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Screening of *Calophyllum inophyllum* L. leaf extracts for cytotoxic, larvicidal, insect repellent and antimicrobial activities

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Abstract
Petroleum ether (Pet. ether) and Chloroform ($CHCl_3$) solvent extracts were prepared from *Calophyllum inophyllum* L. leaves for testing bioactive potentiality and were assessed against brine shrimp (*Artemia* salina) for cytotoxicity, against larvae of *Culex quinquefasciatus* for larvicidal activity; against eggplant aphid (*Aphis gossypii*) and black bean aphid (*Aphis fabae*) for repellent activity; and against five pathogenic bacteria and three pathogenic fungi for anti-microbial activity tests. Both the extracts exposed propitious results through brine shrimp lethality assay where Pet. ether extract found most effective with LC_{50} values 1413.490, 384.766 and 37.562ppm for 12, 18 and 24h of exposure respectively against *A. salina*. These two extracts showed promising larvicidal activity against the first instar larvae of *Culex quinquefasciatus* where $CHCl_3$ extract was more active with LC_{50} values 237.034, 75.492 and 28.783ppm in compare to Pet. ether extract with LC_{50} values 1854.121, 418.648 and 42.675ppm for 18, 24 and 30h of exposure respectively. In case of repellency test Pet. ether extract found moderately active ($P<0.01$) against eggplant aphid (*Aphis gossypii*) and weakly active ($P<0.05$) against black bean aphid (*Aphis fabae*) where $CHCl_3$ extract found weakly active ($P<0.05$) and inactive against eggplant aphid and black bean aphid respectively. The similar extracts were also propitious against five pathogenic bacteria (*Agrobacterium sp., Bacillus cereus, Escherichia coli, Shigella dysenteriae and Staphylococcus aureus*) at concentrations of 200 and 400µg/disc along with a standard Penicillin 30µg/disc and three pathogenic fungi (*Aspergillus niger*, *Candida* sp. and *Saccharomyces* sp.) at concentrations of 50 and 200µg/disc along with a standard Nystatin 10µg/disc. Between the two extracts Pet. ether showed promising antibacterial and antifungal activities with the zone of inhibition 16 and 20mm; and 6 and 14mm against *Sh. dysenteriae* bacterium and *Candida* sp. fungal strains in comparison to standard penicillin (30µg/disc) and standard Nystatin (10µg/disc) with the inhibition zone of 30 and 7mm respectively. The results showed the potential use of *C. inophyllum* leaf extracts to control aphids, mosquito larvae, bacteria and fungi.

Keywords: *Calophyllum inophyllum*, cytotoxicity, larvicidal-activity, repellency, antimicrobial-activity

1. Introduction
Plants have been widely used as a source of inspiration for new drug compounds, since plant derived medicines have made many gifts to human health and its wellbeing. Global plant biodiversity serves as the main source of herbal medicine and almost ¾ th of the world population depends on plant related medicines for basic health care [1]. *Calophyllum inophyllum* L. (Calophyllaceae) commonly called Alexandrian laurel ball tree, Indian doombaoiltre, beauty leaf and Indian-laurel etc. is a medium to large evergreen tree. It is widely distributed in tropical areas and tolerates varied kinds of soil, coastal sand, clay or even degraded soil [2]. The leaves are large, stiff, shiny, leathery and oblong with a blunt tip. They are 3-8 inches long, arranged opposite each other and have closely placed fine parallel veins running from a prominent raised yellow-green midrib to the leaf margin [3]. Traditional Chinese folk medicine employs this for the treatment of wounds, eye diseases, inflammations and rheumatism [4]. The leaves saturated in water are useful for inflamed eyes. The leaf cocktail can be used internally for heatstroke [5]. The chemical literature shows the presence of diverse biomolecules such as flavonoids [6], triterpenes [7] which have assorted bioactive such as anti-microbial [8] and cytotoxic activities [9]. It has been reported the importance of *C. inophyllum* in treatment of HIV by inhibiting the activity of HIV-integrate and protease [10]. Some isolated compounds have been reported to be biologically active, with cytotoxic [8], repellent [11] and anti-inflammatory [12] activities. *C. inophyllum* also shows antiviral activity, especially the anti-HIV activity by inhibiting antiviral replicating and functional enzymes [13]. The anti-termitic assay of wood extracts was also investigated against *Coptotermes curvignathus* [14]. The fruits and leaves of *C. inophyllum* are very poisonous [15].

