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Phytochemical profile, antioxidant and antibacterial activity of four *Hypericum* species from the UK

Zeb Saddiqe[a,*], Ismat Naeem[b], Claire Hellio[c], Asmita V. Patel[d], Ghulam Abbas[e]

[a] Department of Botany, Lahore College for Women University, Lahore, Pakistan
[b] Department of Chemistry, Lahore College for Women University, Lahore, Pakistan
[c] Univ Brest, Laboratoire des Sciences de l'Environnement MARin (LEMAR) CNRS, IRD, Ifremer F-29280, Plouzané, France
[d] School of Pharmacy and Biomedical Sciences, University of Portsmouth, UK
[e] Biological Sciences and Chemistry-Chemistry Section, College of Arts and Sciences, University of Nizwa, Oman

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ABSTRACT

Treatment of skin wounds is an important domain in biomedical research since many pathogenic bacteria can invade the damaged tissues causing serious infections. Effective treatments are required under such conditions to inhibit microbial growth. Plants are traditionally used for the treatment of skin infections due to their antimicrobial potential. The antibacterial activity of different solvent extracts of four *Hypericum* species (*H. androsaemum, H. ericoides, H. x moserianum* and *H. olympicum*) traditionally acclaimed for their wound healing activity was examined in the present study against *Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Enterobacter aerogenes*. In addition the content and types of flavonoids [High Performance Liquid Chromatography (HPLC) analysis], and antioxidant activity [1,1-diphenyl-2-picryl-hydrazyl (DPPH) assay] were evaluated for all the species. The most prominent antibacterial activity was displayed by *H. olympicum* (MIC between 0.001 – 0.1 mg/mL) in particular ethyl acetate and *n*-butanol fractions which were found to be rich in phenolic and flavonoid contents. Strong antioxidant activity was observed for all the species and was associated with the more polar methanol, ethyl acetate and *n*-butanol extracts, with IC_{50} values ranging between 0.093 to 0.3 mg/mL. HPLC analysis of the extracts indicated the presence of different flavonoids in the plants and the highest content of selected flavonoids was determined for *H. olympicum*. The antibacterial activity of the selected *Hypericum* species shown in this study supports the traditional role of using these species for wound healing.

Introduction

Skin infections such as wounds, sepsis, atopic dermatitis, cellulitis, acne and candidiasis are caused by a variety of microorganisms including both bacteria and fungi. The most common bacterial species associated with the various skin infections include *Staphylococcus aureus, Bacillus subtilis, Escherichia coli* and *Pseudomonas aeruginosa* (Ayub et al., 2015; Bessa et al., 2015). These bacteria, particularly *Staphylococcus aureus* and *Bacillus subtilis*, are associated with infections, abscesses, carbuncles, erysipelas, bacteremia, endocarditis, furuncles and impetigo. The Gram-negative bacterium *Escherichia coli*, although part of the physiological intestinal flora, can also cause wound infections and sepsis when residing otherwise (Madigan et al., 2003; Gnanamani et al., 2017).

Plants of the genus *Hypericum* (Hypericaceae) have long been used as traditional wound-healing agents and this potential has been demonstrated in several *in vivo* based studies with their extracts (Suntar et al., 2010; Yadollah-Damavandi et al., 2015; Altan et al., 2018; Altiparmak et al., 2019). The literature also abounds with studies regarding antimicrobial activities of various species of the genus (Naeem et al., 2010; Maltas et al., 2013; Süntar et al., 2016; Lyles et al., 2017). The present study was designed to assess the *in vitro* antibacterial activities of crude extracts and fractions of four *Hypericum* species *viz., H. androsaemum, H. ericoides, H. x moserianum* (*H. calycinum* x *H. patulum*) and *H. olympicum* against bacterial species most commonly associated with wound and skin infections. Only a few studies regarding the *in vitro* antibacterial activities of the selected *Hypericum* species are available (Mazandarani et al., 2007; Radulovic et al., 2007; Saddiqe et al., 2014; Ferreira et al., 2015).

Reactive oxygen species (ROS) are produced in all the living organisms in which there is aerobic mode of respiration. These ROS play an important role in cell metabolism including intercellular signaling, defense responses of cells, phagocytosis and energy production (Dickinson and Chang, 2011; Pizzino et al., 2017). These reactive species are kept in balance by antioxidants (He et al., 2017). Many of

* Corresponding author.
E-mail address: zeb_rukhsana@yahoo.com (Z. Saddiqe).

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the commonly used synthetic antioxidants including propyl gallate, butylated hydroxyanisole and butylated hydroxytoluene are reported to cause toxic effects such as liver damage and carcinogenesis, enzyme and lipid alterations particularly when used for prolonged periods at high concentrations or due to the formation of their degradation products (Gharavi et al., 2007; Atta et al., 2017). A number of studies have shown that the plant-derived antioxidants with free-radical scavenging potential are helpful in counteracting the toxic effects of these reactive species. Several plant secondary metabolites, especially phenols and flavonoids, are not just associated with antioxidant capacity, but have the additional advantage of antibacterial activity (Li et al., 2015; Zhang et al., 2017). Thus the present study was carried out to screen both antibacterial and antioxidant activities of different polarity solvent extracts of selected *Hypericum* species to confirm their potential for treatment of wounds.

Materials and methods

Chemicals

All chemicals including methanol (MeOH), *n*-hexane, dichloromethane (CH_2Cl_2), *n*-butanol (*n*-BuOH), and ethyl acetate (EtOAc) used for the extraction of plant material were of analytical grade purchased from Fisher Scientific Leicestershire (UK). Folin-Ciocalteu reagent, sodium nitrite ($NaNO_2$), aluminium chloride ($AlCl_3$), sodium carbonate anhydrous (Na_2CO_3), sodium hydroxide (NaOH), 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), dimethylsulfoxide (DMSO), propyl gallate, quercetin, myricetin, rhamnetin, isorhamnetin, kaempferol and luteolin were all purchased from Sigma-Aldrich (St Louis, MO, USA). Ferrous sulphate ($FeSO_4$) was purchased from Carl Roth, Karlsruhe (Germany). HPLC grade acetonitrile, methanol and water were purchased from Merck (Germany). Lauria Bertani (LB) broth was purchased from Conda (Spain).

