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Radical Scavenging and Antimicrobial Activities of *Convolvulus microphyllus*

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Convolvulus microphyllus Sieb. (family: *convolvulaceae*) is reported to be brain tonic and useful in CNS disorder. Powdered whole plant material was sequentially extracted with petroleum ether, ethyl acetate, methanol and water, dried *in vacuo* and weighed (%). Later, each of these extracts was tested for antioxidant (DPPH and FRAP method) and antimicrobial potentials (agar well diffusion method). Interestingly, ethyl acetate extract showed appreciable results (IC_{50} 0.07 mg/mL, % inhibition 93.46) followed by the methanol extract (IC_{50} 0.075 mg/mL, % inhibition 92.43) in DPPH method. In FRAP method, water extract was potentially active having antioxidant capacity 460 ± 7.64 ascorbic acid equivalent/mg extract. In antimicrobial activities, the ethyl acetate extract demonstrated appreciable activity against *C. albicans* and *P. chrysogenum* (IZ 20.00 ± 1.52 mm and 19.00 ± 1.00 mm respectively) followed by its methanol extract. Further, from its ethyl acetate and methanolic extracts different phenolic acids namely caffeic acid, *p*-coumaric acid, ferulic acid, gallic acid, vanillic acid and syringic acid were isolated.

Key Words: *Convolvulus microphyllus* sieb., Antioxidant, Antimicrobial potentials.

INTRODUCTION

Convolvulus microphyllus Sieb. ex Spreng (*convolvulaceae*) is perennial herb distributed throughout the plains of North India and Bihar. This plant is useful in depression, epilepsy, nervous debility, insomnia and in improvement of memory[1]. Genus *Convolvulus* consists about 42 species, among these detailed work on secondary metabolites has been carried out only in some species. Glycosides such as microphyllic acid and kaempferol 3-D-glucoside, β-sitosterol[2-5] alkaloids-convolvine, convolamine, phyllabine, convolidine, confoline and phenolic acid scopoline have been isolated from *C. microphyllus*[6,7].

In antimicrobial activity, ethanolic extract has been tested against some Gram +ve bacteria and dermatophytes[8,9]. In the present paper we report the antimicrobial and antioxidant activities and isolation of phenolic acids from *C. microphyllus*.

EXPERIMENTAL

Silica gel, 2,2-diphenyl-1-picrylhydrazyl (DPPH), quercitin, potassium dihydrogen phosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4), methanol, trichloroacetic acid (TCA), ascorbic acid, potassium ferricynide $K_3[Fe(CN)_6]$, ferric chloride ($FeCl_3$) sabouraud dextrose broth (SDA) and Müller-Hinton agar (MH) were purchased from HiMedia and Merck (Mumbai, India). All reagents and solvents used in the experiments were of analytical grade.

Preparation of the extracts for biological activity: During the course of studies, authenticated whole plants of *C. microphyllus* were collected from the campus of University of Rajasthan, Jaipur, during the months of January- February 2010. Voucher specimen was compared with herbarium sheets in the Botany department, University of Rajasthan, Jaipur. Whole plants (500 g) were collected, washed carefully, shade-dried, powdered and extracted in a soxhlet apparatus with 500 mL of ethanol (3 × 24 h). The extract was filtered, concentrated, dried and further fractionated sequentially to petroleum ether, ethyl acetate, methanol and water. Resultant extracts were concentrated, dried, weighed and stored at 4 ºC, till further studies.

Antioxidant activity

DPPH free radical scavenging activity: The effect on DPPH radical was determined using the method by Fogliano *et al.*[10]. Different concentrations of extract (0.8, 0.6, 0.4, 0.2, 0.1 mg/mL) were prepared in methanol and mixed with 2.5 mL of DPPH (2 mg/10 mL methanol). After 0.5 h of incubation time, optical density (OD) was measured at 517 nm using a UV-VIS spectrophotometer (varian type cary PCB 150 water peltier system with standard cuvetts). Data was recorded in

TABLE-1
In vitro ANTIOXIDANT ACTIVITY OF *Convolvulus. microphyllus* BY DPPH AND FRAP METHOD

