European Journal of Medicinal Plants
8(1): 50-59, 2015, Article no.EJMP.2015.089
ISSN: 2231-0894

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In vitro Antioxidant Activities and Phytochemical Analysis of Methanol Extracts of Leaves and Stems of *Lumnitzera racemosa*

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Authors' contributions

This work was carried out in collaboration between all authors. Authors FMQ and BLJ designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author NK managed the analyses of the study and the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/EJMP/2015/16226
Editor(s):
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Complete Peer review History: http://www.sciencedomain.org/review-history.php?iid=1083&id=13&aid=8826

Original Research Article

Received 16th January 2015
Accepted 27th March 2015
Published 15th April 2015

ABSTRACT

Aim: To study antiradical and the reducing power activities in the leaves and stems methanol extracts of *Lumnitzera racemosa* mangrove species.
Design: Soxhlet extraction of leaves and stems using methanol for *in vitro* antioxidant assay.
Place and Duration of Study: Department of Life Sciences, University of Mumbai, Vidyanagari Campus, Santacruz (East), Mumbai, India, April, 2014 to December, 2014
Methodology: The plant branches were collected in the month of May, identified by an expert taxonomist. The leaves and stems were separated, washed, shed dried, powdered and Soxhlet methanol extracts were prepared to study antioxidant properties using DPPH (1, 1-diphenyl-2-picrylhydrazyl) Scavenging and Reducing power assay.

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Results: Both DPPH test and reducing power assay showed better antioxidant activity in leaves than stems. The scavenging activity in the leaves was (23.31 µg/mL) while in stems (111.5 µg/mL) as compared to ascorbic acid (14.98 µg/mL)
Conclusion: Overall *L. racemosa* has shown antioxidant properties in which leaves were more potent than stems.

Keywords: Lumnitzera racemosa; antioxidant; DPPH; reducing power; scavenging activity.

1. INTRODUCTION

Mangroves are halophytic (salt loving) and salt resistant marine tidal forest comprising trees, shrubs, palms etc. found in tropical climate. Several mangrove species are used as folklore medicine to treat various diseases like infectious diseases, diabetes and asthma [1]. Recent research have shown that Indian mangroves contain antimicrobial [2-5] and antiviral activities [6]. *C. tagal* [7] and *K. candel* [8] mangrove species have shown mild to strong antioxidant properties. The *L. racemosa* mangrove is used for antifertility, herpes, treatment of asthma, diabetes, and snake bite and for skin disorder. [9] This species was also tested for antibacterial [10], hepatoprotective, antioxidant [11,12] and cytotoxic activities [12].

2. MATERIALS AND METHODS

2.1 Plant Material

Mangrove plant *L. racemosa was* collected in the month of May from Ratnagiri coast Maharashtra, India and identified by an expert taxonomist. Leaves and stems were separated, washed thoroughly under running tap water to free them from dust and other contaminants, oven dried at 40ºC to remove the moisture content, grinded, resultant powder was individually sieved through a muslin cloth and used for the study.

2.2 Extract Preparation

20 g sample powder were extracted by Soxhlet with 80% aqueous methanol .The extract solvent was completely evaporated by rotary evaporator to obtain sticky gummy residue. In 1g residue 10mL aqueous methanol (10%, v/v) was added and analyzed for free radical scavenging activity and reducing power. Antioxidant assays were carried out in triplicate for each sample.

2.3 DPPH Radical Scavenging Activity

The antioxidant activity of the extracts was measured on the basis of the free radical scavenging activity by as follows [13]. Aliquots of 25 to 500 µg/mL of the test samples were placed in test tubes and added to 3.9 mL freshly prepared DPPH (25 mg/L) solution in methanol, mixed thoroughly and after 30 min absorbance was measured at 517 nm. Standard Ascorbic acid was used as a positive control. The percentage of radical scavenging activity was calculated as:

% Scavenging activity=

$$\frac{\text{Absorbance of Control} - \text{Absorbance of Sample}}{\text{Absorbance of Control}} \text{ X100}$$

Where,

Absorbance of Control= DPPH
Absorbance of Sample = Leaves and stems extracts of plant.