The extracted oil from the fruit is used as a remedy for sciatica, shingles, neuritis, leprous neuritis and rheumatism, ulcers and skin diseases; while the oil from this tree's seed is reported to have medicinal and healing properties [16]. Other uses included gum for treatment of wounds and ulcers, bark for vaginal discharge after childbirth, passing of blood and gonorrhea [17], antiseptic, disinfectant, internal haemorrhages [18] and Calocoumarin-A as an anti-cancer agent [19]. The Japanese believed the tree had diuretic properties but in Samoa the whole tree is considered a virulent poison [2]. Recently, *C. inophyllum* has been identified as the most suitable feedstock for future generation biodiesel [20]. However, to the best of our knowledge, a study of leave extracts of *C. inophyllum* species from Bangladesh, or any other country, is still scanty. A few studies focus on the medicinal properties [2, 21] rather than on leaves preservatives. This is why the aim of this study was to evaluate the *C. inophyllum* leaves extracts for investigating of its bioactive potentials.

2. Materials and methods

2.1 Collection and preparation of test materials

The fresh leaves of *C. inophyllum* were collected from the coastal region of Barisal, identified and kept in the herbarium of the Department of Botany, University of Rajshahi. The leaves were chopped into small pieces, dried under shade and powdered using an electric grinder, weighted and placed in separate conical flasks to add Pet. ether and $CHCl_3$ (Merck, Germany) (100gm × 300ml × 2times) for 48h. Filtration was done by Whatman filter paper (made in USA) at 24h interval in the same flask followed by evaporation until the extract was left. The extracts were then removed to glass vials and preserved in a refrigerator at 4^0C with proper labeling.

2.2 Lethality test on Brine shrimp nauplii

Brine shrimp eggs were purchased from Kalabagan, Dhaka and kept in aerated sea water at room (25-30 °C) temperature and they took 30-48h to give nauplii. The series of concentration were 200, 100, 50, 25 and 12.5ppm for Pet. ether and $CHCl_3$ extracts. Ten freshly hatched nauplii were added to each of the test tubes with different concentrations mentioned earlier and observed mortality after ½, 6, 12, 18 and 24h of exposures. The data was then subjected to Probit analysis.

2.3 Larvicidal activity test

To perform larvicidal activity test mosquito rafts (eggs) were collected from the different drains of Rajshahi University then placed in a new beaker containing normal pond water and kept in a dark place inside the laboratory for hatching. Hatched larvae were collected after 24h and used in the experiment. The leaves extracts were dissolved in 10µl of DMSO for its solubility in water. Ten freshly hatched larvae were treated with the plant extracts in doses 400, 200, 100, 50 and 25ppm for Pet. ether and $CHCl_3$ extracts. The mortality was observed after 6, 12, 18, 24 and 30h of exposure. The data was then subjected to Probit analysis.

2.4 Collection and culture of the test insects

Aphids are very soft and tiny creature and have highly reproducing capability. At first some mature aphids were collected from affected plants and released on new fresh eggplants and bean plants for further production. They multiply in a good number within short time. Aphids were collected repeatedly from the culture field with a fine camel hair-brush in a Petri dish and were used in the experiments.

