Pharmacological Authentication of *Calophyllum inophyllum* Leaf Concerning Diarrhea, Pyrexia, Hepato and Nephro toxicities in Mice Models: A well-known Medicinal Plant of Bangladesh.

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Abstract

The objective of the study was focused to evaluate the anti-diarrheal, anti-pyretic, hepato and nephro protective actions of the ethanolic leaf extracts of Calophyllum inophyllum (EECI) (family - Calophyllaceae), conventionally used in inflammation, rheumatism, hypertension, diabetes. The investigation was conducted in vivo using animal models. EECI was alienated into three doses, 100 mg/kg, 200 mg/kg, 400 mg/kg of body weight and then exposed to animal models(mice) to investigate the anti-diarrheal, anti-pyretic, hepato and nephro protective activities. The antidiarrheal property was determined by castor oil induced diarrhea method using loperamide as standard. The antipyretic test was done by injecting 20% yeast solution at 10 ml/kg using aspirin as standard. CCl_4 induced hepato and nephro toxicity tests were done by 20% v/v CCl_4 solution in mice models at 0.5 ml/kg dose intraperitoneally to investigate protective potential of EECI. Antidiarrheal and antipyretic tests were conducted at the doses of 100 and 200 mg/kg body weight. On the other hand, 100, 200 and 400 mg/kg body weight doses were applied to evaluate hepato and nephro protective properties. EECI inhibited castor oil-induced diarrhea significantly ($P<0.05$) in mice with 42.86% & 57.14% of inhibition at 100 & 200mg/kg body weight respectively as compared with standard drug loperamide (78.57%). EECI also showed significant ($P<0.001$) antipyretic effect in pyrexia induced by yeast at the doses 100 & 200 mg/kg as compared with aspirin 300 mg/kg body weight. The maximum reduction of body temperature was observed at 20hour (91.83 & 92.47°F) for both doses. The EECI exhibited a potential hepato and nephro protective activities against CCl_4 induced hepato and nephro toxicities. The oral administration of EECI at the doses 100, 200, and 400 mg/kg/day for 5 days pointedly ($P < 0.05$) reduced the elevated levels of ALP, AST, Bilirubin, Creatinine compared with CCl_4 treated mice, and these biochemical parameters were comparable with Silymarin. The explored findings of the study authenticate and verify the pharmacological properties of C. inophyllum leaf regarding Bangladeshi species and necessitate the identification and isolation of responsive chemicals with mechanism of action in future.

Keywords: *Calophyllum inophyllum, Antidiarrheal, Antipyretic, Hepato and nephro protective*

Introduction

Medicinal plants are the most valuable gifts of nature. In Bangladesh, 80% people live in rural area and their health issues habitually depend on dietary supplements and folklore medicines. Traditional healers of villages use several types of plant parts and herb for the ailment of health problems. As a gift of nature, plants with medicinal values are believed to possess lesser side or adverse effects. Maximum plants of our country have therapeutic uses with reported medicinal value that necessitate the identification of bioactive entities. Certain public health problems like diarrhea, fever and

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unusual but life-threatening complications as liver, kidney damage may aggravate overall well-being of the country. These health conditions can be lessened and managed by using medicinal plants or by controlling synthetic drugs and environmental toxins with severe side effects.

Calophyllum inophyllum, known as Alexendrial laurel, is native to Africa in Madagascar; Comoros; Kenya; Mozambique; Seychelles; Tanzania; Mauritius; south, east and southeast Asia in: Bangladesh; Cambodia; southern India; China; Andaman and Nicobar Islands Indonesia; Malaysia; Myanmar; Japan and many other countries. As ornamental plant *C. inophyllum* is very famous with beautiful cluster fragrant white flowers and renowned for the essential oil extracted from fruit kernel. In Bangladesh, it is known as Sultan Chapa. The oils, as well as poultices prepared from leaves and flowers, are used for traditional medicine. The dark green oil extracted from seed also possesses medicinal properties with therapeutic and cosmetic uses. *C. inophyllum* had been chemically investigated several times and many vital bioactive secondary metabolites were identified. Three pyranocoumarin derivatives had been identified and isolated from the plant's seeds named as tamanolide, tamanolide D and tamanolide P (Hollister, 1981). Stem and leaves were also chemically investigated and canophyllol, 3β, 23-epoxy-friedelan-28-oic acid, friedelin, canophyllal, canophyllic acid, 3-oxo-friedelan-28-oic acid, epifriedelanol and oleanolic acid named triterpinoids were identified (Li et al., 2010). Four coumarins had been identified and named as (+)-12-methoxyinophyllum A, (+)-12-methoxyinophyllum H-1, (-) - 12-methoxyinophyllum H-2, and inophyllum J, along with two known compounds like 12- ethoxyino-phyllum D and isoinophynone (Li et al., 2016). Phytochemical investigation of methanolic leaf extracts were performed by spectrophotometric method where polyphenol, alkaloid, flavonoid, tannin, triterpenoid, saponin, with three novel compounds as 10c-dimethyl-10b, trans-2-[2-(trifluoromethyl) phenyl]-10b, 10c-dihydropyrene were identified and purified (Idris et al., 2017). *C. inophyllum* had also been subjected to many *in vivo* and *in vitro* biological studies. These study results reveal analgesic, antibacterial, antidiabetic, antifungal, anti-inflammatory, anxiolytic, anticovulsant, antiviral, antioxidant, antiplatelet, antipyretic, anti-HIV, antiulcer, thrombolytic, membrane stabilizing properties of different extracts of this plant (Narayan et al., 2011; Perumal et al., 2017; Aminudin et al., 2015; Léguillier et al., 2015; Mishra et al., 2010; Jaiswal et al., 2015; Jantan et al., 2010; Akter et al., 2017). The properties demand further investigation regarding Bangladeshi species and for this the study was designed to verify and evaluate the pharmacological properties of *C. inophyllum.*