The inhibition curve was plotted and the IC_{50} values were determined in µg/mL

2.4 Reducing Power Assay

The reducing power of the extracts was determined [14] as follows. Various concentration from 0 to 525 µg/mL with regular interval of 75 were prepared in 1 mL distilled water and mixed with phosphate buffer (2.5 mL, 0.2M, pH6.6) and potassium ferricyanide $K_3[Fe(CN)_6]$ (2.5 mL, 1%). The mixture was incubated at 50°C for 20min then 2.5 ml 10% trichloroacetic acid was added to the mixture and centrifuged at 3000 rpm for 10 min. 2.5 mL upper layer of the solution were mixed with 2.5 mL, distilled water and 0.5 ml $FeCl_3$ (0.1%) then absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid was used as a positive control.

2.5 Phytochemical Analysis

2.5.1 HPTLC analysis

Quantitative phytochemical analysis was carried out by HPTLC (High Performance Thin layer Chromatography) Linomat V supplied by CAMAG in Anchrom, R & D laboratory, Mulund, Mumbai [15]. Details of solvent system, extract preparation, detecting reagent and visualization of flavonoids, anthraglycosides, bitter principles,

essential oil, saponins, coumarin triterpenes, phenol carboxylic acid and alkaloids are shown in Table 1.

2.5.2 Chromatographic condition

Each extract was loaded on readymade fluorescent pre coated silica gel G aluminum plate (Supplied by MERCK) and developed using appropriate solvent systems. The resultant chromatograms were illuminated for the characteristic quenching or fluorescence respectively for the particular class of bioactive compounds. The plates were derivatized and heated if necessary on a HPTLC heater for the detection of compounds.

3. RESULTS AND DISCUSSION

Various abiotic stresses lead to the over production of reactive oxygen species (ROS) in plants which are highly reactive and toxic and cause damage to proteins, lipids, carbohydrates and DNA which ultimately results in oxidative stress. Phenolics are often produced and accumulated in the subepidermal layers of plant tissues exposed to stress and pathogen attack. Recent researches have shown that antioxidants of plant origin with free radical scavenging property could have great importance as therapeutic agents in management of oxidative stress. Antioxidants are important in the prevention of human diseases. Naturally occurring antioxidants in leafy vegetables and seeds, such as ascorbic acid, vitamin E and Phenolic compounds possess ability to reduce the oxidative damage associated with many diseases, inducing cancer, cardiovascular disease, cataracts, atherosclerosis, diabetes, arthritis, immune deficiency diseases and aging [16]. Therefore, it is important to evaluate antioxidant activity of the plants either to elucidate the mechanism of their pharmacological action or to provide information on antioxidant activity [17].

Mangroves grow under stressful conditions such as violent environment, high concentration of moisture, high and low tides of water and abundant living microorganisms and insects. Therefore may have ability to produce variety of secondary metabolites.

Polar solvents are used for the extraction of antioxidants due to their polar nature. Soxhlet method extracts large amount of drug with much smaller quantity of solvent. Therefore Soxhlet extracts are used for the evaluation of antioxidant activities.

3.1 DPPH Radical Scavenging Activity Assay

DPPH radical was used as a substrate to evaluate free radical scavenging activities of extract. It involves reaction of specific antioxidant with stable free radical 1,1 –diphenyl-2-picryl-hydrazyl(DPPH). As a result, there is reduction of DPPH concentration by antioxidant, which decreases the optical absorbance of DPPH; this is detected by spectrophotometer at 517 nm. Ascorbic acid was used as standard [18]. The free radical scavenging activity of the extracts was evaluated based on the ability to scavenge the synthetic DPPH. This assay provided useful information on the reactivity of the compounds with stable free radical, because of odd number of electrons. The leaves extracts have shown better free radical scavenging activity 23.31 µg/mL than stems 111.5 µg/mL. The antioxidant activities of many traditional medicinal plants have not been systematically studied due to lack of popularity.

