IJPSR (2013), Vol. 4, Issue 7 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 26 February, 2013; received in revised form, 24 May, 2013; accepted, 21 June, 2013

A COMPARATIVE STUDY OF THE ANTIOXIDANT, ANTIMICROBIAL AND THROMBOLYTIC ACTIVITY OF THE BARK AND LEAVES OF *LANNEA COROMANDELICA* (ANACARDIACEAE)

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Keywords:

L. coromandelica, Antioxidant, DPPH, Antimicrobial, Thrombolytic

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ABSTRACT: The present study was designed to compare the antioxidant, antimicrobial and thrombolytic effects of the *Lannea coromandelica* bark and leave extracts. After the initial phytochemical screening, the ethanolic fractions of the *L. coromandelica* bark and leaves were partitioned by solvents of different polarity. Methods used to evaluate the antioxidant potential of the extracts were total phenolic content, total flavonoid content, free radical scavenging capacity, total antioxidant capacity and reducing power assay. Total phenol and total flavonoid contents were found to be the highest for bark and leaves in the ethyl acetate fraction and lowest in the n-Hexane fraction. In DPPH free radical scavenging test, the lowest IC_{50} value was found in the ethyl acetate fraction of the bark and leaf, resulting IC_{50} value of 3.8±0.14 µg/ml and 6±0.32 µg/ml respectively. In the same vein, ethyl acetate fraction of both leaf and bark showed the highest antioxidant capacity and reducing power. Reducing power of both bark and leaves were found to be concentration dependent and most prominent was observed with the fractions of higher polarity both in case of bark and in case of leaves. Furthermore, the leaf extracts produced moderate antimicrobial activity whereas the bark extracts showed weak antimicrobial activity. Dichloromethane fraction of leaf showed the most potent antimicrobial activity with zone of inhibition ranging from 8 mm to 21 mm at a dose of 400µg/disc and 10 mm to 23 mm at a dose of 800µg/disc. In addition, extracts from both parts of *L. coromandelica* produced good thrombolytic activity compared with streptokinase.

INTRODUCTION: Nowadays, the use of medicinal plants for alleviating diseases are growing day by day around the world especially in Asia. Fewer side effects of medicinal plants play a big role for the popularity of the medicinal plants.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.4(7).2609-14

Article can be accessed online on: www.ijpsr.com

Currently, many established drugs are not working against intended diseases due to drug resistance. In addition, new diseases are emerging which are threatening for human race. Thus, we have to have new arsenals against those threats. Moreover, Plants are the source of versatile chemical compounds. The costs of the drugs are also increasing significantly.

Therefore, the use of traditional medicine and medicinal plants in most developing countries, as a basis for the maintenance of good health, has been widely observed [1]. Previously, many lead compounds were discovered from plants.

Scientists throughout the world are trying to explore the precious assets of medicinal plants to help the suffering of humanity. Furthermore, in the world more than 30% of the pharmaceutical preparations are based on plants [2]**.** Hence, plants can be considered as the prime source of drugs.

L. coromandelica is a medium-sized deciduous tree. The bark is considered as astringent and stomachic; used as a lotion in impetiginous eruptions, leprous and obstinate ulcers; cures sprains, bruises, skin eruptions, heart diseases, dysentery and mouth sores. Decoction of the bark is used for toothache. Its bark is also useful in impotency. Scrapped bark is chewed for 2-3 days to cure glossitis. Boiled leaves are applied as a fomentation for local swelling and pains [3]. Presence of dihydroflavonols in the stem bark of the plant also has been reported [4].

The work described in this study is an attempt to compare the antioxidant, antimicrobial and thrombolytic activity among the different partitionates of the bark and leaves of *L. coromandelica*.

MATERIALS AND METHODS:

The leaves and barks of *L coromandelica* were collected from Rajsahi, Bangladesh in August 2011. The plant was identified in Bangladesh National Herbarium and voucher specimens were deposited there **(DACB accession no. 36703)** and at East West University for future reference.

The dried plant materials (powdered bark and leaves) were macerated in ethanol for 7 days in separate containers. The filtrates of the plant materials were dried by using rotary evaporator and marked as LCB and LCL respectively. Dried crude extract of both laves and barks were subjected to modified Kupchan method[5] and the resultant partitionates i.e. n-hexane (LCBN), dichloromethane (LCBD), ethyl acetate (LCBE) soluble fractions were used for the experimental process.