Collection of plant material

The four *Hypericum* species from UK *viz., H. androsaemum, H. ericoides, H. x moserianum* and *H. olympicum*, were obtained from Perryhill Nurseries in the UK and were grown in the green house of University of Portsmouth, UK for one year. Aerial parts of the plants were used for the study. Herbarium specimens of the four species were deposited in the Herbarium of Hampshire County Council Museum Service, Winchester, Hampshire, UK (Index Herbarium code: HCMS; accession number: Bi 2000 16–370, 371, 372 and 373 for *H. androsaemum, H. ericoides, H. x moserianum* and *H. olympicum*, respectively).

Extraction

The fresh aerial parts of the four *Hypericum* species growing in the green house were harvested and washed to remove dust and dirt and cut into small pieces for effective extraction. A weighed amount of each sample was soaked in a sufficient volume of methanol (100%) to completely dip the material in the solvent for 3 days at 25 °C with frequent agitation. After 3 days solvent-containing extracts were filtered. The process was repeated twice and the extracts from each extraction were combined and methanol was removed under reduced pressure at 35 °C in a rotary evaporator (Laborata 4002, Heidolph, Germany) to obtain crude methanol extracts. The extracts were weighed and the data recorded for each species. The methanol extract of each plant was further dissolved in distilled water and partitioned between *n*-hexane, dichloromethane, ethyl acetate and *n*-butanol sequentially (three times each) to separate the components on the basis of their polarity and solubility in different solvents. All the organic fractions (*n*-hexane, dichloromethane, ethyl acetate and *n*-butanol) were condensed in the rotary evaporator. The inorganic aqueous fraction was dried in Virtis Liter freezer dryer, SL model (Virtis, Gardiner, NY, USA). All the fractions were weighed and stored in tightly sealed dark glass containers at 4 °C until required for further analysis and antibacterial activity testing.

Phytochemical analysis

The crude extracts and fractions were analyzed to detect the presence or absence of selected chemical constituents according to standard methods (Harborne, 1978). Stock solutions of all the samples for phytochemical analysis were prepared by dissolving 0.1 mg/mL of extracts in methanol. All the tests were performed at ambient room temperature.

Tannins: To detect the presence of tannins 1 mL of KOH solution (10% w/v) was added to equal volume (1 mL) of plant extract. The presence of tannins was indicated by the appearance of dirty white precipitates.

***Glycosides*:** For glycosides equal volumes of KOH solution (10% w/v) and plant extract was added (1 mL each). The presence of glycosides was indicated by the formation of brick red precipitates.

Saponins: For saponins, frothing test was performed in which 2 mL of the extract was vigorously shaken in the test tube for 2 min. Formation of persistent froth indicated the presence of saponins.

***Steroids*:** Steroids were identified by adding 3 drops of H_2SO_4 (concentrated) to 1 mL of plant extract. Red coloration indicated the presence of steroids.

Triterpenes: For triterpenes, 5 drops of H_2SO_4 (concentrated) and 1 mL of each plant extract were mixed together. Appearance of blue green color indicated the presence of triterpenes.

Flavonoids: Presence of flavonoids was tested by adding 1 mL of 5% $AlCl_3$ (w/v in methanol) to 1 mL of plant extract. Yellow coloration indicated the presence of flavonoids.

Phenolics: For phenolics, 2 drops of $FeCl_3$ (5% w/v in methanol) was added to 1 mL of the extract. Formation of greenish precipitate confirmed the presence of phenolics.

Determination of total phenolic content (TPC)

For determination of TPC the method of Cliffe et al. (1994) was employed. Each plant sample (20 μL; 1 mg/mL in respective solvent) was mixed with Folin-Ciocalteu reagent (100 μL) and deionized water (1.58 mL). After incubation at 25 °C for 10 min, 25% Na_2CO_3 solution (300 μL; w/v aqueous solution) was added. The mixture was again incubated at 40 °C and after 30 min absorbance at 765 nm was measured against a blank (20 μL of plant sample replaced by 20 μL of extracting solvent) using a UV–Visible double beam spectrophotometer (Hitachi, U2800 Japan). Calibration curve of gallic acid used as standard phenol was prepared by treating different concentrations of gallic acid (1, 0.5, 0.25, 0.1, 0.05 and 0.01 mg/mL) under similar conditions as above and determining their absorbance values at 765 nm (Fig. 1). TPC of each sample was calculated from the calibration curve and results were expressed as mg gallic acid equivalent (GAE)/g dry extract (dE).

Determination of total flavonoid content (TFC)

For determination of TFC the colorimetric method of Dewanto et al. (2002) was used. According to the method 250 μL of the extract was mixed with 500 μL of the deionized water and 90 μL of 5% (w/v) $NaNO_2$ solution and left for 6 min at 25 °C. Then, 180 μL of $AlCl_3$ (10% w/v in methanol) was added to the above mixture and again left to stand for 5 min. Finally 600 μL of 1 M NaOH aqueous solution was added to the reaction mixture and the final volume was made up to 3 mL with deionized water. Absorbance at 510 nm was measured against a blank (250 μL of plant extract was replaced by 250 μL of extracting solvent). Calibration curve of quercetin used as standard flavonoid was prepared by treating different concentrations of quercetin (1, 0.5, 0.25, 0.1, 0.05 and 0.01 mg/mL) under similar conditions

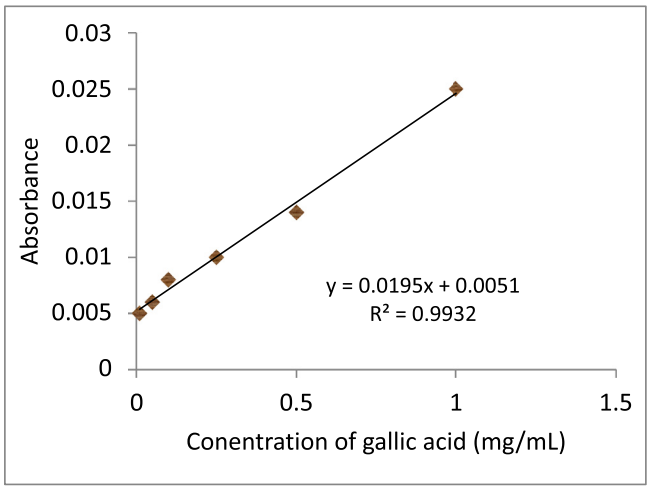

Fig. 1. Calibration curve for Gallic acid.

as above and determining their absorbance values at 510 nm (Fig. 2). TFC was calculated from the calibration curve and the results were expressed as mg quercetin equivalent (QE)/g dE.

