(14.5±2.0)^{a}	(13.4±2.1)^{a}
Fr. 3	(11.6±1.2)^{b}	(12.5±1.4)^{a}	(11.8±1.9)^{a}	(10.2±2.0)^{a}	(11.2±2.2)^{a}	(12.2±2.1)^{a}
Fr. 4	(11.1±1.7)^{b}	(13.2±2.3)^{a}	(12.4±2.0)^{a}	(14.2±1.9)^{a}	(12.5±2.2)^{a}	(14.2±2.0)^{a}
Fr. 5	(23.2±2.0)^{a}	(11.3±2.1)^{a}	(12.1±1.9)^{a}	(14.2±2.3)^{a}	(11.2±2.0)^{a}	(13.3±2.4)^{a}
Streptomycin	30.3±1.8	31.2±2.5	25.3±1.7	30.2±2.3	28.5±2.2	32.1±2.2

Data are expressed as mean values±standard deviations (N=3); values marked by the same letter in the same column of the same species are not significantly different ($p>0.05$)

the diameters of the zone of inhibition ranging between 10 and 23 mm. Of the studied plants, the most active fractions were fraction 1 and fraction 5 obtained from *H. dyeri* against *Escherichia coli*. Fractions 3, 4 and 5 from *H. perforatum* and fractions 1, 3 and 4 from *H. oblongifolium* showed positive antibacterial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa*, and so did fractions 1 obtained from *H. monogynum* and *H. choisianum*. Fractions 3, 4 and 5 from *H. monogynum* showed activity against *Pseudomonas aeruginosa*. All other fractions from all plants exhibited weak activity on the tested strains. All of the bacteria in the study were sensitive to streptomycin with *Staphylococcus aureus* and *Pseudomonas aeruginosa* being the most sensitive (inhibition zone values of 31 and 32 mm, respectively).

Antimicrobial activities of *Hypericum* species have been reported by several authors (*11*,*15*,*25–28*). Radulović *et al*. (*11*) studied the antibacterial activities of nine *Hypericum* species which showed significant activity even at a dose of 5 μg/disc and the zone of inhibition ranged from 12 to 42 mm. In our study, the zone of inhibition ranged between 10 and 23 mm, which is almost the same as that previously reported (*15*,*26*). Our findings correlate with the observations of previous screenings of medicinal plants for antimicrobial activity, where most of the active plants showed activity against Gram-positive strains only (few are active against Gram-negative bacteria) (*15*). Moreover, our current results show an antimicrobial activity against the Gram-negative bacterium *Escherichia coli* and this microorganism has been isolated from infected wounds of humans.

Results reported in Table 4 show that extracts from the five *Hypericum* species examined showed some antifungal activity against the tested fungal strains, with the zone of inhibition ranging between 10 and 52 %. The crude, aqueous, ethyl acetate, and acetone fractions of *H. perforatum* were found most active against *Helminthosporium maydis*, while all fractions of the same plant showed

Table 4. Antifungal activities of phenolic fractions of *Hypericum* species expressed as growth inhibition (in %)

