

Phytochemical Screening, Antimicrobial Activity, Antioxidant Capacity And In Vivo Anticancer Activity Of *Lannea Coromandelica* Bark Extracts

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Abstract: In this study, experiments were performed to evaluate the phytochemical components, antioxidant capacity, antimicrobial activity and in vivo anticancer activity of *Lannea coromandelica* bark extracts. In the phytochemical analysis alkaloids, carbohydrates, tannins, saponins, resins, phenolic compounds, proteins, and steroids were found to be present. An intermediate zone of sensitivity was found in antimicrobial activity assay of crude extract against *Escherichia coli*, *Shigella flexneri*, and *Staphylococcus aureus*. In antioxidant capacity experiments, the chloroform extract showed CUPRAC reducing capacity more than the standard (Butylated hydroxyl toluene), and the total flavonoid content (TFC), total phenolic compound (TPC) and total antioxidant capacity (TAC) showed significant results ($p < 0.01$). In case of in vivo anticancer effect of the chloroform extract, mice were seeded with approximately 1×10^5 Ehrlich ascites carcinoma (EAC) cells and after seven days of consecutive treatment the negative and positive control groups showed an average EAC cell count of 2.4×10^8 and 1.9×10^8 cells respectively and the experimental group showed 1.1×10^8 cells. Mice in the experimental group showed percent increase of life span (%ILS) of 33.3, when compared with the negative control mice. However, treatment in a cyclic manner showed the %ILS to be 88.85 for experimental group when compared negative control. All these results demonstrated the antimicrobial, antioxidant, and anticancer activity of the chloroform extract of *L. coromandelica* barks.

Keywords: *Lannea coromandelica*, Phytochemical screening, antioxidant capacity, Ehrlich ascites carcinoma cells.

Date of Submission: 09-05-2018

Date of acceptance: 24-05-2018

I. Introduction

In many developing countries, medicinal plants are used for the maintenance of good health [1-3] and *Lannea coromandelica* is one of such important plants that are well distributed in India, Bangladesh, and other South Asian countries. This plant belongs to the family Anacardiaceae and an important medicinal plant [4]. In rural areas of Bangladesh the leaf and bark of this plant is prescribed by the village quack to the diarrheal and other patients [5]. However, there is no scientific data on the biological activity of this plant or its compounds. Therefore, in this study, we determined the phytochemical compounds, antioxidant capacity, antimicrobial activity and in vivo anticancer activity of *L. coromandelica* bark extracts, to understand its biological activities using different experimental models.

II. Materials And Methods

2.1. Collection and processing of plant samples

The barks of *L. coromandelica* were collected from Gazipur District, Bangladesh and the plant sample is submitted to the National Herbarium of Bangladesh, Mirpur-1, Dhaka for its identification. Barks were sun dried to remove the moisture and ground into coarse powder. Thirty gram of grinded *L. coromandelica* bark samples were extracted in a soxhlet apparatus and then crude extracts were obtained using four different solvents n-Hexane, Petroleum benzene, Methanol, and Chloroform, according to their increasing solubility. The extracted samples were stored at 4°C for future use.

2.2. Phytochemical analysis

Extracted samples were subjected to identify the presence of different phytoconstituents by using standard laboratory protocols [9-13].

2.3. Antimicrobial activity of samples

Disc diffusion method was applied to evaluate the effectiveness of sample extracts against Gram positive and Gram negative bacteria [14]. To standardize the inoculum density, McFarland standard was used. Bark extracts (500µg dissolved in 10µl of ethanol in each disk) was used to observe the antimicrobial activity of sample extracts. Two Gram negative bacteria, *Escherichia coli* (ATCC-25922) and *Shigella flexneri* (ATCC-12023) and one Gram positive bacteria, *Staphylococcus aureus* (ATCC-25923) were taken and the results were compared with that of amoxicillin, which served as positive control. Respective solvents were served as negative controls.

2.4. Cupric Reducing Antioxidant Capacity (CUPRAC)

The assay was conducted as described previously [15]. A concentration of 100, 50, 25 and 12.5µg/ml of ethanol was taken from both sample extract (5 mg extract in one ml of ethanol), and standard butylated hydroxytoluene (BHT). Then 1ml of each Copper chloride, Ammonium acetate, Neocuproine and 600 µl of dH₂O were added in each test tube and the mixtures were incubated at room temperature (25⁰C) for 60 minutes. Absorbance of each mixture was taken at 450nm.