DPPH radical scavenging assay

Radical scavenging activity of the extracts was determined by DPPH radical scavenging assay as described by Lee et al. (1998). Briefly, 95 μL of DPPH$^{\bullet}$ solution in ethanol (300 μM) and 5 μL of the plant extract (0.5 mg/mL in 100% DMSO) were combined in 96-well microtitre plates (Biopack; Buenos Aires, Argentina) and plates were covered with parafilm. The contents were mixed by shaking the plates for one minute in a microtitre plate shaker (Cole-Parmer^{TM} SSM5) and were incubated for 30 min at 37 °C. Decrease in absorption was recorded on microplate ELISA reader (Spectra Max 340, Molecular Devices, CA, USA) at 515 nm. The reaction mixture containing 5 μL of DMSO in place of plant extract was used as negative control. Propyl gallate was used as a positive control. The reactions were performed in triplicates. Samples where % inhibition was > 50 in initial screening, antiradical activity was also performed at 250, 125, 62, 32 and 16 μg/mL. IC_{50} values were calculated by using EZ-fit software. The extracts were prepared 24 hrs before DPPH measurements. The radical scavenging activities (%) were calculated by using the following formula:

$$\%\text{scavengingactivity} = (1-(\text{Abs}_s/\text{Abs}_c \times 100))$$

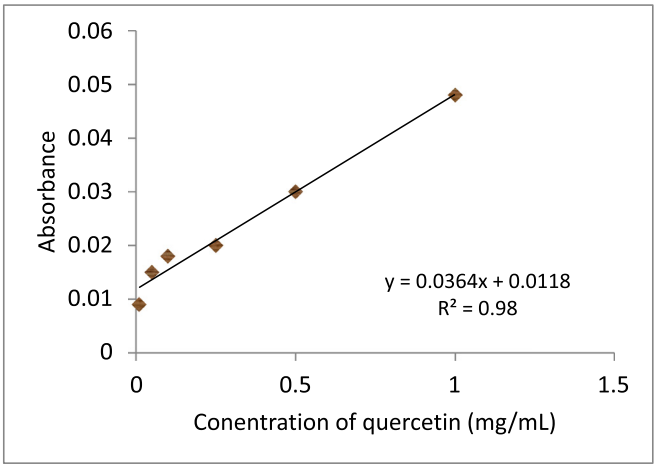

Fig. 2. Calibration curve for Quercetin.

where

Abs_s = absorbance of sample, and

Abs_c = absorbance of control

HPLC analysis

Crude methanol extracts of selected *Hypericum* species were analyzed for the presence of selected flavonoid aglycones (quercetin, myricetin, isorhamnetin, rhamnetin, kaempferol, luteolin and apigenin) through analytical reversed-phase HPLC using a diode array detector. Plant extracts and pure standards of flavonoids were dissolved in HPLC grade MeOH to give a concentration of 100 μg/mL. The mobile phase consisted of water: ACN (1:1) acidified with 1% acetic acid. All the samples and mobile phase were filtered through membrane filters (pore size 0.45 μm, Millipore Corporation, Bedford, MA, USA) before HPLC analysis and the mobile phase was also deaerated using a sonicator bath (BL4–150, Bio-Equip, Shanghai Bilon Instrument Co. Ltd., China). HPLC system (Waters, USA) consisted of a pump (1500 series) and a dual absorbance UV detector (2487). The compounds were separated on a prepacked analytical C18 column (250 mm × 4.6 mm, 5 μm particle size). The flow rate was kept constant at 1.0 mL/min at 25 °C. Throughout the experiment all injection volumes were 10 μL and the compounds were detected at 254 nm. Identification of flavonoids in extracts of different plants was done by comparing retention times of separated flavonoids with those of standard compounds. Variation of the retention time of each peak was less than 1%. The flavonoid content was expressed as quercetin equivalent and the quantification was by peak area measurement of the HPLC chromatograms from the 3 replicate samples. Before analysis, the instrument was calibrated using standards and the response linearity of the detector and stability of baseline was examined. The same operating conditions of the HPLC system were maintained throughout the analysis of all the samples.

Antibacterial activity

Test microorganisms and microbial culture

Bacterial strains used in this study were two Gram-positive strains, *S. aureus* (NCIMB 8625) and *B. subtilis* (NCIMB 1026), and three Gram-negative strains, *E. coli* (B 81), *P. aeruginosa* (NCIMB 1039) and *E. aerogenes* (ATCC 13,048). Bacterial inoculum was prepared by transferring bacteria (cultured on agar plates and stored at −80 °C) into freshly prepared LB medium 24 hrs prior to test setup.

Minimum inhibitory concentration (MIC)

The 96-well microtitre plates (non-tissue cultured Biopack; Buenos Aires, Argentina) were used as the test chambers for the bacterial assay. Plant extracts at different concentrations (0.1, 0.05, 0.025, 0.01 and 0.001 mg/mL) were prepared in their respective solvents and were placed in different wells (100 μL each) with 6 replicates for each sample. Solvent was evaporated from each well by placing the plates in a fume hood at 25 °C. Bacteria, growing in LB broth, were diluted in fresh LB medium and OD of dilution was determined at 630 nm using UV–Visible spectrophotometer. Each dilution was diluted/concentrated accordingly to get an OD between 0.2 and 0.65. Dilutions of bacterial solutions were made according to the method of Amsterdam (1996) to obtain the concentration of 2×10^8 cells/ mL. Bacterial dilutions were placed in each well (100 μL) under aseptic conditions. In growth control wells only bacterial inoculum was added to check the growth of bacteria. Bacterial solution without any extract sample was used as negative control. Plates were covered with sterile plate sealer and incubated in an incubator (Memmert GmbH Incubator Oven INB200) at 37 °C for 48 hrs. Results were evaluated by comparison of the visible growth intensity in the control wells and treated wells as observed by naked eye. The lowest

Table 1
Amount (g) and % yield of methanol extracts of *Hypericum* species.