3.2 Reducing Power Assay

The reduction capacity of a compound may serve as a significant indicator of its potential antioxidant activity [19]. The reductive capabilities of the extracts were compared with ascorbic acid. A higher absorbance indicates a higher ferric reducing power. The reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating hydrogen atom [20].

The reducing activity of ascorbic acid and both the parts of the selected mangrove species was found to be directly proportional to concentration of samples (Table 2; Fig. 1). As concentration increased reducing activity increased. At 525 µg/mL maximum absorbance of the leaves and stem extracts were 1.67 and 0.793 as against ascorbic acid 2.5 and 2.05 respectively. Absorbance of the sample increased linearly with concentration. The colour of the test solution changes from yellow to green when Fe^{3+} reduced to Fe^{2+}. This initiates the compounds to exert an antioxidant response [21]. Coumarin, bitter principle, essential oil, terpenes, flavonoids, anthraglycosides and saponins compounds are capable of donating hydrogen ions thereby exert antioxidant response. These phytochemicals are recorded in the leaves and stems extracts of *L. racemosa which* may be responsible for high reducing ability of the plant.

Table 1. Solvent system, extract preparation, detecting reagent and Visualization for HPTLC

Classes	Solvent system	Extract preparation	Reagent	Visualization wavewlength	Color
Flavonoids	Ethyl acetate: formic acid: glacial acetic acid: Water(10:0.5:0.5:1.3)	Powdered drug(1 gm) in 5 ml methanol was extracted by heating on water bath for 10 min	Anisaldehyde sulphuric acid	365	Blue
Anthraglycosides	Ethyl acetate: methanol :water(100:13.5:10)	Powdered drug(1 gm) in 5 ml methanol was extracted by heating on water bath for 10 min	10% KOH	Visible	Red, yellow
Bitter principle	Ethyl acetate: methanol: water (100:13.5:10)	Powdered drug(1 gm) in 5 ml methanol was extracted by heating on water bath for 10 min	Vanillin sulphuric acid	Visible	Red, violet, yellow, blue, brown, green
Saponin	Chloroform: acetic acid :methanol: water (6.4:3.2:1.2:0.8)	Above extract prepared , filtered and evaporated to 1 ml, mixed with 0.5 ml water and extracted with 3 ml n-butanol	Vanillin sulphuric acid	Visible	Blue, blue violet, red, yellow,brown
Triterpenes	Chloroform: methanol (9.5:0.5)	Powdered drug (1 gm) was extracted by heating under reflux for 15 min with 10 ml dichloromethane. Filtrate was evaporated and residue was dissolved in 0.5 ml toluene	Anisaldehyde sulphuric acid	Visible	Blue violet, red to red violet
Phenol carboxylic acid	n-butanol:acetic acid: water(4:1:1)	Powdered drug (1gm) was extracted by heating under reflux for 15 min with 10 ml dichloromethane. Filtrate was evaporated and residue was dissolved in 0.5 ml toluene	$Fecl_3$	Visible	Brown grey or black
coumarin	Toluene: ethyl acetate (93:7)	Powdered drug (1 gm) was extracted by heating under reflux for 15 min with 10 ml dichloromethane. Filtrate was evaporated and residue was dissolved in 0.5 ml toluene	10% KOH	365	Light blue, brown
Essential oil	Toluene: ethyl acetate (93:7)	Powdered drug (1 gm) was extracted in 5 ml dichloromethane. The suspension was filtered and	Vanillin sulphuric acid	Visible	Red, yellow, blue, brown, green

Classes	Solvent system	Extract preparation	Reagent	Visualization wavewlength	Color
		clear filtrate was evaporated to dryness. The residue was dissolved in 1ml toluene.			
Alkaloids	Toluene :ethyl acetate: diethylamine (7:2:1)	Powder drug (1 gm) was moistened with 1 ml, 10% ammonia solution on water bath.	Dragendroff reagent	Visible	Orange brown