2.5. Total Flavonoid Contents (TFC)

The aluminum chloride colorimetric method was used for Flavonoid content determination [16]. This experiment was done by taking 5mg/ml concentration of each crude extract and Quercetin as standard. Four different concentrations of 100, 50, 25 and 12.5µg/ml (both the sample and standard) were used. 1.5ml of methanol, 100µl of Aluminium chloride, 100µl of Potassium acetate and 2.8ml of dH₂O were added and the mixtures were incubated at room temperature (25⁰C) for 30 minutes. The absorbance was taken at 415nm. Total flavonoid content of sample bark in quercetin equivalents was calculated.

2.6. Total Phenolic Compounds (TPC)

The total phenolic content of samples was measured employing the method involving Folin-Ciocalteu reagent as an oxidizing agent and Gallic acid as standard [13]. This experiment was performed by taking 5mg/ml concentration of crude extracts and standard. Four different concentrations of 100, 50, 25 and 12.5 µg/ml were taken and then 2.5ml of Folin-Ciocalteu reagent, 2ml of sodium carbonate were added in each test tube and incubated at room temperature (25⁰C) for 60 minutes. The absorbance was taken at 765 nm and was calculated.

2.7. Total Antioxidants Capacity (TAC)

The total antioxidant capacity was evaluated by the phosphor molybdenum method [17].

Four different concentrations of 100, 50, 25 and 12.5µg/ml of ethanol (both the sample and standard Ascorbic acid) were used. Then 1 ml of each 0.333% Sulphuric acid, 0.004M Ammonium molybdate, and 0.02295M Monosodium phosphate were added to each test tube. The mixtures were incubated at 95⁰C for 90 minutes. After cooling in room temperature, the absorbance of each sample and standard was measured at 695 nm. The following equation was used to calculate TFC, TPC, and TAC with their respective standards:
 $A=(c \times V)/m$

Where, A = total content of antioxidant compounds in mg of ascorbic acid equivalent/gm plant extract, c=the concentration of standards established from the calibration curve in mg/ ml, V=the volume of extract in ml and, m=the weight of crude plant extract in gm.

2.8. Measurement of Anticancer Activity

Male Swiss albino mice of 25-30 gm which obtained from ICDDR,B were used for this study. Approximately 1.1×10^5 ehrlich ascites carcinoma (EAC) cells were injected intraperitoneally. Positive control, negative control and experimental mice groups were divided into subgroups 1, 2 and 3 respectively. All mice under positive control received bleomycin from a cumulative dose of 0.3 mg per kg body weight per week up to seven days and subgroup 3 received in a cyclic manner (consecutive seven days treatment followed by five days interval and this cycle was repeated until the death of mouse in this subgroup). In the negative control group, all mice were given the solvent Tween 20 and followed the same treatment pattern mentioned above. In the experimental group, all mice in subgroup 1, 2, and 3 received dose of 200 mg per kg body weight per week and subgroup 3 received sample at the same dose in a cyclic manner. Mice in subgroup 2 and subgroup 3 were monitored for survival in terms of days. Another group of mice (n=6) was used to observe the apparent physiological effects of the sample extracts. The mean survival time and percentage increase in lifespan (%ILS) were calculated using following equations [18]:

$$\text{Mean survival time (MST)} = \frac{\text{Survival time (days) of each mouse in a group}}{\text{Total number of mice}}$$

$$\% \text{ Increase in life span (ILS \%)} = \frac{\text{MST of treated group}}{\text{MST of control group}}$$

III. Results

3.1. Phytochemical analysis

Extracts of all four solvents showed the presence of carbohydrates, tannins, and resins whereas, alkaloids were absent in petroleum benzene extract. Reducing sugars were present only in methanol extract but pentose and hexose were absent from all extracts. Glycosides and phenolic compounds were also absent in all samples. Flavonoids were present in both chloroform and methanol extracts. Saponins and proteins were present only in methanol extract, whereas, steroids were absent only in n-hexane extract (table 1).

Table 1: Analysis of phytochemical components of *L. coromandelica* bark extracts

List of phytoconstituents	n-Hexane	Petroleum benzene	Chloroform	Methanol
Alkaloids	+ve	-ve	+ve	+ve
Glycosides	-ve	-ve	-ve	-ve
Flavonoids	-ve	-ve	-ve	-ve
Carbohydrates	+ve	+ve	+ve	+ve
Steroids	-ve	+ve	+ve	+ve
Saponins	-ve	-ve	-ve	+ve
Tannins	+ve	+ve	+ve	+ve
Proteins	-ve	-ve	-ve	+ve
Resins	+ve	+ve	+ve	+ve
Phenols	-ve	-ve	-ve	-ve

3.2. Antimicrobial activity

In antimicrobial activity experiment, the zone of sensitivity was found to be 10.5 mm for *E. coli*, 11 mm for *S. flexneri* and 10 mm for *S. aureus* was found when compared to negative control (8 mm for all solvents) and amoxicillin (22 mm for all species) (table 2).