2.5 Repellent activity test against eggplant aphids and black bean aphids

The methodology for repellency test used in the experiment was adopted from the method (No. 3) of McDonald *et al*. [22] with some modifications by Talukder and Howse [23-24]. For aphids fresh eggplant leaves were used. Stalks of each of the leaves were wetted with water soaked cotton to keep them fresh. A CD marker was used to draw round circle (3.6 cm diam.) on the leaves. A general concentration for each of the leaves extracts was selected as stock dose for repellency and other successive doses were prepared by serial dilution to give 0.079, 0.039, 0.019, 0.009 and 0.004mg/cm^2 concentrations for Pet. ether and $CHCl_3$ extracts. Ten aphids were released in the middle of each of the leaves circle. The orientation was changed in the 2 remaining replicates to avoid the effects of any external directional stimulus affecting the distribution of the test insects.

2.6 Observation and analysis of repellency data

Repellency was observed for one-hour interval and up to five successive hours of exposure, just by counting the number of insects from non-treated part of the restricted circle (3.6 cm) on the eggplant leaf. The average of the counts was converted to percent repellency (*PR*) using the formula of Talukder and Howse: $PR = (Nc\text{-}5) \times 20$, where, Nc is the percentage of insects on the untreated half of the disc or circle [23, 25].

2.7 Growth and maintenance of test microorganisms for antimicrobial studies

Strains of bacteria and fungi, were obtained from the Department of Pharmacy, University of Rajshahi, Bangladesh, were used for antimicrobial test. The bacteria were maintained on nutrient broth (NB) at 37 °C and fungi were maintained on Potato dextrose agar (PDA) at 28 °C.

2.8 Anti-bacterial activity

The Pet. ether and $CHCl_3$ extracts of *C. inophyllum* leaves were tested by the disc diffusion method. Two concentrations (200 and 400µg/disc) of the extracts were prepared by reconstituting with required solvents. The test microorganisms were seeded into respective medium by spread plate method with 24h cultures of bacteria growth in nutrient broth. After solidification the filter paper discs (5mm in diameter) impregnated with the extracts separately were placed on test organism-seeded plates. *Agrobacterium* sp., *Bacillus cereus, Escherichia coli, Shigella dysenteriae* and *Staphylococcus aureus* were used for antibacterial test. Penicillin (30µg/disc) was used as Standard. The antibacterial assay plates were incubated at 37 °C for 24h. The diameters of the inhibition zones were measured in mm.

2.9 Antifungal Activity

The antifungal activity was tested by disc diffusion method. Potato dextrose agar plates were inoculated with each fungal culture (10 days old) by point inoculation. Filter paper discs (5mm in diam.) impregnated with two (50 and 200µg/disc) concentrations of the extracts was placed on test organism-seeded plates. Solvents were used to dissolve the extracts and were completely evaporated before application on test organism-seeded plates. Nystatin (10µg/disc) used as standard. The activity was determined after 24-48h of incubation at 28 °C. Diameters of the inhibition zones were measured in mm.

3. Results and Discussion

3.1 Brine shrimp lethality effect

Ethanol extract of *C. inophyllum* leaves exhibited cytotoxic activity at concentration 20 to 100µg/ml after 24h of exposure against Brine shrimp [1]. Present experiment executed to trace the lethality test of *C. inophyllum* leaves extracts on brine shrimps is in agreement with the above studies where Pet. ether extract offered high toxicity (LC_{50} values 37.562) against *A. salina* followed by $CHCl_3$ extracts (LC_{50} values 287.857) after 24h of exposure. Brine shrimp lethality assay indicates that the bioactive components present in this plant can be accounted for its pharmacological effects. Thereby this results support the uses of these plant species in traditional medicine. Brine shrimp lethality results and LC_{50} values obtained are shown in and Table 1. Previous works on the same supported recent findings. Absolute ethanol extracts of *Phyllanthus niruri* and *Passiflora foetida* were toxic after 24h of exposure against *A. salina* with the LC_{50} values at 251.19µg/ml and 749.89µg/ml respectively [26]. Ethanol extract showed a toxicity effect after 6h and 24h exposures with LC_{50} values at 944.07 and 266.07ppm respectively [27]. The acetone extract of *A. arctotoides* and the hexane extracts of *A. arctotoides* and *G. bicolor* exhibited significant brine shrimp lethality with LC_{50} values of 0.87, 0.89 and 0.82mg/ml, respectively [28]. The Pet. ether, $CHCl_3$ and CH_3OH extracts of *S. nodiflora* showed lethality effect against the brine shrimp with LC_{50} values 140.866, 22.161 and 248.325ppm for 30h of exposures respectively [29].

