Asian Journal of Chemistry; Vol. 24, No. 8 (2012), 3513-3516

ASIAN JOURNAL OF CHEMISTRY

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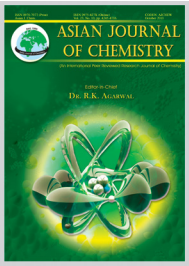

Studies on Antimicrobial and Antioxidant Potentials of *Pergularia daemia* (Forsk.) Chiov.

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(*Received*: 23 July 2011; *Accepted*: 14 March 2012) AJC-11179

Pergularia daemia (Forsk.) Chiov., a perennial twining wild herb is known for its varied medicinal properties. In the present study, whole plant material was successively extracted with pet. ether, hexane, dichloromethane, chloroform, ethyl acetate, acetone and methanol, concentrated and investigated for their phytochemical, antibacterial and antioxidant profile. α-, β-amyrin, β-sitosterol, lupeol, lupeol acetate, oleanolic acid, kaempferol and quercetin was isolated. In antibacterial screening, appreciable activities were demonstrated by hexane extract against *E. aerogenes* (19.66 ± 0.66 mm; MIC 62.5 µg/mL) and ethyl acetate extract against *E. coli* (19.99 ± 0.67 mm; MIC 31.2 µg/mL) whereas in antifungal screening, acetone extract against *A. flavus* (16.33 ± 0.87 mm; MIC 125 µg/mL) and chloroform extract against *C. albicans* (15.66 ± 0.66 mm; MIC 62.5 µg/mL). In antioxidant efficacy, methanol extract demonstrated higher efficacy, *i.e.* with lowest IC_{50} value (maximum antioxidant capacity; 0.075 mg/mL with 363.33 ± 3.33 mg AAE/g antioxidant capacity in FRAP assay).

Key Words: *Pergularia daemia*, Antimicrobial, Antioxidant, Ascorbic acid.

INTRODUCTION

Pergularia daemia a perennial twining herb, grows wildly along the road side. Its latex or leaf paste is applied on boil, sap of the leaves used to cure eye sore, rheumatism, edema, kidney pains, catarrhal affection, infantile diarrhea, asthma and rumen swelling, whereas its root bark is purgative[1,2]. Whole plant is used as antihelmenthic, laxative, antipyretic, treatment of dysentery, malarial intermittent fevers and jaundice[3-5]. Phytochemically, triterpenes, cardenolides, saponins, alkaloids and steroids have been isolated[6,7]. Antiinflammatory, antipyretic, analgesic, antifertility, antidiabetic, antibacterial and hepatoprotective activities[6,8-12] have been demonstrated. In this paper, we report the antimicrobial and antioxidant efficacies of the sequential metabolites rich fractions of whole plant of *Pergularia daemia*.

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, ascorbic acid, potassium ferricynide, ferric chloride, Sabouraud dextrose broth and Müller-Hinton agar 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 *P. daemia* (Forsk.) Chiov. were collected from Campus, University of Rajasthan, Jaipur, during the months of July-September, 2008 and voucher specimen was deposited in the Herbarium, Botany Department, University of Rajasthan, Jaipur. Whole plants (200 g) were collected, washed carefully, shade-dried, powdered and extracted in a Soxhlet apparatus with 500 mL of ethanol (3 × 24 h). Extract were filtered, concentrated, dried and further fractionated sequentially to pet. ether, hexane, dichloromethane (DCM), chloroform, ethyl acetate, acetone and methanol. Resultant extracts were concentrated, dried, weighed and stored at 4 ºC, till studied further. Each of the extract was tested for the metabolites following the standard protocols[13].

Total phenolics contents: The total phenolics content was determined with Folin-Ciocalteau reagent[14]. Optical density (OD) was measured at 750 nm (Shimadzu, Pharmaspec UV-VIS spectrophotometer). A standard calibration curve of gallic acid (10-500 mg/mL) was prepared and total phenolics in the extracts were expressed in mg of gallic acid equivalents (mg GAE/g) of extract.

Total flavonoid contents: Total flavonoids were estimated by $AlCl_3$ spectrophotometic method[15]. Optical density was taken at 510 nm and compared with standard curve of quercetin (10-100 mg/mL). The total flavonoids were expressed as mg of quercetin equivalents (mg QE/g) of extract.