Table 2: Evaluation of the antimicrobial activity of sample extracts

Name of the organisms	Zone of inhibition in diameter (mm)				
	n-hexane	Petroleum benzene	Chloroform	Methanol	Amoxicillin
<i>E. coli</i>	0	0	0	10.5	22
<i>S. flexneri</i>	0	0	0	11	22
<i>S. aureus</i>	0	0	0	10	22

3.3. Cupric Reducing Antioxidant Capacity

In this experiment, Cu^{2+} to Cu^{1+} reducing antioxidants were found in all samples. Reducing components were found to rise with the increasing concentrations of different extracts. Here the chloroform extracts showed highest reducing capacity which was more than that of standard (BHT) (figure 1).

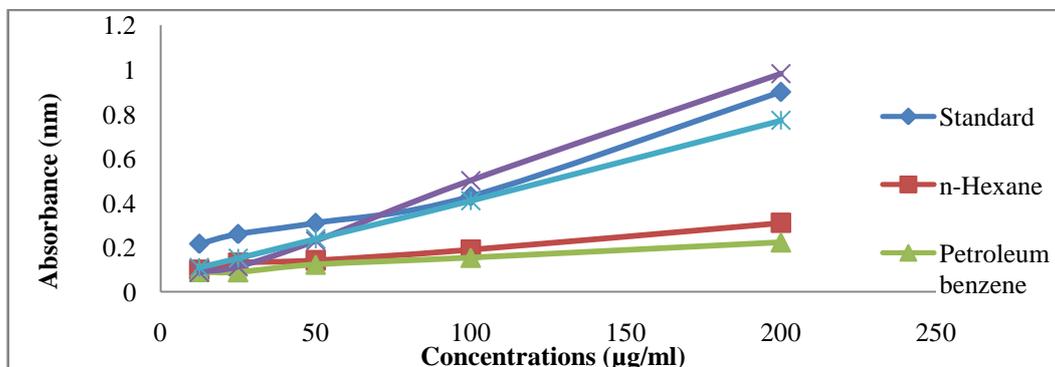


Figure1: Cupric reducing capacity of the sample extracted by different solvent and butylated hydroxyl toluene (BHT) was used as a standard.

3.4. Total flavonoid content (TFC)

Total flavonoid contents were calculated using the standard curve of quercetin ($Y=0.002x-0.021$; $R^2=0.871$) and were expressed as quercetin equivalents (QE) per gram of the sample extract. Chloroform extract of the sample was found to contain the highest amount of flavonoid and all the extracts showed significant

($p < 0.01$) result (table 3). Flavonoid contents of the extracts were found to decrease in the following order: Chloroform extract > Methanol extract > n-hexane extract > Petroleum benzene extracts.

Table 3: Total flavonoid contents in different extracts of samples.

Extract	TFC contents, mg/gm
n-Hexane	151.96±18.20**
Petroleum benzene	151.95±13.84**
Chloroform	347.35±95.91**
Methanol	151.98±15.25**

Values are expressed as mean ± SD, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; Significant when compared with the corresponding value of the standard Quercetin; done by independent t-test.

3.5. Total phenolic contents (TPC)

All the extracts of samples were tested for total phenolic content and results were expressed as Gallic acid equivalent (GAE) per gram of sample extract. The total phenolic content was calculated using the standard curve of Gallic acid ($Y = 0.331x + 0.073$; $R^2 = 0.866$). All the extracts showed significant ($p < 0.01$) result. Total phenolic contents of the extracts were found to decrease in the following order: Chloroform extract > n-hexane extract > Petroleum benzene > Methanol extract (table 4).

Table 4: Total phenolic content in different sample extracts.

Extract	TPC contents, mg/gm
n-Hexane	151.99± 19.24**
Petroleum benzene	151.98±14.46**
Chloroform	152.95±13.65**
Methanol	151.96±18.39**

Values are expressed as mean ± SD, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; Significant when compared with the corresponding value of the standard Gallic acid; done by independent t-test.

3.6. Total antioxidants capacity (TAC)

The total antioxidant capacity of the different extracts of the sample was expressed as ascorbic acid equivalents (AAE) per gram of sample extract. The total antioxidant capacity of the test samples was calculated using the standard curve of ascorbic acid ($Y = 0.443x + 0.025$; $R^2 = 0.888$). Chloroform extract of the sample was found to possess the highest total antioxidant capacity and all the extracts showed significant ($p < 0.01$) result (table 5).