Chemicals: DPPH (1, 1-diphenyl, 2-picrylhydrazyl), TCA (trichloroacetic acid) and ferric chloride were obtained from Sigma Chemical Co. USA; Ascorbic acid was from SD Fine Chem. Ltd. India, ammonium molybdate from Merck, Germany. Streptokinase was from CSL Behring GmbH, Germany.

Phytochemical Screening: The freshly prepared crude extracts (LCB and LCL) were qualitatively tested as described by Kokate [6, 7] for the presence of Alkaloids, Flavonoids, Steroids, Terpenoids, Saponins, Tannins and Cardiac glycosides.

In-vitro Antioxidant Activity:

1. **Total Phenolic Content Assay:** Folin-Ciocalteu method [8, 9] was used to determine the total phenolic content of the partitionates; 0.5 ml of a methanol solution of the fractions having concentration of 1 mg/ml was mixed with 5 ml Folin ciocalteu reagent (1:10 v/v distilled water) and 4 ml (75g/L) of Sodium carbonate. The mixtures were vortexed for 15 seconds and allowed to stand for 30min at room temperature in dark place for color development and the absorbance was measured at 760 nm against methanol as blank by using a UV- visible spectrophotometer. All the tests were carried out in triplicate and average absorption was noted for each time. The total phenolic content was calculated by using the standard Gallic acid calibration curve and expressed as mg of GAE (gallic acid equivalent)/gm of the dried extract.

2. **Total Flavonoid Content Assay:** Aluminum chloride ($AlCl_3$) colorimetric method [10] was applied to determine the total flavonoid contents of the extracts and quercetin was used as the reference compound. 0.5 ml of a methanol solution of each extract (10 mg/ml) was mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water. Blank was prepared in the same way without the sample. Both sample and blank solution were allowed to stand for 30 min at room temperature. After the incubation the absorbance of the reaction mixtures were measured at 415 nm. All the tests were carried out in triplicate and average absorption was noted for each time. The total flavonoids was expressed as mg of Quercetin equivalent/gram of dried extract by using the standard quercetin calibration curve.

3. **Free Radical Scavenging Activity:** Free radical scavenging activity of the different fractions was determined by decrease in the absorbance of methanol solution of DPPH (2, 2-Diphenyl-1-picrylhydrazyl) [11].

Antioxidant activities of the different fractions were determined on the basis of their scavenging potential of the stable DPPH free radical. 2.0 ml of various concentrations of each sample were mixed with 3.0 ml of a DPPH methanol solution (20 µg/ml).

After 30 min reaction period at room temperature in dark place, the absorbance was measured at 517 nm against methanol as blank by using a UV- visible spetrophotometer. Inhibition free radical DPPH in percent (I %) was calculated as follows:

$$\text{Percentage inhibition (I \%)} = (1 - A_{sample}/A_{blank}) \times 100$$

A_{blank} = Absorbance of the control reaction (containing all reagents except the test material).

A_{sample} = Absorbance of the test sample.

L-Ascorbic acid was used as positive control. Tests carried out in triplicate and average value was taken. Extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotted inhibition percentage against extract concentration.

4. **Total Antioxidant Capacity:** The total antioxidant activity was evaluated by the phosphomolybdenum assay method as described by Prieto *et al* [12]. 0.3 ml of ethanolic solution of the fraction having concentration of 2 mg/ml was mixed with 3 ml of reagent solution (0.6 M Sulfuric acid, 28mM Sodium Phosphate and 4mM Ammonium molybdate) (all of them were taken in equal volume). Sample blank was prepared in similar way by replacing sample extract with ethanol. The reaction mixture was incubated at 95°C for 90 min.

   After that, the samples were allowed to cool to room temperature. The absorbance of the reaction mixture was measured at 695 nm against a blank by using a UV-visible spectrophotometer. All the tests were carried out in triplicate and average absorption was noted for each time.

   The total antioxidant activity of the crude extract was expressed as the number of mg of ascorbic acid equivalents per gram of dried extract by using the equation obtained from a standard Ascorbic acid calibration curve.

5. **Reducing Power Assay:** The reducing power assays of the partitionates were determined according to the method previously described by Oyaizu *et al* [13]. 1 ml of the methanol solution of the fraction of different concentrations (1, 5, 10, 50, 100 µg/ml) was mixed with 2.5 ml phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and 2.5 ml potassium ferricyanide $[K_3Fe(CN)_6]$ (1%). The mixture was incubated at 50°C for 20min.