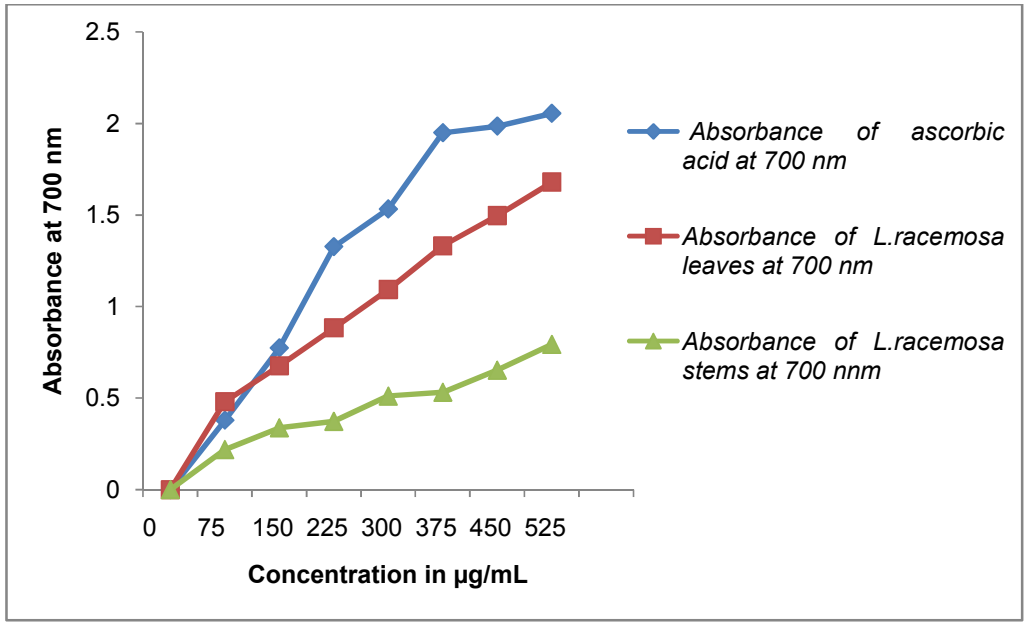

Fig. 1. Reducing power of *L. racemosa* leaves and stems

Table 2. IC_{50} for free radical scavenging by DPPH radical

Sample	Parts	IC_{50}µg/mL
Ascorbic acid	-	14.99 µg/mL
L. racemosa	Leaves	23.31 µg/mL
	Stems	111.5 µg/mL

3.3 Phytochemical Analysis

3.3.1 HPTLC analysis

Plant produces various classes of secondary metabolites like alkaloids, flavonoids, phenol, terpenes, steroids and quinines. The medicinal properties of plant depend upon quality and quantity of these phytochemicals.

Different composition of solvent systems for HPTLC analysis was studied to obtain high resolution and reproducible peaks of various classes of secondary metabolites. The aim was to determine number of compounds in each class of secondary metabolites. The leaves have shown 4 different types of coumarins with 4 different Rf (Retention factor) values between 0.22 to 0.62 while stem showed 3 types with Rf values 0.22, 0.38 and 0.82. Among these coumarin 1 and 3 of leaves has similar Rf value to that of coumarin 1 and 2 of stem indicating they were same compounds. This suggests leaves and stem contain all together 5 different types of coumarins (Table 9; Fig 2, supplementary). Beside this leaves recorded 4 different types of bitter principles with 4 different Rf values from 0.15 to 0.40 while stem showed 3 different types with Rf values between 0.12 to 0.74. All the Rf values of bitter principles in leaves and stem were different from each other, indicating 7 types of bitter principles in the plant (Table 4; Fig. 4 supplementary).

The leaves and stem contain 3 different types of essential oils in each with different Rf values, indicating 6 types of essential oils in the plant (Table 7; Fig. 3 supplementary).