Table 5: Total antioxidant capacity in different sample extracts.

Extract	TAC contents, mg/gm
n-Hexane	151.71±16.01**
Petroleum benzene	151.88±17.26**
Chloroform	152.96±15.18**
Methanol	151.96±14.72**

Values are expressed as mean ± SD, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; Significant when compared with the corresponding value of the standard Ascorbic acid; done by independent t-test.

3.7. Evaluation of in vivo anti tumor activity

3.7.1. EAC cells count after treatment (subgroup 1)

After seven days of treatment, EAC cells were counted on day 8. Subgroup 1 of negative and positive control group showed an average EAC cell count of 2.4×10^8 and 1.9×10^8 cells respectively and that of experimental group showed a cell count of 1.1×10^8 cells.

3.7.2. Percent increased in life span (%ILS) of the subgroup 2 of positive, and experimental groups of mice

The positive control, negative control, and experimental group were observed for survival. %ILS of subgroup 2 of positive and experimental groups was determined to be 18.7 and 33.3 respectively (figure 2).

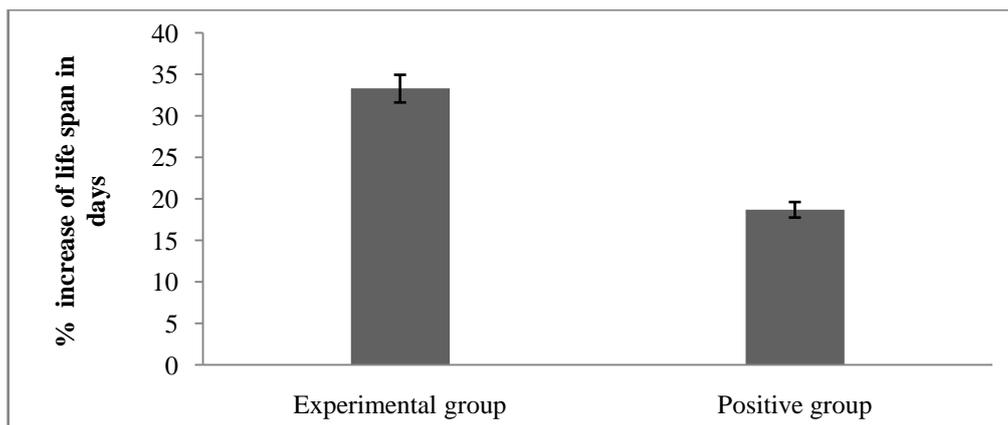


Figure 2: Percent increased survival (%ILS) of the experimental and control group of mice after seven consecutive days of treatment.

3.7.3. Percent increased in life span (%ILS) of experimental and control group mice (subgroup 3) after cyclic treatments

Subgroup 3 of the positive control, negative control, and experimental control groups were observed for survival. Therefore, %ILS of subgroup 3 of the positive control was 35.79 and then that of subgroup 3 of the experimental group was 88.85 (figure 3).

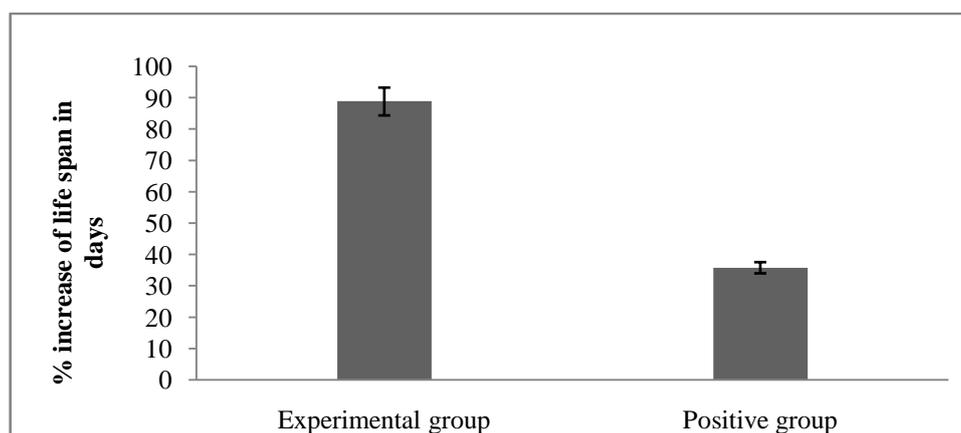


Figure 3: Percent increased in life span (%ILS) of subgroup 3 of positive and experimental group mice after the treatment in a cyclic manner until the death of all mice.