Table 1: LC_{50} values of Pet. ether and $CHCl_3$ extracts of *C. inophyllum* leaves against *A. salina* nauplii.

Solvents	LC_{50} values (ppm) at different exposures (in hours)			
	6	12	18	24
Pet. ether	-	1413.490	384.766	37.562
$CHCl_3$	-	303.296	289.913	287.857

3.2 Effect of larvicidal activity

The present study well presented the larvicidal capability of *C. inophyllum* leaves as a novel source against *C. quinquefasciatus*, the most crucial filarial vector, under laboratory conditions for the first time. The results of this experiment indicate that the two solvent extracts of the *C. inophyllum* leaves can be used as potential larvicide in vector control programs as field application of these extracts can be done. The larvicidal activity for Pet. ether and $CHCl_3$ extracts of *C. inophyllum* against *C. quinquefasciatus* represented in Table 2. Singh *et al*. 2015 reported that ethyl acetate extractives *of Nicotiana plumbaginifolia* leaf exhibited larvicidal activity against 3rd instar larvae of *Anopheles stephensi* with LC_{50} and LC_{90} values 17.07 and 99.33 ppm respectively after 24h of exposure [30]. Toward 3rd instar *Aedes aegypti* larvae *Zanthoxylum piperitum* bark XDA (LC_{50}, 0.24 mg/l) was the most toxic compound, followed by pellitorine (LC_{50}, 0.98 mg/l), as judged by the 24 h LC_{50} values [31]. In our experiment, LC_{50} values were found 28.783 and 42.675ppm for the $CHCl_3$ and Pet. ether extracts respectively after 24h of exposure for the 1st instar larvae of *C. quinquefasciatus*. The LC_{50} values of the methanol extract of *Murraya exotica* and *Lawsonia inermis* against the 3rd and 4th instar larvae and pupae were 135.539, 154.361, 178.571ppm and 139.057, 163.630, 188.151ppm respectively after 24h of exposure [31]. Naser *et al*. revealed that Pet. ether, $CHCl_3$ and CH_3OH extracts of *Phyllanthus niruri* offered cytotoxic activity against the *Culex quinquefasciatus* with the LC_{50} values 3.39, 3.42 and 259.86ppm after 30h of exposure respectively [32].

Table 2: LC_{50} values of Pet. ether and $CHCl_3$ extracts of *C. inophyllum* leaves against *Culex quinquefasciatus* larvae.

Solvents	Duration of exposure in hours			
	12	18	24	30
Pet. ether	-	1854. 121	418.648	42.675
$CHCl_3$	28607.520	237.034	75.492	28.783

3.3 Repellent effect against eggplant and black bean aphid

The present study was executed to evaluate the repellent capability of *C. inophyllum* leaves against eggplant aphids (*A. gossypii*) and black bean aphid (*Aphis fabae*), the most crucial sap-sucking true-bugs, under laboratory conditions. This study is a preliminary investigation in aphid control and more studies are needed to bioassay the activity of specific compounds against aphid species and other pests. The repellent results have been showed in Table 3 and 4. Naser *et al*. (2014) reported that $CHCl_3$ and CH_3OH extracts of *Phyllanthus niruri* offered a promising repellent effect against eggplant aphids at the level of significance $P<0.01$, and $P<0.05$ respectively while Pet. ether extract did not offered repellent activity [32]. In the current investigation the Pet. ether and $CHCl_3$ extracts showed repellent activity against *A. gossypii* with the level of significance $P<0.01$ and $P<0.05$ respectively while only Pet. ether extract exposed repellent activity ($P<0.05$) against *Aphis fabae*. The crude leaf extract of *T. minuta* and *T. vogelii* was significantly repellent against red spider mites with the level of significance (Fisher's LSD test $P<0.05$) [33].