Leaves and stem recorded 3 different types of terpenes in each with different Rf values. Among these one Rf value 0.57 was common in both the parts. This shows presence of 5 types of terpenes in the plant (Table 8; Fig. 8 supplementary). 2 types of saponins were seen in leaves with Rf values 0.33 and 0.45 (Table 10; Fig. 6 supplementary). Similarly 3 different types of flavonoids with Rf values 0.29, 0.74, 0.87 were recorded in stem (Table 6; Fig. 5 supplementary) while 2 different types of anthraglycosides with 2 Rf 0.47, 0.52 were seen in leaves (Table 5; Fig. 7 supplementary).

The overall results of phytochemical studies have confirmed the presence of several types of bitter principle, anthraglycosides, flavonoids, essential oils, terpenes, coumarins and saponins in leaves and stem (Table. 3). Among these flavonoids, saponins, terpenes and other phenolic compounds are reported to have antioxidant activity [22]. The antioxidant properties in the *L. racemosa* leaves and stem can be related to the presence of these classes of compounds.

Table 3. Number of constituents in various classes of secondary metabolites in mangrove plant

Secondary metabolites	No of constituents (Leaves)	No of constituents (Stems)
Bitter principle	04	03
Anthraglycosides	02	-
Flavonoids	-	03
Essential oil	03	03
Terpenes	03	03
Coumarin	04	03
Saponins	02	-
Phenol carboxylic acid	_	_
Alkaloids	_	_

Table 4. Bitter principle profile of methanol extracts of leaves and stem of *L. racemosa*

	Peak	Retention factor (Rf)	Height	Area	Assigned substance
	1	0.05	10.3	89.3	unknown
	2	0.11	14.4	155.0	unknown
	3	0.15	22.1	312.9	Bitter principle 1
	4	0.19	56.7	950.6	Bitter principle 2
	5	0.21	53.3	879.0	unknown
Leaves	6	0.32	44.6	904.1	unknown
	7	0.36	88.4	2339.0	Bitter principle 3
	8	0.40	89.6	2001.4	Bitter principle 4
	9	0.45	27.5	622.9	unknown
	10	0.65	42.6	1613.8	unknown
	11	0.73	136.2	6267.9	unknown
	12	0.81	162.9	5931.3	unknown
	1	0.08	12.3	130.1	unknown
	2	0.09	13.2	120.4	unknown
	3	0.12	14.2	156.1	Bitter principle 1
Stems	4	0.14	13.7	172.7	Bitter principle 2
	5	0.41	51.5	863.4	unknown
	6	0.65	23.3	854.1	unknown
	7	0.74	81.5	3610.4	Bitter principle 3
	8	0.81	61.5	2534.0	unknown
	9	0.89	11.7	280.2	unknown

Table 5. Anthraglycosides profile of methanol extracts of leaves of *L. racemosa*

	Peak	Retention factor (Rf)	Height	Area	Assigned substance
	1	0.09	205.5	2615.2	unknown
	2	0.18	11.3	111.1	unknown
	3	0.23	40.9	957.4	unknown
	4	0.30	91.7	4441.6	unknown
	5	0.36	57.3	861.8	unknown
	6	0.39	62.4	1029.1	unknown
Leaves	7	0.47	174.9	7616.6	Anthraglycosides 1
	8	0.52	152.1	3822.9	Anthraglycosides 2
	9	0.60	44.5	1113.3	unknown
	10	0.83	26.3	947.3	unknown

Table 6. Flavonoids profile of methanol extracts of stems of *L. racemosa*

	Peak	Retention factor (Rf)	Height	Area	Assigned substance
	1	0.15	13.1	454.5	unknown
	2	0.29	64.8	2678.4	Flavonoids 1
Stem	3	0.74	16.7	468.1	Flavonoids 2
	4	0.82	97.9	1902.7	unknown
	5	0.87	10.2	103.8	Flavonoids 3
	6	0.91	39.7	543.0	unknown

