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“Determination of Chemical Groups and Investigation of Anthelmintic, Cytotoxic, and Antibacterial Activities of Leaves of *Cinnamomum Tamala* (Family: Lauraceae)”.

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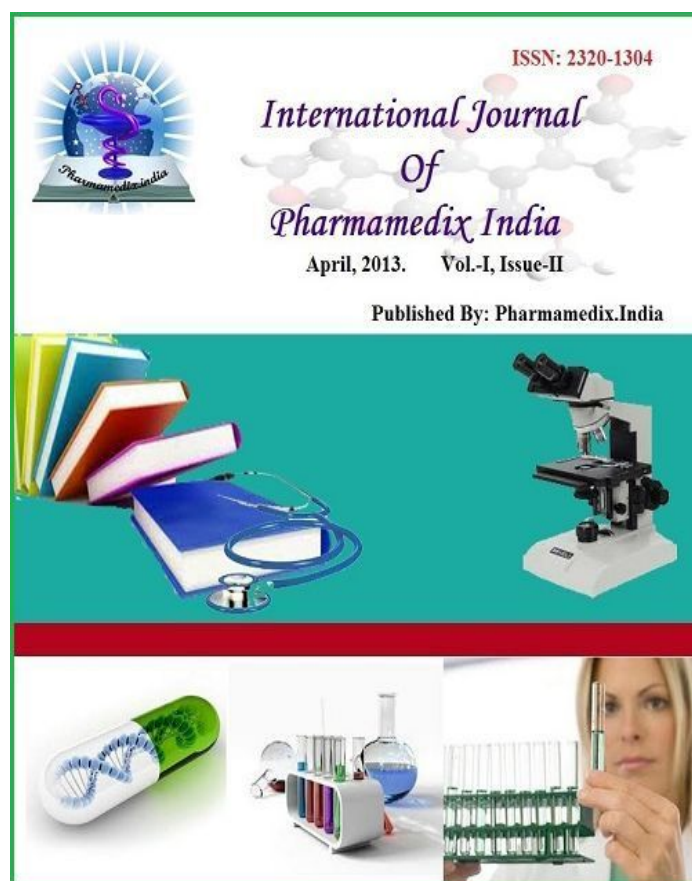
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Abstract:

The present study was conducted for the characterization of possible chemical groups, evaluation of anthelmintic, cytotoxic and antibacterial activities of crude methanolic extract of leaves of *Cinnamomum tamala*. The study revealed the presence of alkaloids, reducing sugar, tannin, amino acids, glycosides and steroid in the crude extract. The extract showed very potent anthelmintic activity while compared with the standard albendazole. To investigate the cytotoxic activity, brine shrimp lethality bioassay was conducted, and the extract showed significant activity while compared with the standard vincristine sulphate (LC₅₀ value 1.007 and 0.839µg/ml respectively). To evaluate the antibacterial activity, disc diffusion method was followed, and the extract showed activity against *Bacillus subtilis*, *Staphylococcus aureus*, *Bacillus cereus*, and *Vibrio cholera*, and resistant to *Escherichia coli* and *Salmonella typhi*.

Keywords: *Cinnamomum tamala*, Lauraceae, cytotoxic, anthelmintic, antibacterial.

Introduction:

Cinnamomum tamala Fr. Nees is an evergreen tree up to 8.5m in height, belonging to family lauraceae. Lauraceae is a vast economically important family consisting mostly of ethnobotanical knowledge from ancient trees or tree-like shrubs. The genus *Cinnamomum* is represented by about 350 species worldwide. It is native to South-east Asia, some Pacific Islands and Australia growing mainly in tropical rain forests at varying altitudes ^[1].

Due to its aroma, the leaves are kept in clothes and also chewed to disguise bad

mouth odor. The leaves of this tree have a clove like taste and a faintly pepper like odor. It is also used in Indian system of traditional medicines. Different extracts from leaves of *C. tamala* have shown anti-inflammatory ^[2], antioxidant ^[3], antiulcer ^[4], anticarcinogenic ^[5], antidiarrhoeal effects ^[6], antidiabetic which is mainly contributed by Cinnamaldehyde (3-phenyl-2-propenal), a potential antidiabetic agent. It is also used medicinally as a carminative, an anti flatulent, a diuretic, treatment of cardiac disorders ^[7] analgesic in dental preparations, due to presence of eugenol (4-hydroxy-3-methoxy allylbenzene).