Nature of extract	[a]% Inhibition (DPPH method) (Concentration in mg/mL)						Antioxidant activity in [b]AAE /mg dw (Concentration in µg/mL)				
	IC_{50}	0.1	0.2	0.4	0.6	0.8	62.5	125	250	500	1000
Pet. ether	0.085	61.69	63.99	65.09	65.24	66.11	24.83±0.55	31.66±6.66	35.00±0.00	46.66±1.66	65.00±5.79
Ethyl acetate	0.07	76.84	83.68	90.97	92.23	93.46	50.00±0.00	43.33±1.67	65.00±5.79	105±10.01	178.33±1.66
Methanol	0.075	71.22	84.79	92.02	91.97	92.43	28.33±4.41	33.33±1.66	48.33±1.66	58.33±7.27	203.33±3.33
Aqueous	0.103	39.17	65.72	71.51	88.34	91.36	33.33±1.66	45.00±2.89	63.33±3.33	188.33±1.07	460.00±7.64
Quercetin	0.04	62.42	80.58	93.38	93.82	94.71	-	-	-	-	-
Ascorbic acid	-	-	-	-	-	-	62.5	125	250	500	1000

[a]% Inhibition = 1-(Absorbance of the sample/Absorbance of the control) × 100; [b]AAE/g = Ascorbic acid equivalent/ mg extract.

triplicate and processed using EXCEL. The concentrations that cause 50 % reduction in absorbance (IC_{50}) were calculated. Percent inhibition of DPPH was calculated by following equation.

$$\% \text{ Inhibition} = 1-(OD_{Sample}/ OD_{Control}) \times 100$$

where, OD_{Sample} is the absorbance of the test sample and $OD_{Control}$ as the absorbance of the test control.

FRAP activity: Total reducing power of extracts was determined according to FRAP (Ferric ion reducing antioxidant potentials) method[11]. Specific concentration of extracts (ascorbic acid) and extract (62.5-1000 µg/mL) was prepared in 1 mL ethanol separately, mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and $K_3[Fe(CN)_6]$ (2.5 mL, 1 %). After incubation at 50 ºC (20 min), 2.5 mL of 10 % TCA, 2.5 mL of distilled water and $FeCl_3$ (0.5 mL, 1 %; chromogenic reagent) were added in the sequence and absorbance was measured at 700 nm. Standard calibration curve of ascorbic acid was prepared using 10-500 mg/L concentrations and total antioxidant activity was calculated in mg of ascorbic acid equivalents (mg AAE/g of extract). All determinations were carried out in triplicate and statistically analyzed.

Antimicrobial activity: For antibacterial screening, pure cultures of test bacteria, *Bacillus subtilis, Enterobacter aerogenes, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Raoultella planticola* and *Staphylococcus aureus* and for antifungal screening *Aspergillus flavus*, *A. niger, Candida albicans, Penicillium chrysogenum* and *Tricophyton rubrum* obtained from obtained from the IMTECH, Chandigarh, India. Antimicrobial assay was performed by agar well diffusion method[12]. Inoculums were prepared by suspending bacteria in nutrient broth (NB) and fungus in sabouraud dextrose broth medium overnight (10^6-10^7 CFU/ mL concentration). Bacterial and fungal suspensions were inoculated in Muller-Hinton agar and sabouraud dextrose borth poured plates respectively along with the test extracts. Theses plates were incubated at 37 ºC for bacteria and 25 ºC in case of fungi for appropriate time periods under aerobic conditions. The diameter of the inhibition zone (IZ) around each well was measured and recorded by inhibition zone recorder (HiMedia, India).

Isolation and identification of compound: Thin layer chromtography (TLC) of ethyl acetate and methanol extracts was carried out on silica G plates (0.4-0.5 mm) and developed in different solvent systems such as methanol:acetic acid:water (18:1:1), methanol:water (4:1), isopropanol:water (4:1), ethahnol:water (3:2), 10 % acetic acid and ethyl acetate:toluene :acetic acid (5:4:2) for better resolution and sprayed with vanillin-sulfuric acid and Folin's reagent reagent[13] seperatly. Later, in the presence of phenols from the extract spots coinciding to the reference markers (caffeic acid, *p*-coumaric acid, ferulic acid, gallic acid, vanillic acid and syringic acid) were scrapped from unsprayed plates, eluted with methanol, filtered, evoparated to dryness, reconstituted and crystallized. The melting point of the isolated compounds was determined in capillary tubes (toshniwal melting point apparatus) and subjected to IR (KBr pellets on A 400S Shimadzu FTIR spectrometer) and NMR (Jeol AL 300 MHz instument using $CDCl_3$ and DMSO-d_6 as solvent and TMS as internal reference) spectral studies.