Plant species	Fresh weight (g)	Dry weight (g)	MeOH extract (g)	Percentage Yield
H. androsaemum	2000	820	291	35.50
H. ericoides	700	270	62	22.00
H. x moserianum	2000	1050	216	20.00
H. olympicum	800	453	220	48.50

concentration of the test solution that led to an inhibition of growth was the MIC. The total antibacterial activity (TAA) of each extract was calculated by dividing the quantity extracted (in mg) from 1 g of plant material by the MIC value (mg/mL) towards a specific bacterium.

Results and discussion

Extraction yield

All the plants gave high extraction yields for crude methanol extract with *H. olympicum* providing the highest yield (48.50%) (Table 1). Fractionation of the crude extracts with solvents of different polarities gave the highest yields for the ethyl acetate and aqueous fractions while respective dichloromethane fraction was in minor quantity in all the species (Table 2). Extraction yield is very important to have an idea regarding the potential of any plant to be used as an economical source of plant-based drugs. Extraction yield greatly affects the overall efficacy of the plant and is also important in calculating the TAA (Eloff, 2000). Hence, the results of extraction yields indicated that all the *Hypericum* species were rich in medium polar and polar components.

Phytochemical analysis of extracts

Phytochemical analysis of *Hypericum* species indicated the presence of glycosides, steroids, flavonoids and phenols in abundance while tannins and triterpenes were scarcely present. Polar fractions of all the plants were rich in contents of different classes of compounds (Table 3).

Analysis of total phenolic and flavonoid contents

High phenolic and flavonoid contents were determined for the selected *Hypericum* species (Table 4). The highest phenolic content in crude methanol extract was observed for *H. x moserianum* (245.00 ± 2.08 mg GAE/g dE) and the least for *H. olympicum* (95.00 ± 1.37 mg GAE/g dE). Total amount of flavonoids ranged between 20.81 ± 1.05 mg QE/g dE (*H. olympicum*) to 29.64 ± 0.02 mg QE/g dE (*H. x moserianum*). After fractionation, the highest contents

Table 3
Phytochemical analysis of extracts and fractions of four *Hypericum* species.

Plant species	Solvent	Tan	Gly	Tri	Ste	Fla	Phe
H. androsaemum	MeOH	–	++	+	++	+	+
	n-Hexane	–	–	+	–	–	–
	CH_2Cl_2	–	+	–	+++	+	–
	EtOAc	–	++	–	++	+++	++
	n-BuOH	–	+++	–	+++	++	++
	Aqueous	–	+	–	+	+	+
H. ericoides	MeOH	+	+	–	–	+	++
	n-Hexane	–	–	++	–	–	–
	CH_2Cl_2	+	–	++	–	–	–
	EtOAc	–	++	–	+	++	++
	n-BuOH	–	++	–	+	++	++
	Aqueous	–	+	–	–	+	++
H. x moserianum	MeOH	+	+	–	+++	+	–
	n-Hexane	–	+	–	+	–	–
	CH_2Cl_2	+	–	–	–	+	++
	EtOAc	–	++	–	++	++	++
	n-BuOH	–	+	–	++	++	–
	Aqueous	–	+	–	+	+	+
H. olympicum	MeOH	+	+	–	–	+	+
	n-Hexane	–	–	+	–	–	–
	CH_2Cl_2	+	+	–	++	+	–
	EtOAc	–	+	–	++	++	+++
	n-BuOH	–	+	–	+++	++	+++
	Aqueous	–	+	–	+	+	+

Tan = Tannins, Gly = Glycosides, Tri = Triterpenes, Ste = Steroids, Fla = Flavonoids, Phe = Phenolics.
–, not detected; +, minimum content; ++, moderate content; +++, maximum content.

of phenols and flavonoids were in the EtOAc fractions. *n*-Hexane and dichloromethane fractions of all the plants were relatively poor in phenolics and flavonoids. Plant polyphenols possess significant biological activities such as antioxidant, anticancer and antimicrobial. Among the phenolic compounds flavonoids are of particular importance and can act as antioxidants due to their ability to chelate transition metals, scavenge free radicals and maintaining endogenous antioxidants such as glutathione and superoxide dismutase (Agati et al., 2012; Kumar and Pandey, 2013). Many plant extracts have bactericidal and bacteriostatic properties that are mainly due to the presence of phenols and flavonoids in these extracts that possess antibacterial as well as antioxidant properties (Venkata et al., 2012).

Antioxidant activity

High radical scavenging potential was displayed by all the plants in the DPPH radical scavenging assay at 0.5 mg/mL concentration. Plant samples with more than 50% scavenging efficiency at 0.5 mg/mL were further screened for antioxidant activity at lower concentrations and their IC_{50} values were also calculated (Table 5). The antiradical properties varied among different plants and among different extracts of the

Table 2
Amount (g) and % yield of different solvent fractions of four *Hypericum* species.

Plant species		Fraction				
		n-Hexane (*)	CH_2Cl_2 (*)	EtOAc (*)	*n*-BuOH (*)	Aqueous (*)
H. androsaemum	Amount (g)	9.00	2.00	86.05	7.32	179.60
	% Yield	(3.10)	(0.70)	(29.60)	(2.51)	(61.72)
H. ericoides	Amount (g)	5.75	6.61	19.84	3.72	24.80
	% Yield	(9.27)	(10.66)	(32.00)	(6.00)	(40.00)
H. x moserianum	Amount (g)	5.57	3.95	48.77	2.68	154.77
	% Yield	(2.60)	(1.82)	(22.60)	(1.24)	(71.60)
H. olympicum	Amount (g)	58.12	4.95	110.00	5.30	36.70
	% Yield	(26.42)	(2.25)	(50.00)	(2.41)	(16.70)

* % Values between parentheses indicate the percentage of mass in the corresponding extract, relative to the amount present in crude extract.

Table 4
Total phenolic and flavonoid content in extracts and fractions of four *Hypericum* species.