Table 7. Essential oil profile of methanol extracts of leaves and stem of *L.racemosa*

	Peak	Retention factor (Rf)	Height	Area	Assigned substance
	1	0.06	16.2	228.2	unknown
Leaves	2	0.09	10.9	117.1	unknown
	3	0.20	96.8	2224.7	Essential oil 1

	Peak	Retention factor (Rf)	Height	Area	Assigned substance
	4	0.30	141.5	4192.1	unknown
	5	0.33	73.9	1335.4	unknown
	6	0.50	129.7	19665.6	Essential oil 2
	7	0.77	198.9	11574.9	unknown
	8	0.88	342.8	13749.0	Essential oil 3
	1	0.09	19.3	249.5	unknown
Stem	2	0.15	22.2	403.4	Ess.oil 1
	3	0.20	75.6	1574.8	unknown
	4	0.30	88.6	2106.5	Essential oil 2
	5	0.50	26.5	1476.6	unknown
	6	0.78	80.4	5580.9	Essential oil 3
	7	0.90	18.6	350.1	unknown

Table 8. Terpenes profile of methanol extracts of leaves of *L. racemosa*

	Peak	Retention factor (Rf)	Height	Area	Assigned substance
	1	0.31	27.7	1037.8	Terpenes 1
	2	0.47	76.6	3153.0	unknown
Leaves	3	0.57	119.6	5152.4	Terpenes 2
	4	0.75	220.5	14354.1	Terpenes 3
	5	0.84	199.4	8756.6	unknown
	6	0.88	230.9	6094.6	unknown
	7	0.90	278.3	3901.4	unknown
	1	0.33	17.0	797.1	Terpenes 1
	2	0.41	32.6	1287.1	unknown
	3	0.48	55.0	2045.2	Terpenes 2
	4	0.57	53.2	2587.0	Terpenes 3
Stems	5	0.69	45.3	1663.9	unknown
	6	0.75	61.6	3315.4	unknown
	7	0.83	37.8	986.4	unknown
	8	0.88	68.6	1356.7	Unknown
	9	0.90	48.4	533.5	unknown

Table 9. Coumarin profile of methanol extracts of leaves and stem of *L. racemosa*

	Peak	Retention factor (Rf)	Height	Area	Assigned substance
	1	0.22	736.8	16148.9	Coumarin 1
	2	0.32	43.6	896.1	Coumarin 2
Leaves	3	0.38	634.1	20357.9	Coumarin 3
	4	0.46	32.3	670.1	unknown
	5	0.62	28.7	1030.6	Coumarin 4
	1	0.22	85.2	1728.8	Coumarin 1
Stem	2	0.38	42.3	1067.1	Coumarin 2
	3	0.56	13.8	534.3	unknown
	4	0.72	16.6	864.7	Coumarin 3
	5	0.82	11.5	338.8	unknown

Table 10. Saponins profile of methanol extracts of leaves of *L. racemosa*

	Peak	Retention factor (Rf)	Height	Area	Assigned substance
	1	0.10	23.0	321.5	unknown
Leaves	2	0.33	14.3	473.2	Saponin1
	3	0.45	19.6	839.1	Saponin 2
	4	0.56	85.6	3475.6	unknown

4. CONCLUSION

In the present study, antioxidant activities of the methanol extract of leaves and stems obtained from *L. racemosa* were investigated. The extract demonstrated effective antioxidant properties, as determined by the scavenging assay and reducing power assay, the leaves were more potent than stems. The phytochemical analysis indicated that leaves and stems contain bitter principle, essential oil, terpenes, and coumarin. Therefore, it can be inferred that the phenolics present in the *L. racemosa* plant might be responsible for its antioxidant properties presenting meaningful information for the collection and application of *L. racemosa* in both healthcare and food industry.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

ACKNOWLEDGEMENTS

We thank University Grant Commission, India for providing Non NET and RGNF Fellowship and funding for this research.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
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