Table 3: Repellent effect of Pet. ether and $CHCl_3$ extracts of *C. inophyllum* leaves against eggplant aphid (*A. gossypii*).

Solvents	Between doses (df =4)		Between time interval (df=4)	
	F-value	Level of significance	F-value	Level of significance
Pet. ether	23.703	$P<0.01$	0.261	-
$CHCl_3$	13.640	$P<0.05$	0.709	-

Table 4: Repellent effect of Pet. ether and $CHCl_3$ extracts of *C. inophyllum* leaves against black bean aphid (*A. fabae*).

Solvents	Between doses (df =4)		Between time interval (df=4)	
	F-value	Level of significance	F-value	Level of significance
Pet. ether	13.638	$P<0.05$	0.465	-
$CHCl_3$	2.241	-	3.102	-

3.4 Effect of antimicrobial activity

The present study was also applied to evaluate the antimicrobial properties of Pet. ether and $CHCl_3$ extracts of *C. inophyllum* leaves. The present study offered a promising result against five pathogenic bacteria and three pathogenic fungi; where Pet. ether extract showed the highest antibacterial and antifungal activities with the zone of inhibition 16 and 20mm; and 6 and 14mm against the *Sh.*

dysenteriae bacterium and *Candida* sp. fungal strains comparing to standard Penicillin (30µg/disc) and standard Nystatin (10µg/disc) with the inhibition zone of 30 and 7mm respectively. This indicates that the test material is a valuable source of antimicrobial compounds that are potent and further investigation is needed to identify the pure components. The results of antimicrobial activity of *C. inophyllum* leaf extract against the selected bacterial and fungal strains are shown in Table 5 and 6.

This finding receives supports from the previous researchers' research outcome. Sundur *et al.* reported that the presence of phenolic compounds in *C. inophyllum* gave acidic properties and could possibly be responsible for the antimicrobial activities [13]. Pathogenic bacteria like *Pseudomonas aeruginosa*, *Staphylococcus aureus* and fungus *Aspergillus niger* were inhibited in presence of the ethanolic extract of *Hildegardia Populiolia* [34]. The methanolic extracts of *Amoora cucullata* stems and leaves showed potent antimicrobial activity against *Escherichia coli, Shigella dysenteriae, Salmonella typhi* and *Salmonella paratyphi* with the zone of inhibition 9, 8, 7 and 10; and 7, 9, 7 and 9mm respectively in comparison to the standard Kanamycin(30µg/disc) with inhibition zone 13, 19, 20 and 32mm respectively [35].

Table 5: Antibacterial activity of *C. inophyllum* leaf extracts and the standard Penicillin

Zone of inhibition in mm					
Bacterial strains	**Pet. ether extract $CHCl_3$ extracts**				**Standard Penicillin (30µg/disc)**
	200µg/dis	**400µg/disc**	**200µg/disc**	**400µg/disc**	
Agrobacterium sp.	8	14	11	12	6
Bacillus cereus	7	8	8	10	6
Escherichia coli	15	18	11	12	11
Shigella dysenteriae	16	20	12	14	30
Staphylococcus aureu	15	16	8	10	16

Table 6: Antifungal activity of *C. inophyllum* leaf extracts and the standard Nystatin

Zone of inhibition in mm					
Fungal strains	**Pet. ether extract $CHCl_3$ extracts**				**Standard Nystatin (10µg/disc)**
	50µg/dis	**200µg/disc**	**50µg/disc**	**200µg/disc**	
Aspergillus niger	6	8	6	12	7
Candida sp.	6	14	6	8	7
Saccharomyces sp.	6	10	6	11	7

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