Hypericum species	Solvent	Total Phenolics mg GAE/g dE*	Total Flavonoids mg QE/g dE*	Flavonoids/Phenolics
H. androsaemum	MeOH	182.50 ± 3.38	27.00 ± 0.30	0.147
	n-Hexane	32.33 ± 1.23	4.36 ± 0.17	0.135
	CH_2Cl_2	15.23 ± 0.78	3.01 ± 0.53	0.197
	EtOAc	198.76 ± 2.69	42.29 ± 0.11	0.212
	n-BuOH	125.41 ± 3.45	23.50 ± 1.21	0.187
	Aqueous	55.37 ± 2.95	10.32 ± 0.24	0.186
H. ericoides	MeOH	190.00 ± 2.13	23.62 ± 0.15	0.124
	n-Hexane	19.75 ± 1.48	1.57 ± 0.30	0.079
	CH_2Cl_2	22.31 ± 2.17	7.04 ± 0.15	0.315
	EtOAc	202.86 ± 2.83	58.63 ± 1.26	0.289
	n-BuOH	100.39 ± 2.57	25.30 ± 0.23	0.252
	Aqueous	45.15 ± 2.11	13.02 ± 0.24	0.288
H. x *moserianum*	MeOH	245.00 ± 2.08	29.64 ± 0.02	0.121
	n-Hexane	25.36 ± 1.74	2.15 ± 0.13	0.084
	CH_2Cl_2	35.71 ± 1.57	11.47 ± 0.68	0.321
	EtOAc	305.83 ± 4.16	65.74 ± 0.17	0.214
	n-BuOH	103.66 ± 2.42	35.86 ± 0.75	0.345
	Aqueous	65.78 ± 2.15	20.54 ± 1.32	0.312
H. olympicum	MeOH	95.00 ± 1.37	20.81 ± 1.05	0.220
	n-Hexane	17.36 ± 1.46	1.36 ± 0.03	0.078
	CH_2Cl_2	29.83 ± 0.27	10.84 ± 0.82	0.363
	EtOAc	125.32 ± 1.77	68.94 ± 1.03	0.550
	n-BuOH	85.36 ± 1.63	29.71 ± 0.57	0.348
	Aqueous	47.24 ± 1.28	18.05 ± 1.04	0.382

Table 5
Antioxidant activity of crude extracts and fractions of four *Hypericum* species.

Plant species	Solvent*	Radical Scavenging (%)[a]	IC_{50} (mg/mL)[b]
H. androsaemum	MeOH	96.197 ± 2.34	0.093
	n-Hexane	41.467 ± 2.76	–
	CH_2Cl_2	21.650 ± 1.83	–
	EtOAc	88.427 ± 1.25	0.20
	n-BuOH	81.368 ± 1.69	0.145
	Aqueous	54.122 ± 2.13	–
H. ericoides	MeOH	51.596 ± 1.45	–
	n-Hexane	23.165 ± 2.73	–
	CH_2Cl_2	23.979 ± 2.68	–
	EtOAc	73.732 ± 3.21	0.295
	n-BuOH	47.339 ± 1.66	–
	Aqueous	55.090 ± 1.43	–
H. x moserianum	MeOH	70.960 ± 1.51	0.232
	n-Hexane	44.868 ± 2.36	–
	CH_2Cl_2	53.802 ± 1.42	–
	EtOAc	92.528 ± 2.53	0.13
	n-BuOH	45.898 ± 2.11	–
	Aqueous	70.630 ± 2.67	0.27
H. olympicum	MeOH	82.295 ± 2.39	0.098
	n-Hexane	19.404 ± 2.04	–
	CH_2Cl_2	32.749 ± 2.47	–
	EtOAc	87.098 ± 2.80	0.121
	n-BuOH	73.917 ± 1.58	0.30
	Aqueous	40.983 ± 1.27	–
Propyl gallate	–	90.341 ± 1.03	0.034

* Scavenging concentration = 0.5 mg/mL.
[a] Mean ± SD (n = 3).
[b] IC_{50} = concentration at which 50% DPPH radical was scavenged, nd = not determined.

same plant. For crude methanol extracts the highest activity was determined in *H. androsaemum* (IC_{50} value 0.093 mg/mL) and *H. olympicum* (0.098 mg/mL). After fractionation, the highest antioxidant activity was recorded in the ethyl acetate fractions of all the plants possessing the highest phenol and flavonoid contents. Ethyl acetate fraction of *H. x moserianum* had the highest antioxidant potential with 92.528 ± 2.53% inhibition of DPPH radical (IC_{50} value 0.13 mg/mL) while the lowest activity was observed for *H. ericoides* (73.732 ± 3.21% inhibition; IC_{50} value 0.295 mg/mL). *n*-Hexane and dichloromethane fractions in all the plants had the lowest antioxidant activity that might be due to low phenolic and flavonoid contents in these fractions. Propyl gallate used as standard antioxidant gave IC_{50} value of 0.034 mg/ml. A strong correlation was determined for the phenol and flavonoid contents and antioxidant activity of the plants (Fig. 3). Bacterial infections usually cause production of ROS from oxygen metabolism thus damaging cells and tissues (Kamlesh et al., 2007). In these conditions plant extracts with antibacterial as well as antioxidant potential can serve two ways, *i.e.*, inhibit bacterial growth and reactive species simultaneously. Besides, the antioxidants also help in wound healing by scavenging ROS produced by neutrophils and monocytes during the wound healing process (Pereira et al., 2016).

Identification of selected flavonoids through HPLC in four Hypericum *species*

Seven pharmacologically active flavonoids commonly present in plants were identified and quantified in the crude methanol extracts of the four *Hypericum* species using HPLC. Among the selected flavonoids quercetin and apigenin were present in all the plants with the highest content of quercetin observed in *H. ericoides* (855.0 mg/kg fresh weight) and of apigenin observed in *H. olympicum* (2013.0 mg/kg fresh weight). *H. olympicum* also had the highest total content of detected flavonoids (6726.52 mg/kg fresh weight) while *H. x moserianum* had the least content (816.69 mg/kg fresh weight) (Table 6). The method used for HPLC analysis was optimized for simultaneous detection of seven flavonoids in the crude extracts of the selected plants in minimum run time (7 min) which is important for optimum equipment use and reduced solvent consumption. All the identified flavonoids have antibacterial activity against Gram-positive, Gram-negative and even against multidrug-resistant bacterial strains. These compounds exhibit various modes of action to inhibit bacterial growth such as formation of complexes with bacterial cell wall components, inhibiting bacterial enzymes, biofilm eradication, inhibition of efflux pumps of bacterial cells and induction of depolarization of bacterial cell membrane thereby increasing the susceptibility of clinically used antibiotics (Farhadi et al., 2018).