Isolation of compounds: Thin layer chromatography of pet. ether and hexane extracts (triterpenoids-rich fraction) was

TABLE - 1
TOTAL PHENOLICS, FLAVONOIDS AND ANTIOXIDANT EFFICACY BY FRAP METHOD

Nature of extract	Total phenolics (GAE/gdw)[a]	Total flavonoids (QE/gdw)[b]	Antioxidant activity (AAE/gdw[-c])				
			62.5	125	250	500	1000
Pet. ether	55.66 ± 0.52	27.13 ± 0.37	133.33 ± 8.34	266.66 ± 1.67	271.66 ± 3.33	271.66 ± 3.33	331.66 ± 6.01
Hexane	58.33 ± 0.34	28.33 ± 0.33	116.66 ± 3.38	123.33 ± 1.66	210.00 ± 0.00	258.33 ± 3.22	285.00 ± 7.64
DCM	63.00 ± 0.57	25.66 ± 0.50	120.00 ± 0.00	141.66 ± 6.17	246.66 ± 6.17	275.00 ± 0.00	315.00 ± 1.66
Chloroform	53.66 ± 1.20	26.33 ± 0.67	95.00 ± 2.89	103.33 ± 1.66	118.33 ± 1.66	210.00 ± 0.00	273.33 ± 1.66
Acetone	76.66 ± 0.64	26.33 ± 0.88	106.66 ± 6.67	110.00 ± 7.64	218.33 ± 8.34	273.33 ± 1.66	311.66 ± 1.66
Ethyl acetate	103.33 ± 0.05	32.33 ± 0.88	103.33 ± 1.67	103.33 ± 1.67	120.00 ± 0.00	176.66 ± 1.66	363.33 ± 3.33
Methanol	109.00 ± 1.00	9.76 ± 1.48	105.00 ± 2.89	120.00 ± 2.89	121.66 ± 3.33	240.00 ± 7.96	298.33 ± 3.72
Ascorbic acid	-	-	62.5	125	250	500	1000

[a]GAE/gdw = Gallic acid equivalents/g dry weight extract; [b]QE/gdw = Quercetin equivalents/g dry weight extract; [c]AAE/g = Ascorbic acid equivalents/g dry weight extract.

carried out on Silica gel G pre-coated TLC plates (Merck; 20 × 20 cm; thickness 0.2-0.3 mm) activated at 100 ºC for 0.5 h and brought to room temperature, just before use. Extract and standard compound were applied on these plates, developed in solvent mixture of benzene-heptane-alcohol (100:100:1). The developed plates were air-dried, sprayed with 10 % alcoholic H_2SO_4 and anisaldehyde reagent, separately and heated to 100 ºC for few min until characteristics colours developed. Later, chromatograms were visualized under UV light (366 nm).

Methanol and ethyl acetate extracts (flavonoids-rich fractions), were acid hydrolyzed (7 % H_2SO_4; 10 mL/g) for 2 h. Each of the hydrolyzates was filtered, extracted thrice with ethyl acetate, pooled, washed thoroughly with distilled water till neutrality and concentrated *in vacuo*. The fraction was discarded and remaining fraction was reconstituted in ethanol before chromatographic analysis. Each of the extracts was applied on TLC plates along with the reference flavonoid markers and developed in selected solvent systems (benzene-acetic acid-water 125:72:3). Developed and air dried plates were sprayed with 5 % alcoholic $FeCl_3$ as also with 1 % alcoholic $AlCl_3$ separately for flavonoids and heated to 100 ºC for few min, until characteristic colours developed and their R_f values calculated.

Using preparative TLC, triterpenes and flavonoids were separated in previously mentioned solvent systems (benzene-heptane-alcohol 100:100:1 for triterpenoids and benzene-acetic acid-water 125:72:3 for flavonoids), after visualized under I_2 vapours, spots coinciding to reference compounds were marked, scrapped, eluted with chloroform and ethyl acetate. Each of elutes was dried, reconstituted in chloroform and crystallized using methanol. Later, the isolated compounds were subjected to melting point in capillary tubes (Toshniwal melting point apparatus) and IR (KBr pellets on A400S Shimadzu FTIR spectrometer) spectral studies.