RESULTS AND DISCUSSION

Variable yield of the various fractions were recorded (petroleum ether 2.22 %, ethyl acetate 1.40 % and aqueous extract 2.72 %), where the maximum yield was recorded in the methanol extract (7.00 %).

DPPH free radical scavenging activity: The stable DPPH radical model is a widely-used, relatively quick method for the evaluation of free radical scavenging activity. DPPH is a stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule[14]. The reduction capability of the DPPH radical is determined by the decrease in its absorbance at 517 nm, induced by antioxidants. The absorption maximum of a stable DPPH radical in ethanol was at 517 nm. The decrease in absorbance of DPPH radical is caused by antioxidants, because of the reaction between antioxidant molecules and radical, progresses. It is visually noticeable as a change in colour from purple to yellow and lower the absorbance higher the antioxidant capacity[15]. In several studies on antioxidant activity, ethyl acetate and methanolic extracts demonstrated similar IC_{50} value (0.07 and 0.075 mg/ mL respectively). At its 0.8 mg/mL concentration, % inhibition of ethyl acetate and methanol extract was 92.23 and 91.97 respectively (Table-1 and Fig. 1). These results indicated that DPPH activity is concentration dependent. Likewise, free radical scavenging activity also increased with increasing concentration.

FRAP method: This is a novel method for assessing antioxidant power of various antioxidants. The FRAP assay directly measures the ability of antioxidant to reduce ferric tripyridyltriazine complex (Fe^{3+}-TPTZ) to ferrous complex (Fe^{2+}-TPTZ) at low pH produces blue ferrous form, with an increase in absorbance at 700 nm. The final results were expressed as mg ascorbic acid equivalents (AAE/ gm) on dried basis. In FRAP method, aqueous extract showed 460.00 ± 7.64 AAE/mg dw of antioxidant potentials and 203.33 ± 3.33 AAE/ mg dw of methanol extract (Fig. 2).

TABLE-2
ANTIMICROBIAL ACTIVITY OF *C. microphyllus* WHOLE PLANT SEQUENTIAL EXTRACT AGAINST VARIOUS PATHOGENIC MICROORGANISMS

Test microorganisms	Sequential extracts							
	Petroleum ether		Ethyl acetate		Methanol		Aqueous	
	IZ[a]	AI[b]	IZ[a]	AI[b]	IZ[a]	AI[b]	IZ[a]	AI[b]
E. aerogenes	15.00 ± 0.57	1.02	13.00 ± 1.00	0.92	12.00 ± 0.81	0.85	10.00 ± 0.00	0.71
E. coli	16.66 ± 0.73	0.87	16.00 ± 0.57	0.84	14.33 ± 0.33	0.75	12.66 ± 0.33	0.66
P. aruginosa	15.33 ± 0.88	0.76	14.00 ± 0.57	0.70	13.00 ± 0.00	0.65	11.00 ± 0.57	0.55
R. planticola	14.66 ± 0.88	0.66	14.66 ± 0.88	0.66	10.00 ± 0.00	0.45	11.00 ± 0.00	0.50
S. aureus	17.66 ± 1.20	0.84	16.33 ± 0.66	0.71	19.33 ± 1.45	0.92	13.33 ± 0.87	0.63
A. flavus	15.00 ± 0.57	0.55	17.66 ± 0.33	0.65	11.66 ± 0.33	0.43	12.33 ± 0.32	0.45
A. niger	–	–	–	–	–	–	–	–
C. albicans	16.33 ± 0.33	0.74	20.00 ± 1.52	0.90	16.00 ± 0.57	0.72	11.00 ± 0.00	0.50
P. chrysogenum	15.00 ± 0.57	0.71	19.00 ± 1.00	0.90	15.00 ± 1.00	0.71	10.33 ± 0.88	0.49
T. rubrum	15.66 ± 0.33	0.54	17.00 ± 0.57	0.80	17.33 ± 0.66	0.82	12.33 ± 0.88	0.58

Test samples 4 mg/well. Standard test drugs: Gentamycin for bacteria, Ketonocozole for fungi (10 mcg/disc). [a]IZ=Inhibition zone (in mm) including the diameter of well (6 mm). AI[b] = Activity index = Inhibition zone of the sample/Inhibition zone of the standard.