The main goal of our study was to evaluate the presence of possible chemical groups, cytotoxic, anthelmintic, and antibacterial activities of the crude methanolic extract of the leaves of *Cinnamomum tamala* to validate its folkloric use.

Materials and Methods:

Plant Material Collection

The leaves of *Cinnamomum tamala* were collected by the authors from the surrounding area of Noakhali, a coastal region of Bangladesh in September, 2012. The plant was identified and authenticated by expert botanist of Bangladesh National Herbarium (DACB Accession No. 39290), Mirpur, Dhaka.

Crude Extraction

300 gm of the dried and powdered sample was soaked in 1300 ml of 99.8% methanol (Merck KGaA, Germany). After 15 days the solution was filtered using filter cloth and Whatman[®] filter paper No. 1. The resulting filtrates were then evaporated in water bath maintained at 40°C to dryness and thus a blackish green semisolid mass of the extract was obtained.

Chemical Group Test

Small quantity of freshly prepared methanolic extract of *C. tamala* leaves were subjected to

preliminary quantitative phytochemical investigation for the detection of chemical constituents using the following standard methods^[8].

i. Detection of alkaloids

Extract was dissolved individually in dilute Hydrochloric acid and the solutions were filtered.

a) Mayer's Test: Filtrates were treated with Mayer's reagent (Potassium Mercuric Iodide). Formation of a yellow colored precipitate marked the presence of alkaloids.

b) Hager's Test: Filtered solutions were taken in a test tube and Hager's reagent (saturated picric acid solution) was added with it. Presence of alkaloids was confirmed by the formation of yellow colored precipitate.

ii. Detection of carbohydrates

Extract was dissolved individually in 5 ml distilled water and filtered. The filtrates were evaluated for the presence of carbohydrates.

a) Benedict's test: Filtrates were treated with Benedict's reagent and heated gently. Orange red precipitate pointed the presence of reducing sugars.

b) Fehling's Test: Filtered solutions were hydrolyzed with dil. HCl,

neutralized with alkali and heated with Fehling's A & B solutions. Formation of red precipitate specified the presence of reducing sugars.

iii. Detection of glycosides

Extract was hydrolyzed with dil. HCl, and then subjected to test for glycosides.

a) Legal's Test: Extracts were mixed with sodium nitropruside in pyridine and sodium hydroxide. Formation of pink to blood red color indicated the presence of glycosides.

b) Modified Borntrager's Test: Extracts were treated with Ferric Chloride solution and immersed in boiling water for about 5 minutes. The mixture was cooled and extracted with equal volumes of benzene. The benzene layer was separated and treated with ammonia solution. Formation of rose-pink color in the ammoniacal layer showed the presence of glycosides.

iv. Detection of saponins

a) Froth Test: Extract was diluted with distilled water to 20 ml and this was shaken in a graduated cylinder for 15 minutes. Formation of 1 cm layer of foam expressed the presence of saponins.

b) Foam Test: 0.5 gm of extract was shaken with 2 ml of water. Foam was

produced which remained for 10 minutes and pointed the presence of saponins.

v. Detection of phytosterols

a) Salkowski's Test: Extract was treated with chloroform and filtered. The filtrates were treated with few drops of conc. sulphuric acid, shaken and allowed to stand. Appearance of golden yellow color showed the presence of triterpenes.

b) LibermannBurchard's test: Extract was mixed with chloroform and filtered. The filtrates were treated with few drops of acetic anhydride, boiled and cooled and then conc. sulphuric acid was added. Formation of brown ring at the junction confirmed the presence of phytosterols.

vi. Detection of phenols

Ferric Chloride Test: Extract solution was taken in test tubes and 3-4 drops of ferric chloride solution were added to them. Formation of bluish black color indicated the presence of phenols.

vii. Detection of tannins

Gelatin Test: To the extract, 1% gelatin solution containing sodium chloride was added. Formation of

white precipitate confirmed the presence of tannins.