   2.5 ml of 10% trichloroacetic acid (TCA) was added to the mixture, which was then centrifuged at 3000rpm for 10min. The upper layer of the solution was separated and mixed with 2.5 ml distilled water and 0.5ml $FeCl_3$ (0.5 ml, 0.1%). The absorbance was measured against a blank at 700nm. All the tests were carried out in triplicate and average absorption was noted for each time. L-Ascorbic acid was used as positive control. The intensity of the absorbance could be the measurement of the extract.

Antimicrobial Assay: The antimicrobial assay was performed by using the disc diffusion method [14-16] where thirteen microorganisms were used as test organisms for antimicrobial activity of dried sample extract. The microbial strains were collected from East West University microbiology lab. 400 µg/disc and 800 µg/disc of the sample extract were used to observe the antibacterial activity of the plant extract and compared with the standard cephradin disc (30 µg/disc) and antifungal activity was observed by comparing with nystatin disc (30 µg/disc).

The test organisms were inoculated on 10 ml previously sterilized nutrient agar media, mixed thoroughly and transferred immediately to the sterile petri dish under an aseptic condition using a sterile loop. Prepared sample and standard solutions were applied to the corresponding Petri dish. The plates were incubated for overnight at 37^0C. After proper incubation, clear zone of inhibition around the point of application of sample solution were measured which is expressed in millimeter (mm).

Thrombolytic Activity: The *in vitro* thrombolytic activity was determined according to the method reported earlier by Prasad, S. *et al* [17, 18]. 5 mL venous blood drawn from healthy volunteer was transferred to 8 pre-weighed sterile eppendorf tubes (500 µL/tube) and incubated at 37^oC for 45 minutes.

After clot formation, serum was completely removed without disturbing the clot formed. Each tube having clot was again weighed to determine the clot weight (Clot weight = weight of clot containing tube - weight of tube alone).

Each eppendorf tubes containing clot was properly labeled and 100 µL of LCBN, LCBD, LCBE, LCLN, LCLD and LCLE extracts (100 mg/mL) were added to the tubes separately. As a positive control, 100 µL of streptokinase (CSL Behring GmbH, Germany) (3000 000 IU/mL) and as a negative control, 100 µL of normal saline (0.9% NaCl) were separately added to the control tubes. All the tubes were then incubated at 37°C for 90 minutes and observed for clot lysis. After incubation, fluid obtained was removed and tubes were again weighed to observe the difference in weight after clot disruption. Difference obtained in weight taken before and after clot lysis was expressed as percentage of clot lysis. The experiment was conducted on the blood samples of 10 volunteers (male=5; female=5) without a history of oral contraceptive or anticoagulant therapy since two weeks.

RESULTS AND DISCUSSION: The present study was performed to evaluate the *in-vitro* antioxidant potential (**Table 2**), antimicrobial activity (**Table 3**) and thrombolytic activity (**Table 4**) of the different solvent partitionate of the ethanolic extract of the leaves and bark of *L. coromandelica.* In doing so, initial phytochemical screenings of the crude ethanolic extracts were performed also and it revealed the absence of saponins in both the bark and leaves of *L. coromandelica* (**Table 1**).

TABLE 1: COMPARISON OF PHYTOCHEMICAL SCREENING OF THE CRUDE EXTRACTS OF BARK AND LEAVES OF *L COROMANDELICA*.

Crude Extracts	Carbohydrate	Saponin	Steroid	Alkaloid	Cardiac Glycoside	Terpenoids	Tannin	Flavonoid
LCB	+	-	+	+	+	+	+	+
LCL	+	-	+	+	+	+	+	+

See text for the identification of extracts and fractions.

The total phenolic content of the different fractions of leaves and barks of *L coromandelica* differed fraction to fraction widely (**Table 2**) and the highest amount of phenolics was found in the ethyl acetate fractions of both the leaves and bark (430±0.36 and 349±0.16 mg GAE/gm of dried extract). The variability is observed according to the polarity of solvent used in extraction.

High solubility of phenols in polar solvents justifies its maximum amount in a polar solvent compared to others [19]. Like the total phenolic content, ethyl acetate soluble partitionate of the bark exhibited the highest flavonoid content. Total flavonoids content were determined using spectrophotometric method with aluminum chloride and the values ranged from 80.2 to 190.6 mg of quercetin equivalent/gm of dried extract (Table 2).