Antibacterial activity of extracts

Strong antibacterial activity was recorded in all the plants against all the bacterial strains with MIC values ranging between 0.001 –

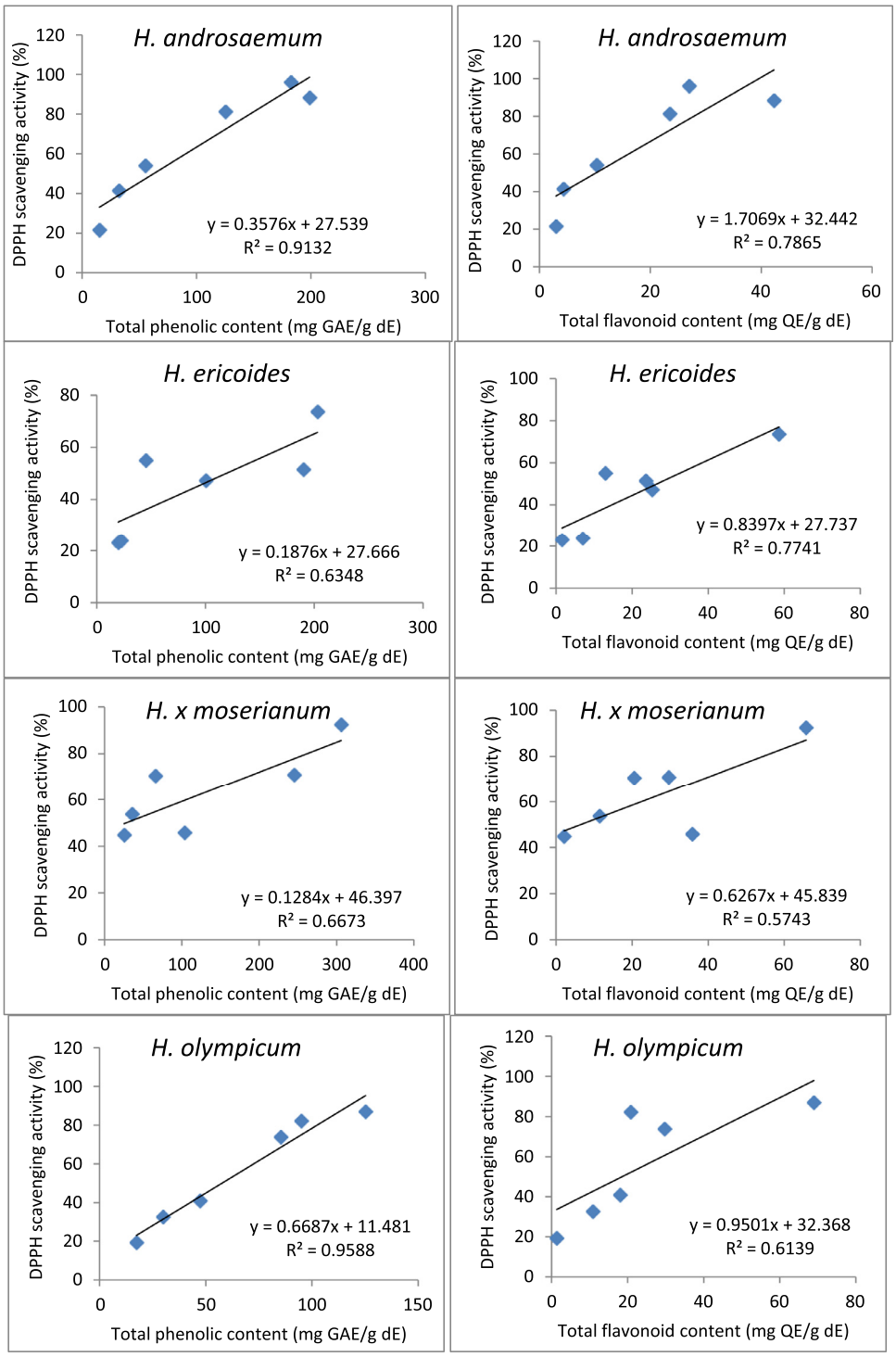

Fig. 3. Correlation between total phenolic and flavonoid contents and radical scavenging activity of four *Hypericum* species.

Table 6
Contents of flavonoid aglycones (mg/kg fresh weight) in methanol extracts of six *Hypericum* species.

	M	L	Q	A	K	I	R	Total content
t_R (min)*	1.787	1.966	2.059	2.487	2.639	2.740	3.750	
H. androsaemum	nd	nd	405.0	482.0	nd	10.50	383.65	1281.15
H. ericoides	nd	nd	855.0	0.67	nd	13.72	4.25	873.64
H. x moserianum	540.0	0.50	9.83	0.33	266.03	nd	nd	816.69
H. olympicum	nd	4.57	48.00	2013.0	nd	1038.95	3622.0	6726.52

* retention time, nd = not detected
M = Myricetin, *L* = Luteolin, *Q* = Quercetin, *A* = Apigenin, *K* = Kaempferol, *I* = Isorhamnetin, *R* = Rhamnetin.

Table 7
MIC (mg/mL) and TAA (mL/g) of extracts of four *Hypericum* species against test bacterial strains.