viii. Detection of flavonoids

- a) Alkaline Reagent Test:** Extract was treated with 4-5 drops of sodium hydroxide solution. Formation of intense yellow color, which becomes colorless on addition of dilute acid, indicated the presence of flavonoids.
- b) Lead acetate Test:** 4-5 drops of lead acetate solution was added to the extract solution. Formation of yellow color precipitate marked the presence of flavonoids.

ix. Detection of proteins and amino acids

- a) Xanthoproteic Test:** The extract was treated with 4-5 drops of conc. Nitric acid. Formation of yellow color indicated the presence of proteins.
- b) Ninhydrin Test:** To the extract, 0.25% w/v ninhydrin reagent was added and boiled for few minutes. Formation of blue color indicated the presence of amino acid.

x. Detection of fixed oils and fats

A few drops of 0.5N alcoholic potassium hydroxide were added to a small quantity of extract along with a drop of phenolphthalein. The mixture was heated on a water bath for 1-

2 hours. Formation of soap or partial neutralization of alkali pointed the presence of fixed oils and fats.

xi. Detection of gums and mucilages

1 ml of the extract was hydrolyzed using dil. HCl (3ml). Then Fehling's solution was added drop by drop till the appearance of red. Test for mucilages were carried out by treating 1 ml of extract with 2 ml of ruthenium red solution to get red coloured solution.

In-vitro Anthelmintic Study

The anthelmintic study was carried out by the method of Ajaiyeoba *et al.* ^[9] with minor modifications. Adult earthworms were selected for the study of anthelmintic activity because of their anatomical and physiological resemblance with the intestinal roundworm parasites of human being ^[10]. Earthworms are widely used as effective tools for anthelmintic study due to their availability ^[11]. Adult earthworm (*Pheretima posthuma*) were collected (3-5 cm in length and 0.1- 0.2 cm in width weighing about 0.8-3.04 g) from moist soil of a road side field of Noakhali Science and Technology University, Sonapur, Noakhali. All the worms were properly washed with normal saline in order to remove all fecal materials.

Extract was weighed and dissolved in 10 mL of distilled water to obtain the solution of 20,

40, 60, and 80 mg/ml. Albendazole was used as reference standard (20 mg/mL). Earthworms were divided into seven groups (each containing three worms) in petridish. In five groups extract solution was applied, one is for reference and one is for negative control. Observations were made for the determination of paralysis time and death time of the worm. Paralysis was designated as the occurrence where the worms do not move even in normal saline and death was confirmed when the worms lose their motility followed with fading away of their body color.

***In-vitro* Cytotoxic Study**

The cytotoxic activity of the extract was examined using brine shrimp lethality bioassay^[12]. In this study vincristine sulphate was used as the positive control. Measured amount of the vincristine sulphate was dissolved in DMSO to get an initial concentration of 40µg/ml from which serial dilutions were made using DMSO to get 20µg/ml, 10µg/ml, 5µg/ml, 2.5µg/ml, 1.25µg/ml, 0.625µg/ml, 0.3125 µg/ml, 0.15625µg/ml and 0.78125µg/ml solution from the extract. Then the positive control solutions were added to the pre-marked vials containing ten living brine shrimp nauplii in 5 ml simulated sea water to get the positive control groups. 100µl of DMSO was added to each of three pre-marked glass vials containing 5 ml of simulated sea water and 10

shrimp nauplii to use as control groups.

Counting of Nauplii

After 24 hours, by using a magnifying glass, the vials were inspected and the number of survived nauplii in each vial was counted. From this data, the percent (%) of lethality of the brine shrimp nauplii was calculated for each concentration.

Antibacterial Activity Test

Test Organisms

Three strains of Gram-positive (*Bacillus cereus*, *Staphylococcus aureus*, and *Bacillus subtilis*), and three strains of Gram negative bacteria (*Escherichia coli*, *Salmonella typhi*, *Vibrio cholerae*) were used to evaluate the antibacterial activity. The strains were collected from the Department of Microbiology, Chittagong Veterinary and Animal Sciences University. For the experiment, the organisms were sub-cultured in nutrient broth and nutrient agar medium.