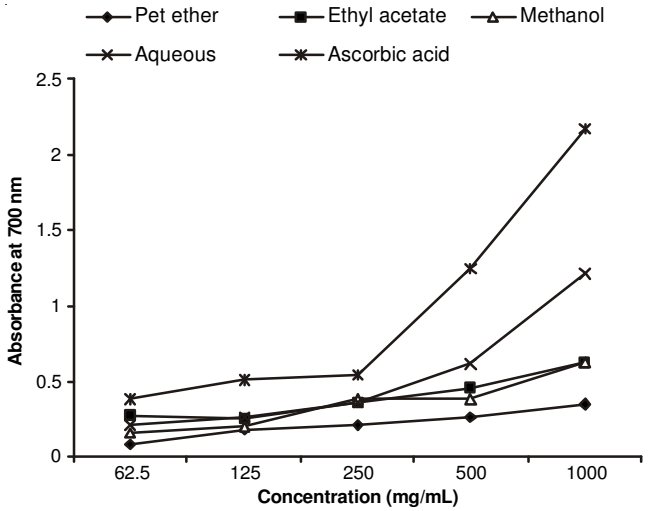

Fig. 1. Antioxidant activity of *C. microphyllus* by DPPH method.

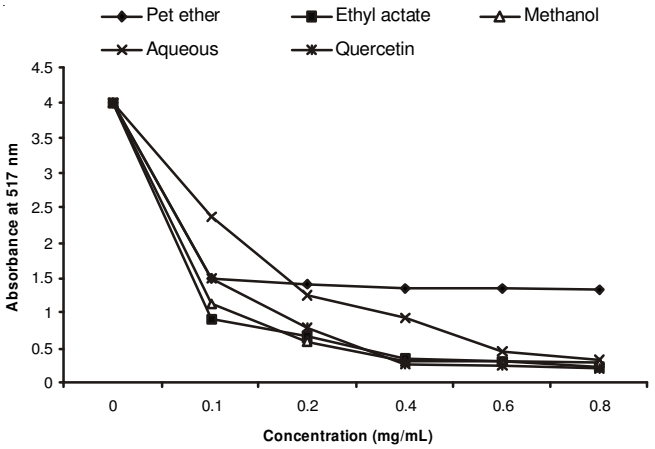

Fig. 2. Antioxidant activity of *C. microphyllus* by FRAP method.

Antimicrobial activity: *C. microphyllus* demonstrated different antimicrobial behaviours. Appreciable antibacterial activity was documented by methanolic extract against *S. aureus* (IZ 19.33 ± 1.45 mm), followed by pet. ether extract, active against *S. aureus, E. coli* and *P. aeruginosa* (IZ 17.66 ± 1.20, 16.66 ± 0.73 and 15.33 ± 0.88 mm respectively). Maximum antifungal activity demonstrated by ethyl acetate extract against *C. albicans* and *P. chrysogenum* (IZ 20.00 ± 1.52 and 19.00 ± 0.57 mm respectively). Ethyl acetate and methanol extracts proved more effective in antimicrobial efficacy as compared to others (Table-2).

Isolation and identification of compounds: Phenolic acids were isolated from ethyl acetate and methanol extract using various TLC solvent systems. On the basis of IR and NMR spectra isolated compounds are documented in Table-3, Fig. 3.

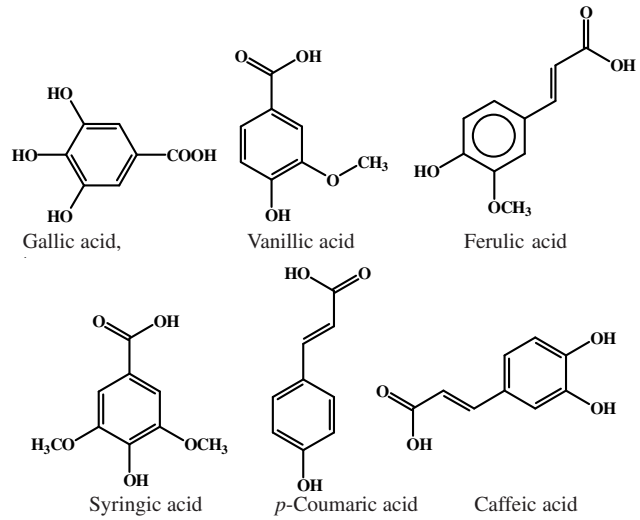

Fig. 3. Structure of the isolated phenolic acids.