Total reduction capability by ferric ion reducing antioxidant potentials method: Total reducing power of extracts was determined according to ferric ion reducing antioxidant potentials (FRAP) method[16]. The optical density was measured at 700 nm using a UV-VIS spectrophotometer. Ascorbic acid was used as positive control. A standard calibration curve of ascorbic acid (10-500 mg/mL) was prepared and antioxidant activity was expressed in mg of ascorbic acid equivalents (mg AAE/g) of extract.

Free radical scavenging activity by 2,2-diphenyl-1-picryl-hydrazyl: The effect on DPPH radical was determined using the method by DPPH reagent[17]. The optical density was measured at 517 nm using a UV-VIS spectrophotometer where quercetin was used as a standard. The capability to scavenge the DPPH radical was calculated using following equation:

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

where, $OD_{Control}$ is the absorbance of the control (containing all reagents except the test compounds) and OD_{Sample} is the absorbance of test sample. The optical density of control was considered as 100 % unreduced DPPH and IC_{50} values were determined as the concentration of the extract required to achieve 50 % reduction in DPPH radicals.

Antimicrobial efficacy: For antibacterial screening, pure cultures of test bacteria and fungi were obtained from IMTECH, Chandigarh and IARI, New Delhi, respectively (Table-1). Bacterial cultures were grown and maintained on nutrient broth (NB) medium at 27 ºC for 48 h and fungal cultures on Sobouraud dextrose agar medium at 37 ºC for 48 h. Antimicrobial assay was performed by agar well diffusion method[18]. Inoculums were prepared by suspending the bacteria in nutrient broth medium and fungus in Sobouraud dextrose agar medium overnight at 37 ºC (10^6-10^7 CFU/mL concentration). 20 µL of bacterial and 80 µL of fungal suspensions were inoculated in nutrient agar and Sobouraud dextrose agar plates respectively. 4 mg extract concentration was used for each well. To ensure diffusion of sample into agar, the plates were incubated at 4 ºC for 1 h. Later, such 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 around each well was measured. Agar well diffusion method[18] was used for the determination of MIC of crude plant extracts. Serial dilutions of the extracts ranging 2000 µg to 20 µg were prepared, inoculated the plates and the zone was measured followed by statistical analysis of the data (Mean ± S.E.). Gentamycin (10 µg/mL) in case of bacteria and ketoconozole (100 units/mL) in case of fungi were used as standard antibiotics.

All experiments were performed in triplicates and data were analyzed according to the one-way ANOVA method using SSPS 10.0 (USA) program. Mean values (three replicates ± S.E.) were statistically compared with Duncan test, at probability level $P \geq 0.05$.

RESULTS AND DISCUSSION

All the successive extracts were found to be rich in one or other metabolites *viz*., petroleum ether and hexane rich in triterpenoids, dichloromethane in steroids, chloroform in cardenolides, ethyl acetate in flavonoids, acetone fraction in flavonoids and cardenolides and methanolic fraction in alkaloids, phenolics and saponins.

Phytochemical studies: Likewise, in the entire test extracts, higher levels of total phenolics were detected in methanol extract (109.00 ± 1.00 mg GAE/g) while total flavonoids in ethyl acetate extract (32.33 ± 0.88 mg QE/g; Table-1). On the basis of TLC behaviour of pet. ether and hexane extracts, major spots (R_f 0.06, 0.20, 0.26, 0.32 and 0.36 in solvent system of benzene-heptane-alcohol, 100:100:1) were observed similar to the position as of markers used under UV light, to α-amyrin, β-amyrin, oleanolic acid, β-sitosterol, lupeol and lupeol acetate. Later, these spots gave similar colour reactions with anisaldehyde reagent. On ethyl acetate and methanol extract analysis, two spots coincided to the reference kaempferol and quercetin (R_f 0.86 and 0.78 in solvent system of benzene-acetic acid-water, 125:72:3). These spots gave positive reactions to the spray reagents used. The spots corresponding to standard markers were isolated by PTLC and dissolved in methanol. A comparison of TLC behaviour, physical data and spectral properties were made and found to be in accordance with those reported for the authentic samples.

Ferric ion reducing antioxidant potentials method: In ferric ion reducing antioxidant potentials antioxidant assay methanol and ethyl acetate extracts due to higher phenolic and flavonoid levels demonstrates better antioxidant potentials (363.33 ± 3.33 and 298.33 ± 3.27 mg AAE/g respectively; Table-1).