Disc Diffusion Assay (DDA)

Disc diffusion method is widely acceptable for the evaluation of antimicrobial activity^[13, 14].

In this method, an antibiotic was diffuse from a reliable source through the nutrient agar and a concentration gradient was created. Dried, sterilized filter paper discs (6 mm diameter,

HI-Media, China) containing the known amounts of test samples (400 µg/disc) are placed on nutrient agar medium consistently seeded with the test bacteria. As positive and negative control, Standard antibiotic of Ciprofloxacin (5 µg/disc) and blank discs were used. For the maximum diffusion of the test materials to the surrounding media these plates were reserved at low temperature (4°C) for 24 hours. The plates were then incubated at 37°C for 24 hours to allow optimum growth of the organisms. The test materials having antibacterial property inhibit microbial

growth in plates and thereby yield a clear, distinct zone defined as zone of inhibition. The activity of the test sample was then determined by measuring the zone of inhibition expressed in millimeter^[15].

Results and Discussion:

Chemical Group test

Phytochemical analysis of methanolic extract of leaves of *C. tamala* revealed the presence of some important chemical constituents, e.g., alkaloids, carbohydrates, glycosides, etc (Table 1).

Table 1: Phytochemical screening of the methanolic extract of *C. tamala* leaves.

Sl. No	Group of phytoconstituents	Methanolic extract
1.	Alkaloids	+
2.	Carbohydrates	+
3.	Glycosides	+
4.	Saponins	-
5.	Phytosterols	+
6.	Phenols	-
7.	Tannins	+
8.	Flavonoids	-
9.	Proteins and amino acids	+
10.	Fats & fixed oils	-
11.	Gum and mucilages	-

(+) = presence of constituents; (-) = absence of constituents

Brine Shrimp Lethality Bioassay

LC₅₀ (lethal concentration of half of the test organisms) and LC₉₀ (lethal concentration of 90% of the test organisms) data (for establishing therapeutic index) of vincristine sulphate and all three extracts have been given in table 2, and figure 1.

Table 2: Cytotoxic effect of the test sample of *C. tamala*

Sample	LC ₅₀ (µg/ml)	LC ₉₀ (µg/ml)
Vincristine sulphate	0.839	12.59
Crude extract	1.007	68.53

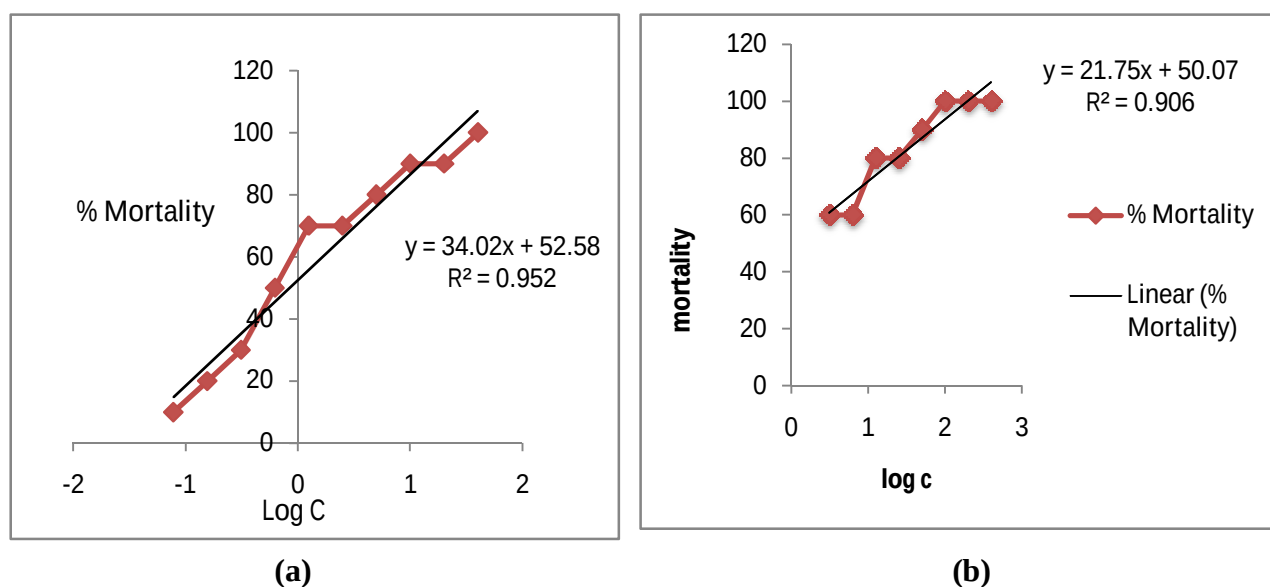


Figure 1: Effect of (a) vincristine sulphate, (b) crude methanolic extract on brine shrimp nauplii.