Plant	Extract	*S. aureus*		*B. subtilis*		*E. aerogenes*		*E. coli*		*P. aeruginosa*	
		MIC	TAA	MIC	TAA	MIC	TAA	MIC	TAA	MIC	TAA
H. androsaemum	MeOH	0.1	3550	0.1	3550	0.1	3550	0.1	3550	0.1	3550
	n-Hexane	0.1	110	0.1	110	0.1	110	0.1	110	0.1	110
	CH_2Cl_2	0.1	24	0.1	24	0.1	24	0.1	24	0.1	24
	EtOAc	0.1	1050	0.1	1050	0.1	1050	0.1	1050	0.1	1050
	n-BuOH	0.1	89	0.1	89	0.1	89	0.1	89	0.1	89
	Aqueous	0.1	219	0.1	219	0.1	219	0.1	219	0.1	219
H. ericoides	MeOH	0.1	2296	0.1	2296	0.1	2296	0.1	2296	0.1	2296
	n-Hexane	0.1	219	0.1	219	0.1	219	0.1	219	0.1	219
	CH_2Cl_2	0.1	244.8	0.1	244.8	0.1	244.8	0.1	244.8	0.1	244.8
	EtOAc	0.1	734.8	0.1	734.8	0.05	1469.6	0.1	1469.6	0.05	734.8
	n-BuOH	0.01	1377	0.025	1377	0.01	1377	0.01	550.8	0.025	550.8
	Aqueous	0.1	918.5	0.1	918.5	0.1	918.5	0.1	918.5	0.1	918.5
H. x moserianum	MeOH	0.1	2057	0.1	2057	0.1	2057	0.1	2057	0.1	2057
	n-Hexane	0.001	5300	0.01	530	0.01	530	0.001	530	0.01	530
	CH_2Cl_2	0.05	37	0.1	18.5	0.05	37	0.1	37	0.05	18.5
	EtOAc	0.1	464	0.1	464	0.1	464	0.1	464	0.1	464
	n-BuOH	0.025	100	0.1	100	0.1	25	0.025	100	0.025	25
	Aqueous	0.1	1493	0.1	1493	0.1	1493	0.1	1493	0.1	1493
H. olympicum	MeOH	0.025	19,424	0.025	19,424	0.05	9712	0.025	9712	0.05	19,424
	n-Hexane	0.01	12,830	0.01	12,830	0.001	128,300	0.01	128,300	0.001	12,830
	CH_2Cl_2	0.1	109.2	0.1	109.2	0.1	109.2	0.1	109.2	0.1	109.2
	EtOAc	0.05	4856	0.025	4856	0.05	4856	0.05	4856	0.05	9172
	n-BuOH	0.025	4676	0.025	4676	0.01	1169	0.025	1169	0.01	4676
	Aqueous	0.05	1620	0.05	810	0.1	810	0.1	810	0.1	1620

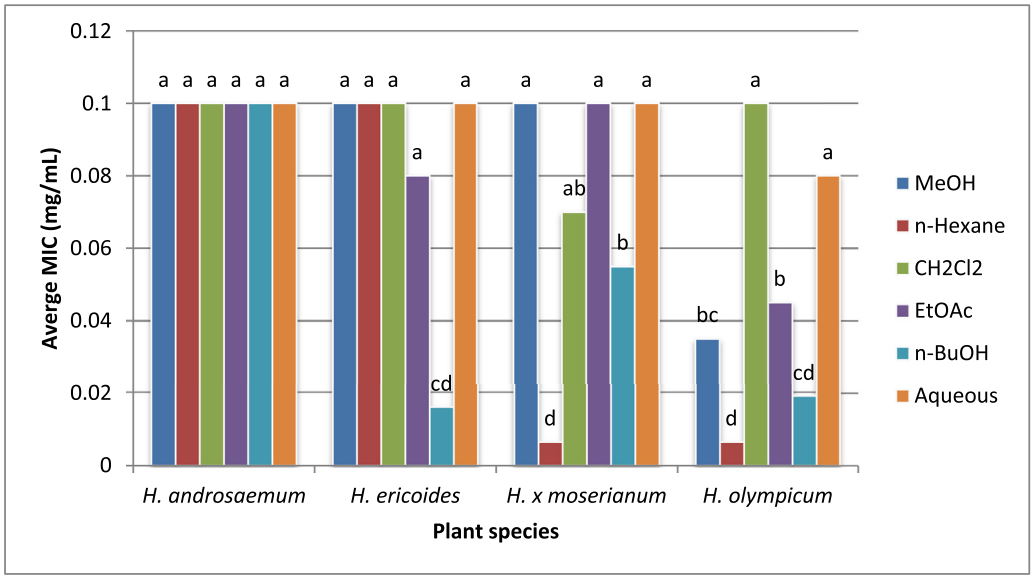

Fig. 4. Average MIC values (mg/mL) of different solvents extracts against five bacterial strains.
Bars with different alphabets are statistically significantly different from each other at $p < 0.05$.

0.1 mg/mL (Table 7). The highest activity was observed for the extracts of *H. olympicum* (MIC values 0.001 – 0.1 mg/mL) with *n*-hexane fraction being the strongest inhibitor of bacterial growth (MIC values between 0.01 to 0.001 mg/mL). The lowest antibacterial activity was recorded for extracts of *H. androsaemum* (MIC value 0.1 μg/mL). The plants were equally effective against both Gram-positive and Gram-negative bacteria. Plants and their extracts were also compared on the basis of their average MIC values. *H. olympicum* extracts had the lowest average MIC values (0.04–0.052 mg/mL) against all the bacterial strains that were significantly lower than the other plants ($p < 0.05$). The type of solvent used for extraction had a strong effect on antibacterial potential of the extract indicated in terms of variations in their average MIC values. Thus the lowest average MIC value indicating the highest antibacterial potential was observed for *n*-hexane fractions of *H. x moserianum* and *H. olympicum* (0.0064 mg/mL) followed by *n*-butanol fractions of *H. ericoides* and *H. olympicum* (0.016 and 0.019 mg/mL respectively). Significant difference was observed between the average MIC values for different solvent extracts ($p < 0.05$) in all the plants except *H. androsaemum* for which the MIC values of different extracts were same for all the bacterial strains (Fig. 4). This reflects the broad spectrum of antibacterial agents present in these plants ranging from non-polar to highly polar compounds. The Gram-negative bacteria were more sensitive to the extracts than Gram-positive ones. However, no significant difference was observed between average MIC values for Gram-positive and Gram-negative bacteria ($p > 0.05$) (Fig. 5).