Phenolic acids are a class of plant secondary metabolites that contain one or more hydroxyl derivatives of benzene rings. Phenolics are widely distributed in plants and are used for defensive functions[13]. Phenolics in plants are mainly synthesized from the phenylpropanoid pathway. *In vitro* antimicrobial activities of the phenylpropanoid pathway intermediates, including *p*-coumaric acid, caffeic acid, ferulic acid and other pathway derivatives have been demonstrated experimentally[10,11]. Flavonoids and phenolic acids have antioxidative properties and anticarcinogenic effects[12,14-16]. Inverse relationships between the intake of flavonoids/ phenolic acids (flavonols and flavones) and the risk of coronary heart disease, stroke, lung cancer and stomach cancer[16-19] have been shown in epidemological studies.

TABLE -3
COMPOUND ISOLATION USING DIFFERENT TLC SOLVENT SYSTEMS

Isolated compounds	Solvent systems						m.p. (^{0}C)	IR spectra (cm^{-1})	H^1 NMR Spectra (δ)
	I	II	III	IV	V	VI			
Caffeic acid	-	-	-	-	-	0.96	195-198	3440, 1640, 1448, 1212, 1172, 1118, 972, 899, 849, 812	11.92 (1H, s, COOH), 8.29, (1H, br, s, OH), 8.07 (1H, br s, OH), 7.52 (1H, d, *J*=15.9 Hz, H-7), 7.08 (1H, s, H-2), 6.91 (1H, d, *J*=8.1 Hz, H-5), 6.83 (1H, d, *J*=8.1 Hz, H-6).
Ferulic acid	0.73	0.53	0.53	0.52	-	-	168-171	3427, 2921, 1620, 1516, 1432	12.21 (1H, s, COOH, 1H), 9.08 (1H, s, OH), 7.59 (1H, d, *J*=15.9 Hz, H-7), 7.36 (1H, s, H-2), 7.04 (1H, d, *J*=8.1 Hz, H-5), 6.93 (1H, d, *J*=8 Hz, H-6), 6.32 (1H, d, *J*=15.9 Hz, H-8), 3.93 (3H, s, OCH_3).
Gallic acid	-	-	-	-	0.46	-	250-252	3367, 3064, 2654-2907, 1702, 1618, 1541, 1246, 1026.	9.21 (1H, s, COOH), 6.96 (2H, s, H-2, 6), 3.86-3.37 (4H, br, OH).
p- Coumaric acid	0.67	0.55	0.04	0.45	-	-	210-213	3384, 2910, 1672, 1627, 1602, 1512, 1450, 1245, 977, 833, 516.	12.09 (1H, s, COOH, 1H), 9.18 (1H, s, OH), 7.59(1H, d, *J*=16.2 Hz, H-7), 7.00 (2H, d, *J*=7.8 Hz, H-2, 6), 6.86 (2 H, d, *J*=7.8 Hz, H-3, 5), 6.29 (1H, d, *J*=16.2 Hz, H8).
Syringic acid	0.72	0.16	0.60	0.67	-	-	206-209	3470, 2926, 1682, 1520, 1463, 1382, 1264, 1200, 1028, 760	12.32 (1H, s, COOH), 9.68 (1H, s, OH), 7.20 [20 (2H, s, H-2, 6), 3.80 (3H, s,-OCH_3].
Vanillic acid	0.71	0.55	0.60	0.50	-	-	210-213	3484, 3097, 1680, 1597, 1522, 1434, 1298, 1284, 1238, 1203, 1028, 757	12.48 (1H, s, COOH), 9.85 (1H, s, OH), 7.45 (1H, d, *J*=8.7 Hz, H-5), 7.43 (1H, s, 2H), 3.80 (3H, s, OCH_3).

I- Methanol: acetic acid : water (18:1:1); II- Methanol : water (4:1); III- Isopropanol : water (3:2); IV- Ethanol : water (3:2); V- Water : acetic acid (9:1); VI- Ethyl acetate : toluene : acetic acid (5:4:2).

Conclusion

Ethyl acetate and methanol fractions-rich in phenolic acids exhibited pronounced antioxidant and antibacterial activities. As global antibiotic resistance by bacteria is becoming an interesting public health concern and the race to discover the new antibacterial agent is on, *C. microphyllus* along with its neuroprotective activity demonstrated promising antibacterial activity, could be used for further studies as an alternative herbal remedy.

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