In-vitro Anthelmintic Activity

From the data (Table 3), we see that, the methanolic extract of *C. tamala* demonstrated paralysis as well as death of worms in fewer times with the gradual increase of the sample concentration.

Table 3: Anthelmintic activity of crude methanolic extract of leaves of *C. tamala* against *Pheretima posthuma*.

Group	Concentration (mg/ml)	Paralysis time (min.)	Death time (min.)
		Mean $\pm$ S.E.M.	Mean $\pm$ S.E.M.
Sample I	20	19.00 $\pm$ 0.45	44.50 $\pm$ 0.22
Sample II	40	12.00 $\pm$ 0.36	19.33 $\pm$ 0.21
Sample III	60	8.33 $\pm$ 0.21	14.00 $\pm$ 0.33
Sample IV	80	5.33 $\pm$ 0.21	8.50 $\pm$ 0.22
Standard	20	14.00 $\pm$ 0.37	43.83 $\pm$ 0.54

n = 5, S.E.M. = Standard Error Mean

Antibacterial Activity Test

The methanolic extract of the leaves showed moderate antibacterial activity against several test organisms. The result of the antimicrobial activity in term of diameter of zone of inhibition in mm is shown in Figure 2. The zone of inhibition varied from 5 to 10 mm at this concentration. This extract did not show any activity against *E. coli* and *Salmonella typhi*.

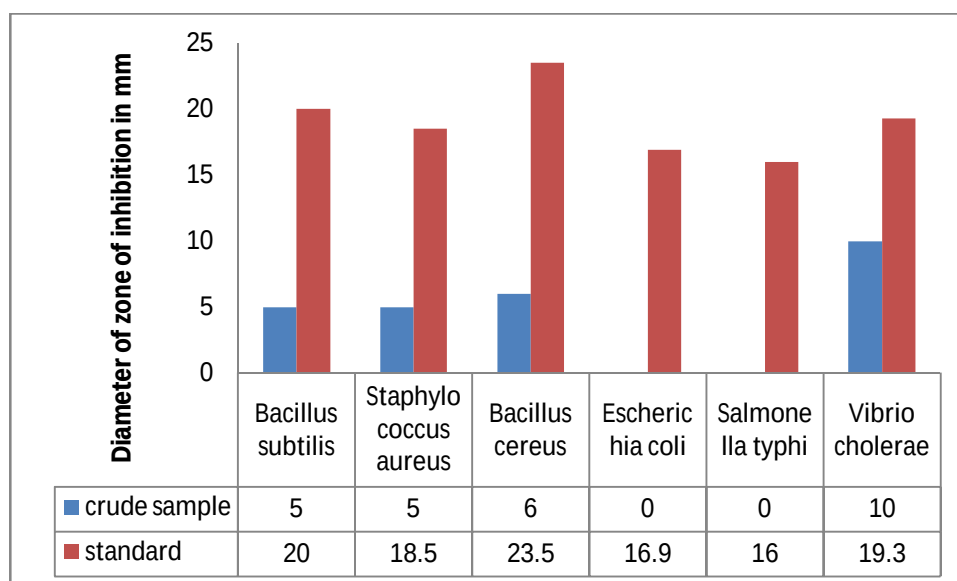


Figure 2: Antibacterial activity of crude extract of *Cinnamomum tamala*

Conclusion:

From the above discussion it can be suggested that further *in vivo* investigation is needed to ensure anthelmintic, antimicrobial, and anticancer activities of the leaves of *C. tamala*, and also it would be interesting to find out responsible compound(s) and relative mechanisms for the mentioned activities.

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