Compare to the commercially available organic nitrogen radicals, DPPH is one of the stable radical. This assay is based on the proportional antioxidant effect to the disappearance of DPPH in test samples. A freshly prepared DPPH solution exhibit a deep purple color with absorption maximum at 517nm. The purple color generally fades or disappears when an antioxidant is present in the medium.

Results were reported as IC_{50}, which is the amount of antioxidant necessary to decrease the initial DPPH concentration by 50%. The lower the IC_{50}, the higher is the antioxidant power.

In the assay for free radical scavenging property the most potent fraction was found to be the ethyl acetate soluble partitionate of the bark with the lowest IC_{50} value of 3.8 µg/ml (Table 2). Among all the partitionates, the dichloromethane soluble partitionate of the leaves possess the least antioxidant potential.

Total antioxidant capacity by Phosphomolybdenum method assay is based on the reduction of Mo (VI) to Mo (V) by the sample analyte and the subsequent formation of green phosphate/Mo (V) complex at acidic pH. The phosphomolybdenum method is quantitative since the total antioxidant activity is expressed as mg of L-ascorbic acid per gm of dried extract.

Ethyl acetate soluble partitionates of both leaves and bark showed greatest total antioxidant capacity and the values were 46.67 mg and 33.78 mg of L-ascorbic acid per gm of dried extract respectively (Table 2).

TABLE 2: COMPARISON OF TOTAL PHENOLIC CONTENT, TOTAL FLAVONOID CONTENT, FREE RADICAL SCAVENGING CAPACITY AND TOTAL ANTIOXIDANT CAPACITY.

Extracts	Total phenol (mg of GAE/gm of dried extract)	Total flavonoid (mg of Quercetin equivalent/gm of dried extract)	DPPH radical scavenging capacity (IC_{50} µg /ml)	Total antioxidant (mg of L-ascorbic acid equivalent/ gm of dried extract)
LCBN	135.89±0.23	148.6±0.02	8±0.11	33.32
LCBD	280±0.59	80.2±0.03	15±0.24	24.32
LCBE	349±0.16	190.6±0.07	3.8±0.14	33.78
LCLN	85±0.29	129.2±0.21	13±0.27	17.48
LCLD	251±0.12	95.6±0.28	18±0.22	11.14
LCLE	430±0.36	182.1±0.34	6±0.32	46.67

Values are the mean of three determinations ± standard deviation (SD). See text for the identification of extracts and fractions.

The reducing capacity of the extracts Fe^{3+}/ ferricyanide complex to the ferrous form may serve as a significant indicator of its antioxidant capacity. The existence of reductones are the key of the reducing power, which exhibit their antioxidant activities through the action of breaking the free radical chain by donating a hydrogen atom.

The reduction of the Fe^{3+}/ ferricyanide complex to the ferrous form occurs due to the presence of reductants in the solution. Among the fractions, like the DPPH radical scavenging activity, the reducing power ability of the extracts increase in a dose dependent manner. Among all the fractions and like the other tests for antioxidant potentials, the ethyl acetate partitionates exhibit the highest reducing power (**Figure 1**).

Antimicrobial activities of the extracts were tested against eleven bacteria and were compared with the standard antibiotic cephradin disc by measuring the zone of inhibition diameter and expressed in millimeter (mm) showed in **table 3**. Antifungal activity was tested also against two fungi and compared with the nystatin.