Plant species and fraction number	*Aspergillus niger*	*Helminthosporium maydis*	*Aspergillus flavus*	*Alternaria solani*
H. perforatum				
Fr. 1	(35.3±2.1)^{ab}	(47.3±4.3)^{ab}	(35.3±3.3)^{a}	(27.2±2.3)^{a}
Fr. 2	(29.4±3.1)^{ac}	(51.8±5.5)^{ab}	(23.5±4.4)^{b}	(23.5±2.4)^{a}
Fr. 3	(41.2±5.4)^{b}	(47.1±4.5)^{ab}	(31.8±4.5)^{ab}	(29.4±4.1)^{a}
Fr. 4	(35.3±2.7)^{ab}	(52.9±3.1)^{a}	(23.5±3.2)^{b}	(29.4±2.3)^{a}
Fr. 5	(27.1±2.3)^{c}	(40.0±5.6)^{b}	(28.8±2.8)^{ab}	(25.9±2.4)^{a}
H. oblongifolium				
Fr. 1	(29.7±4.1)^{a}	(11.8±3.2)^{a}	(23.5±4.6)^{a}	(23.5±2.1)^{a}
Fr. 2	(31.5±3.3)^{a}	(29.4±4.4)^{b}	(23.5±2.9)^{a}	(31.8±2.3)^{b}
Fr. 3	(41.2±2.2)^{b}	(44.7±2.5)^{c}	(22.4±4.3)^{a}	(29.4±4.1)^{ab}
Fr. 4	(35.3±2.8)^{ab}	(30.6±2.9)^{b}	(22.4±5.2)^{a}	(35.3±3.5)^{b}
Fr. 5	(35.3±3.5)^{ab}	(43.5±2.0)^{c}	(22.4±3.3)^{a}	(31.8±2.6)^{b}
H. monogynum				
Fr. 1	(29.4±3.4)^{ac}	(30.6±3.4)^{a}	(21.2±2.3)^{a}	(29.4±3.1)^{a}
Fr. 2	(30.5±2.8)^{a}	(11.8±4.3)^{b}	(21.2±2.9)^{a}	(27.1±2.4)^{a}
Fr. 3	(40.0±2.2)^{bc}	(17.7±3.6)^{bc}	(28.2±4.2)^{a}	(29.4±4.1)^{a}
Fr. 4	(29.4±4.2)^{ac}	(17.7±3.6)^{b}	(22.4±3.2)^{a}	(41.2±3.2)^{b}
Fr. 5	(35.3±3.1)^{abc}	(35.3±2.6)^{a}	(22.4±3.5)^{a}	(29.4±1.9)^{a}
H. choisianum				
Fr. 1	(23.5±3.5)^{a}	(23.5±5.1)^{a}	(11.8±3.2)^{a}	(29.4±2.3)^{ad}
Fr. 2	(35.3±2.6)^{b}	(8.2±4.1)^{b}	(23.5±3.6)^{bc}	(31.8±2.4)^{a}
Fr. 3	(29.4±3.3)^{ab}	(11.8±3.1)^{b}	(31.8±2.3)^{c}	(41.2±3.5)^{b}
Fr. 4	(31.8±2.1)^{b}	(23.5±2.1)^{a}	(17.7±2.5)^{ab}	(8.2±1.3)^{c}
Fr. 5	(29.4±2.9)^{ab}	(23.5±5.4)^{a}	(22.4±6.3)^{bc}	(22.4±2.9)^{d}
H. dyeri				
Fr. 1	(41.2±4.2)^{a}	(31.8±2.1)^{a}	(22.4±5.3)^{a}	(23.5±3.1)^{ad}
Fr. 2	(15.3±3.1)^{b}	(43.5±2.1)^{b}	(41.2±3.2)^{b}	(30.6±2.2)^{ab}
Fr. 3	(38.8±2.4)^{a}	(11.8±2.1)^{c}	(28.2±4.1)^{a}	(27.1±4.1)^{ad}
Fr. 4	(35.3±2.4)^{a}	(35.3±2.1)^{a}	(22.4±4.2)^{a}	(35.3±3.3)^{b}
Fr. 5	(35.3±2.3)^{a}	(11.8±2.1)^{c}	(8.2±1.3)^{c}	(21.2±1.9)^{d}
Fuconazol	76.5±4.5	70.6±5.1	72.9±4.1	74.1±4.9

Data are expressed as mean values±standard deviations (N=3); values marked by the same letter in the same column of the same species are not significantly different ($p>0.05$)

moderate activity against the tested fugal strains. Aqueous, ethyl acetate, acetone, and final residue of *H. oblongifolium* showed moderate activity against *Aspergillus niger*, *Helminthosporium maydis* and *Alternaria solani*, and weak activity against *Aspergillus flavus.* Aqueous, ethyl acetate, and acetone extracts of *H. monogynum* showed moderate activity against *Aspergillus niger* and *Alternaria solani*, and weak activity against *Helminthosporium maydis* and *Aspergillus flavus*. Moderate activity against *Aspergillus niger*, *Helminthosporium maydis* and *Aspergillus flavus* was also noted for fractions 1 and 5 of the same plant. Activity was also found in acetone fractions of *H. choisianum* and final residue against *Aspergillus niger.* The extract obtained from *H. dyeri* also had an activity up to some extent against *Aspergillus niger*; water and ethyl acetate fractions showed activity against *Helminthosporium maydis*, *Alternaria solani* and *Aspergillus flavus*. All the fungi tested in the study were sensitive to fuconazole, with *Aspergillus niger* and *Alternaria solani* being the most sensitive (inhibition zone values of 76 and 74 %, respectively), which is significant. None of the studied samples showed high activity, and most of them exhibited moderate or weak activity.

With regard to the components responsible for the antimicrobial activity shown, several compounds of distinct nature must be acting as antimicrobial agents in these plants. This is not unexpected: in the majority of *Hypericum* species studied so far, there are large varieties of active compounds, including naphthodianthrones, flavonoids, xanthones, tannins, essential oils and phloroglucinols (*15*). Because the antimicrobial activity in other species of this genus has been found to be closely related to the levels of flavonoids and phloroglucinol derivatives (*9*), it is reasonable to assume that these compounds are responsible for the antimicrobial activities reported here. Results suggest that further work is needed to locate the bioactive compounds from various extracts and that such efforts could result in the discovery of new compounds possessing a wide range of bioactivity.

Conclusions

From the achieved results, it can be concluded that the phenolic fractions from five *Hypericum* species from Pakistan possess antioxidant and antimicrobial activities. After toxicological studies of some potentially harmful compounds present in the extracts or their fractions, we suggest that these materials be used as natural antioxidants in food, functional food, and nutraceuticals.

Acknowledgements

Higher Education Commission of Pakistan is acknowledged for financial support. We thank Mr Momin Khan, Lecturer, Centre of Biotechnology and Microbiology, University of Peshawar, Pakistan, for his help in antimicrobial studies.

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