DPPH free radical scavenging activity: Higher anti-oxidant potentials were demonstrated by methanol extract with lower IC_{50} value (maximum antioxidant capacity 0.075 mg/mL) followed by ethyl acetate extract (0.085 mg/mL; Table-2). However the promising antioxidant activity was demonstrated by acetone extract (IC_{50} 0.10 mg/mL and 311.66 ± 1.66 mg AAE/g).

TABLE - 2
ANTIOXIDANT EFFICACY BY DPPH METHOD

Nature of extract	IC_{50} (mg/mL)	% Inhibition (mg/mL)				
		0.1	0.2	0.4	0.6	0.8
Pet. ether	0.55	44.18	48.76	48.76	49.68	52.13
Hexane	0.60	43.57	43.64	44.46	50.75	52.13
DCM	0.45	43.57	43.64	44.64	63.32	64.34
Chloroform	0.55	42.55	45.79	49.01	49.52	53.74
Acetone	0.10	66.49	68.25	70.30	73.19	75.93
Ethyl acetate	0.085	66.49	68.25	70.30	73.19	74.49
Methanol	0.075	51.41	54.15	59.87	72.68	75.94
Quercetin	0.06	62.42	80.58	93.38	93.82	94.71

% Inhibition = 1-(Absorbance of the sample/Absorbance of the control) × 100

Antimicrobial activity: Among all the test extracts, in antibacterial screening, hexane extract showed appreciable activity against *E. aerogenes* (19.66 ± 0.66 mm, MIC 62.5 µg/mL), ethyl acetate extract against *E. coli* (19.99 ± 0.67 mm, MIC 31.2 µg/mL) and methanol extract against *R. planticola* (19.00 ± 0.57 mm, MIC 62.5 µg/mL). In antifungal screening, acetone extract demonstrated maximum inhibition against *A. flavus* (16.33 ± 0.87 mm, MIC 125 µg/mL), chloroform extract against *C. albicans* (15.66 ± 0.66 mm, MIC 62.5 µg/mL) and hexane extract against *P. chrysogenum* (15.66 ± 0.32 mm, MIC 125 µg/mL; Table-3).

The ethyl acetate and methanol extract appear to have the highest concentrations of the total phenolics and flavonoids. Plant also showed presence of pharmacologically active secondary metabolites. All the extracts showed variable results of antimicrobial activity, having inhibition zones ranging from 10 to 19 mm. It is interesting to note that hexane, ethyl acetate and chloroform extracts considerably inhibit the growth of all the test microbes. Many of the test microbes develop resistance to commonly employed antibiotics which cause nosocomial infection[19,20]. Thus, the data on antimicrobial efficacies of *P. daemia* might be useful in the development of further such effects. Traditional healers also use the plant concoctions, in treating inflammation, pain and depression may be due to high amount of antioxidant compounds.

Exogenous and endogenous metabolic processes in human body produce highly reactive free radicals resulting in cell death, tissue damage, cancer, emphysema, cirrhosis, arteriosclerosis and arthritis[21]. Mixtures of dietary antioxidants or foods-rich in antioxidants help the human body in reducing oxidative damage, protective against ischemic heart disease and progression of many neurodegenerative diseases[22-28]. Empirical evidence suggests that *P. daemia*, due to its high antioxidant activity, could be useful in the development of medicine with antioxidant-based neuroprotective therapeutics.

Conclusion

This plant has been in use for many years as decoction or infusions prepared in water to treat various ailments where plant demonstrated broad-spectrum antibacterial and anti-oxidant efficacies. These results provide a scientific basis for the use of plant extracts in home-made remedies and their possible application against microorganisms.

ACKNOWLEDGEMENTS

The authors are thankful to Indian Council of Medical Research, New Delhi, India for partial financial support.