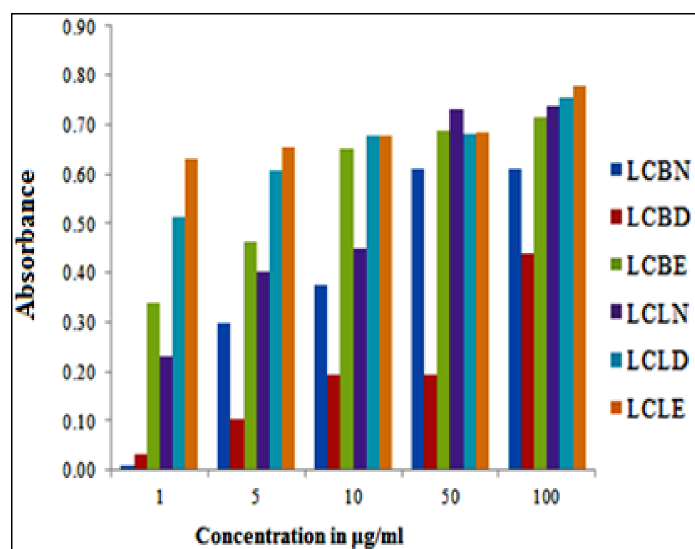

FIGURE 1: REDUCING POWER ASSAY OF *L. COROMANDELICA*

TABLE 3: RESULTS OF ANTIMICROBIAL TEST BY DISC DIFFUSION METHOD

Microorganisms	LCBN800	LCBN400	LCBD800	LCBD400	LCBE800	LCBE400	LCLN800	LCLN400	LCLD800	LCLD400	LCLE800	LCLE400	Control
Bacillus subtilis	10	8	15	10	0	0	0	0	22	18	0	0	26
Bacillus cereus	0	0	8	0	0	0	11	8	19	18	0	0	25
Sarcina lutea	0	0	8	0	0	0	0	0	16	15	0	0	21
Staphylococcus aureus	0	0	9	0	0	0	0	0	14	12	0	0	15
Shigella boydii	0	0	18	12	0	0	0	0	22	20	0	0	23
Vibrio mimicus	0	0	10	0	0	0	0	0	19	18	0	0	22
Shigella dysentery	8	8	12	10	9	10	14	9	17	10	11	10	22
Escherichia coli	0	0	10	0	0	0	10	8	16	10	0	0	28
Salmonella typhi	0	0	12	0	0	0	0	0	18	15	0	0	20
Salmonella paratyphi	0	0	11	0	0	0	0	0	23	21	0	0	25
Pseudomonas aeruginosa	12	8	12	11	10	8	10	0	19	13	11	8	25
Candida albicans	0	0	0	0	0	0	0	0	15	12	0	0	28
Saccharomyces cerevisiae	0	0	8	0	0	0	0	0	10	8	0	0	20

Results are expressing the zone of inhibition in mm. See text for the identification of extracts and fractions.

Dichloromethane fractions of both bark and leaves showed good antimicrobial activity against both gram positive and gram negative bacteria compared to the standard. However, fractions of leaves produced higher antimicrobial activity than the fractions of bark and the highest activity was exhibited by the dichloromethane soluble partionate of leaves against *Salmonella paratyphi* (23mm in 800μg/disc, 21 mm in 800μg/disc). On the other hand, almost all the fractions had weak or no antifungal activity.

In the determination of thrombolytic activity, streptokinase was used as the standard and the average clot lysis was 55.36±0.45 %. The streptokinase and all the fractions, except n-hexane soluble fractions exerted statistically significant thrombolytic activity ($P<0.001$) compared to negative control (normal saline) and the values obtained were 34.81%, 27.41%, 23.98%, 18.27% and 56.46% for LCBD, LCBE LCLD, LCLE and streptokinase respectively.

TABLE 4: RESULTS OF THROMBOLYTIC ACTIVITY TEST

Name	Mean ± SD (% clot lysis)
Blank	4.24 ± 0.97
Streptokinase	56.46 ± 2.14***
LCBN	5.49 ± 0.99**
LCBD	34.81 ± 3.02***
LCBE	27.41 ± 2.18***
LCLN	7.19 ± 2.09**
LCLD	23.98 ± 2.71***
LCLE	18.27 ± 3.91***

Values are expressed in mean ± SD. **$P<0.05$ and ***$P<0.001$ when compared with negative control.

CONCLUSION: From this experiment, it can be concluded that the both extracts showed good antioxidant capacity. The antioxidant activity was found to slight increase with increasing concentration of the extracts. As apparent from our results it can be revealed that the plant extract has good antimicrobial activity against few species but it exhibited good thrombolytic activity. This is only a preliminary study and to make final comment the extract should thoroughly investigated phytochemically and pharmacologically to exploit their medicinal and pharmaceutical potentialities.

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How to cite this article:
Manik MK, Wahid MA, Islam SMA, Pal A and Ahmed KT: A comparative study of the antioxidant, antimicrobial and thrombolytic activity of the bark and leaves of *Lannea coromandelica* (Anacardiaceae). *Int J Pharm Sci Res* 2013: 4(7); 2609-2614. doi: 10.13040/IJPSR. 0975-8232.4(7).2609-14

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