The prevention of wound infections by herbal medicines is based on their antibacterial activity. A delay in wound-healing activity of

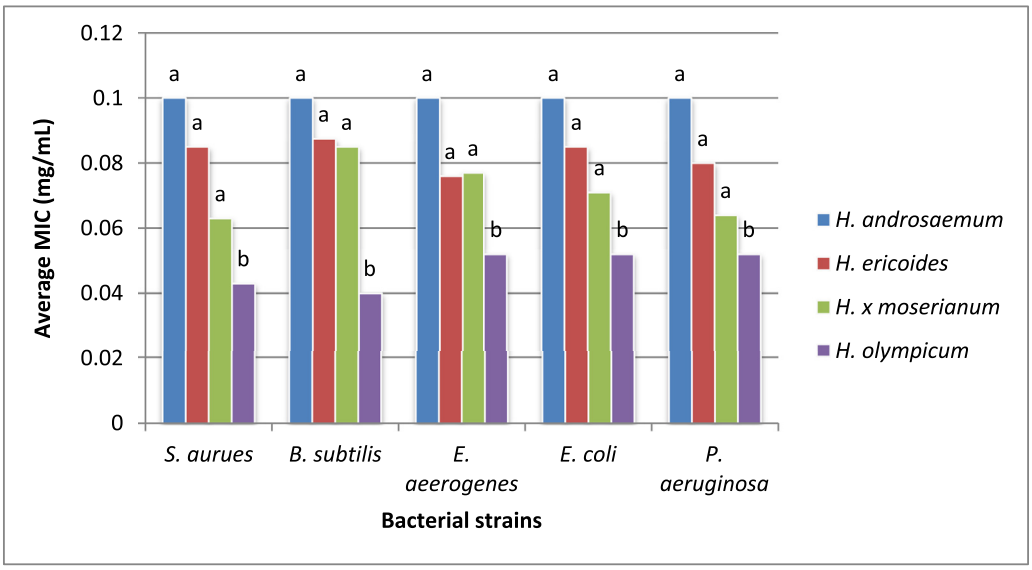

Fig. 5. Average MIC values (mg/mL) of different plants against five bacterial strains.
Bars with different alphabets are statistically significantly different from each other at $p < 0.05$.

Table 8
Average TAA (mL/g) of different solvent extracts of four *Hypericum* species against five bacterial strains.

	H. androsaemum	*H. ericoides*	*H. x moserianum*	*H. olympicum*
MeOH	3550	2296	2057	15,539.2
***n*-Hexane**	110	219	1484	59,018
CH_2Cl_2	24	244.8	29.6	109.2
EtOAc	1050	1028.72	464	5719.2
***n*-BuOH**	89	1046.52	70	3273.2
Aqueous	219	918.5	1493	1134

Table 9
Average TAA (mL/g) of four *Hypericum* species against five bacterial strains.

	S. aureus	*B. subtilis*	*E. aerogenes*	*E. coli*	*P. aeruginosa*
H. androsaemum	840.33	840.33	840.33	840.33	840.33
H. ericoides	965.01	965.01	1087.48	949.78	827.31
H. x moserianum	1575.16	777.08	767.66	780.16	764.58
H. olympicum	7252.53	7117.53	24,159.4	24,159.4	7971.86

these herbal medicines might be due to the failure of plant extracts to inhibit the growth of microorganisms causing these infections such as *E. coli, Staphylococcus, Bacillus, Pseudomonas* and *Enterobacter*. Previous antimicrobial studies of the *Hypericum* species against pathogenic bacteria have shown significant activity against *S. aureus, B. subtilis, E. coli* and *P. aeruginosa* (Özkan et al., 2018; Turker et al., 2018). The crude plant extracts are considered as potentially useful therapeutically only if they have MIC values < 8 mg/mL while isolated plant components should have MIC < 1 mg/mL (Gibbons, 2005). In the present study the extracts of selected *Hypericum* species had MIC values in the range 0.001 – 0.1 mg/mL against different bacterial strains indicating a high potential of these plants in combating infections caused by these bacteria. Compounds have been isolated from *Hypericum* species that caused bacterial cell death through apoptosis (Li et al., 2015).

MIC values indicate minimum amount of plant extract required to inhibit bacterial growth. However, these values do not indicate the actual inhibition potential of a plant against a particular bacterial strain and can often lead to wrong conclusion (Eloff, 2004). "Antibacterial activity" expressed as "total activity" is the tool for comparing different plants using the same measuring instruments. The TAA of any extract (mL/g) indicates the volume to which the active compounds in one gram of plant material can be diluted and still inhibit the growth of the microorganisms (Eloff, 2004). Thus a high TAA means that less plant material is required to inhibit bacterial growth. TAA of all the plants was calculated for each bacterial strain and highest values were recorded for *H. olympicum* (Table 7). The average TAA of *H. olympicum* extracts was higher for Gram-positive (4157.64 mL/g) than Gram-negative ones (3827.37 mL/g). For *H. androsaemum* the average total activity was the same for Gram-positive and Gram-negative bacteria (840.33 mL/g). *H. ericoides* gave a higher TAA for Gram-positive bacteria (965.01 mL/g) than Gram-negative ones (918.55 mL/g) and the average total activity of extracts of *H. x moserianum* was much higher for Gram-positive (3223.21 mL/g) than Gram-negative ones (929.15 mL/g) (Tables 8 and 9). On the basis of results of MIC values as well as total antibacterial activity, *H. olympicum* was found to be the strongest inhibitor of all the bacterial strains and thus is helpful in treating wound infections. These days some antibiotic wound dressings are being used for immediate treatment of the wounds to avoid infections. These dressings carry some natural antibacterial agents mainly essential oils of plants including essential oil of *H. perforatum* (Negut et al., 2018). Extracts of the plant species used in our study can be tested for their use as antibacterial agents in preparation of such dressings. The plants can also be considered for isolation of compounds with antibacterial and wound healing activities.

Conclusion

The plant species used in the present study particularly *H. olympicum* showed strong antibacterial activity against Gram-positive and Gram-negative bacterial strains associated with wound infections. *n*-Hexane and *n*-butanol extracts were particularly effective in inhibiting bacterial growth. Plants also had strong antioxidant activity and possessed high phenolic and flavonoid contents.

Declaration of Competing Interest

The authors of the submitted manuscript hereby submit that there is no conflict of interest.

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