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TABLE-3
ANTIMICROBIAL ACTIVITY BY AGAR WELL METHOD (MIC VALUE) OF *P. daemia* SEQUENTIAL EXTRACTS

Test Bacteria		Sequential extracts						
		Pet. ether	Hexane	DCM	Chloroform	Acetone	Ethyl acetate	Methanol
B.subtilus (MTCC 441)	IZ[a]	13.33 ± 0.32	10.00 ± 0.57	14.00 ± 0.00	10.33 ± 0.32	13.66 ± 0.66	11.66 ± 0.74	12.66 ± 0.88
	MIC[b]	31.25	250	250	500	125	250	125
	AI[c]	0.60	0.45	0.63	0.74	0.62	0.58	0.57
E. aerogenes (MTCC 111)	IZ	15.33 ± 0.32	19.66 ± 0.66	16.66 ± 0.66	14.33 ± 0.32	14.00 ± 0.57	10.33 ± 0.32	11.33 ± 0.32
	MIC	62.5	62.5	250	125	125	500	250
	AI	1.09	1.40	1.19	1.02	1.00	0.73	0.80
E. coli (MTCC 443)	IZ	11.33 ± 0.87	15.66 ± 0.67	15.66 ± 0.33	15.33 ± 0.37	13.66 ± 0.66	19.66 ± 0.67	14.00 ± 0.57
	MIC	500	125	31.2	62.5	125	31.2	250
	AI	0.59	0.82	0.82	0.80	0.71	1.03	0.73
P. aeruginosa (MTCC 741)	IZ	14.00 ± 0.57	13.66 ± 0.66	12.00 ± 0.00	15.66 ± 0.66	12.66 ± 0.66	11.66 ± 0.74	12.33 ± 0.32
	MIC	31.25	62.5	125	62.5	250	125	250
	AI	0.70	0.68	0.60	0.78	0.63	0.58	0.61
S. aureus (MTCC 740)	IZ	11.00 ± 0.57	10.33 ± 0.87	10.00 ± 1.00	11.33 ± 0.37	10.33 ± 0.32	11.33 ± 0.76	11.00 ± 0.00
	MIC	125	250	250	125	250	250	125
	AI	0.52	0.49	0.41	0.53	0.49	0.53	0.56
R. planticola (MTCC 530)	IZ	12.00 ± 0.00	15.33 ± 0.32	15.66 ± 0.66	18.66 ± 0.66	13.00 ± 0.57	12.66 ± 0.66	19.00 ± 0.57
	MIC	62.5	500	500	31.25	1000	250	62.5
	AI	0.54	0.69	0.74	0.84	0.59	0.57	0.86
A. flavus (ATCC 16870)	IZ	10.00 ± 0.57	12.66 ± 0.88	11.33 ± 0.87	18.33 ± 0.32	16.33 ± 0.87	15.33 ± 0.67	11.00 ± 0.81
	MIC	250	62.5	250	125	125	250	1000
	AI	0.37	0.46	0.41	0.67	0.60	0.56	0.40
A. niger (ATCC 322)	IZ	-	-	12.00 ± 0.00	14.66 ± 0.66	11.66 ± 0.74	14.33 ± 0.32	10.00 ± 0.00
	MIC	-	-	500	500	250	250	500
	AI	-	-	0.44	0.54	0.43	0.53	0.37
C. albicans (ATCC 4718)	IZ	12.00 ± 0.00	10.33 ± 0.32	14.33 ± 0.32	15.66 ± 0.66	13.66 ± 0.88	11.00 ± 0.57	10.00 ± 0.00
	MIC	250	500	500	62.5	250	500	500
	AI	0.54	0.46	0.65	0.71	0.62	0.50	0.45
P. chrysogenum (ATCC 5476)	IZ	10.66 ± 0.66	15.66 ± 0.32	13.33 ± 0.32	13.00 ± 0.57	14.33 ± 0.32	12.00 ± 0.57	14.00 ± 0.88
	MIC	250	125	125	62.5	62.5	1000	500
	AI	0.50	0.74	0.63	0.61	0.68	0.57	0.66
T. rubrum (ATCC 2327)	IZ	12.3 ± 0.32	9.66 ± 0.56	15.00 ± 0.57	12.66 ±1.17	14.00 ± 0.57	13.33 ± 0.76	13.00 ± 0.00
	MIC	62.5	1000	62.5	125	125	250	125
	AI	0. 42	0.33	0.51	0.43	0.53	0.45	0.44

[a]IZ = Inhibition zone (in mm) including the diameter of well (6 mm); [b]MIC= Minimum inhibitory concentration in µg/mL; [c]AI = Activity index = Inhibition zone of the sample/Inhibition zone of the standard. Standard test drugs: Gentamycin for bacteria, Ketonocozole for fungi (10 mcg/disc)

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