IV. Discussion

Previous studies on *L. coromandelica* leaves demonstrate that the plant contains mainly polyphenols including flavonoids and tannins, terpenoids, gums, and polysaccharides [5]. In this study, the biochemical components of *L. coromandelica* bark samples showed the presence of carbohydrates, tannins, and resins whereas, alkaloids were absent in petroleum benzene extracts. Reducing sugars were present only in methanol extracts. Flavonoids were present in both chloroform and methanol extracts. Saponins and proteins were present only in methanol extract, whereas, steroids were absent only in n-hexane extract. Hence, results of this study correlates with all previous studies.

In this antimicrobial activity assay, the experimental samples showed bacterial growth inhibition against two Gram negative bacteria (*E.coli* and *S. flexneri*) and one of Gram positive bacteria (*S. aureus*). Zone of inhibition of the methanol extract was found (10.5 mm for *E.coli*, 11 mm for *S. flexneri* and 10 mm for *S. aureus*) when compared to amoxicillin (22 mm for all species). According to FDA standard <13 mm in diameter is resistant and 14-17 mm is intermediate whereas, >18 mm of zone of inhibition is sensitive for amoxicillin [19]. So, the chloroform extracts showed least antimicrobial activity against *E.coli*, *S. flexneri* and *S. aureus*.

Bark extracts of *L. coromandelica* were used to observe the antioxidant capacity. In this study, the chloroform extraction showed highest reducing capacity than standard BHT. In another study, the highest quantity of flavonoid content (41.4 ± 0.12 mg QE/g) was found in n-hexane extract of its leaf [20]. In our experiment, chloroform extract showed the maximum value (347.35 ± 95.91 mg). Total flavonoid contents were calculated using the standard curve of quercetin and were expressed as quercetin equivalents (QE) per gram of

the plant extract. The extracts result showed significant ($p < 0.01$) result. The study of its leaves extract showed that TPC of $798.5 \pm 0.19 \text{ mg/g}$ in aqueous extract followed by methanol extract of $342 \pm 0.21 \text{ mg/g}$ [20]. In our study, total phenolic content was calculated using the standard curve of Gallic acid. Amongst the four extracts, the chloroform extract of *L. coromandelica* bark exhibited the highest total phenolic content ($152.95 \pm 13.65 \text{ mg}$) with significant ($p < 0.01$) result. Another investigation of *L. coromandelica* leaves extracts revealed that, n-hexane showed TAC of $88.6 \pm 0.04 \text{ mg/g}$ Gallic acid equivalents (GAE) per gram of plant extract [20]. In this study, the total antioxidant capacity of the samples was calculated using the standard curve of ascorbic acid. The chloroform extract was found to possess the highest total antioxidant capacity ($152.96 \pm 15.18 \text{ mg}$) and all the extracts showed significant ($p < 0.01$) results.

This experimental sample was also subjected to observe the anticancer activity. Mice were divided into three groups, namely positive control, negative control and experimental groups. Each of these groups was further subdivided into 3 subgroups (each subgroup contained 6 mice). Mice in all subgroup 1 were used to observe the number of EAC cells present after the treatment with chloroform extracts from day-1 to day-7. So, the results showed that the sample extracts reduced the EAC cells 2.2 times (2.2x) when compared with negative and 1.73 times (1.73x) when compared with positive control. Mice in all subgroup 2 were used to observe the survival. The MST of subgroup 2 of negative and positive control groups were 32 and 27.8 days respectively whereas, the subgroup 2 of the experimental group survived for 37 days. %ILS of subgroup 2 of positive and experimental groups was found to be 18.7 and 33.3 respectively. The MST calculation showed the subgroup 3 of positive control survived 37.5 days whereas, the subgroup-3 of the experimental group survived 52 days. In this calculation, the percent increase in life span (%ILS) of subgroup 3 of the positive control was found to have 37.5 and subgroup 3 of experimental group showed 88.85. After seven days treatment by the crude sample extracts, apparently, no significant changes was found in their movement, food uptake, enlargement of the peritoneal cavity.

V. Conclusion

From above results, it can be inferred that mainly chloroform extract of sample bark has the potent antioxidant capacity and in vivo anti-cancer effect to some extent in terms of the capacity of the extract to reduce intra peritoneal cancer cell count and increased survival of the experimental animal. Further study should include isolation of pharmacologically active ingredients from *L. coromandelica* bark and evaluation of each active ingredient.

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Md. Jakir Hossain "Phytochemical Screening, Antimicrobial Activity, Antioxidant Capacity And In Vivo Anticancer Activity Of *Lannea Coromandelica* Bark Extracts " *IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS)* 13.3 (2018): 19-25.