Methods and Materials

Animals

Male Swiss albino mice weighted 12-14g were obtained from icddrb, Mohakhali, Dhaka Bangladesh. Mice were housed under basic laboratory specifications (22-25°C, moisture 40- 60%, 12 h light: 12 h dim cycle). The mice were kept in typical size plastic box in properly ventilated room. The mice were given food for one month for making the weight 25-35gram. During experiment all animals were handled with care. All methods were completed conferring institutional standards of animal research.

Preparation of Plant Extract

Leaves of *C. inophyllum* were collected from Botanical Garden, Dhaka, Bangladesh January 2019. Plant specimen was detected by an expert taxonomist from National Herbarium of Bangladesh and a specimen was preserved there with Accession number 45309. The collected leaves were sun dried and powdered. The leaf powder was soaked in ethanol and extracted by occasional shaking for 14

days. The ethanolic leaf extract (EECI) was filtered and reduced using a rotary evaporator at 55°C temperature to be ready for concentrated crude extract. Then the gummy or semisolid filtrate dried at low temperature (390C).

Pharmacological Investigations

Antidiarrheal Activity Evaluation

Castor oil induced diarrheal models were used modified by Shoba and Franca (Shoba & Thomas, 2001; Franca et al., 2008). Healthy animals were separated and distributed to four groups of four animals each. Mice were fasted overnight with the supply of water before treatment. For the stimulation of diarrhea, each mouse was treated with 0.5 ml castor oil 30 minutes before treatment. At zero-hour saline water to Group I as normal control, standard drug Loperamide (5mg/kg) to Group II and 100 & 200 mg/kg EECI were administered to Group III & IV orally(1ml). Each mouse was then housed in distinct cage, transparent paper lining was used on the cage floor to count feces per hour. Beginning of diarrhea, overall quantity of feces for 4 hours was noted. The diarrheal tendency was measured each hour for 4 hours.

Antipyretic Activity Evaluation

The Antipyretic property of EECI was investigated by Brewer Yeast Stimulated Pyrexia method in mice models. Healthy male mice models (16) were selected and adapted with research laboratory environment before starting of the experiment. The animal models were separated into four groups consisting of four mice per group. At the very beginning, the body temperature of each mouse was noted using digital thermometer and then fever was tempted by injecting Brewer's yeast (20% aqueous suspension) at the dose of 10ml/kg SC in all mice. All mice were free access to drinking water without any food and after 24h rectal temperature of each mouse was verified. The initiation of pyrexia was confirmed by increased rectal temperature more than 0.5°C and mice with pyrexia were separated from other mice to conduct further study (Kang et al., 2008). Group I was treated with saline (10ml/kg) as a normal control, Group II received Aspirin (300mg/kg) as a standard drug (Jena et al., 2012; Perianayagam et al., 2004) while groups III and IV treated with 100 and 200 mg/kg of extract intraperitonially. After treatment, rectal temperature of each mice model was again documented periodically at 1, 2, 3, 4 and 5h. Antipyretic properties of the extracts were evaluated by comparing the result with the normal control and standard control groups.

Hepatoprotective and Nephroprotective Activities Evaluation

Hepatotoxicity and nephrotoxicity are the serious complications associated with adverse effects of synthetic drugs. This study was planned to explore the protective effect in carbon tetrachloride (CCl_4)-induced hepatotoxicity and nephrotoxicity using mice models (Umer et al., 2010). Twenty-four experimental animals were distributed into six groups with four mice of each. Group 1 (Normal Control) did not receive any treatments, Group 2 (Positive Control) received saline water for five days. This group only received CCl4 at the end of the experiments. Group 3 (Standard Control) received 200mg/kg silymarin and Group 4, 5 & 6 (Positive Control) received 100, 200 and 400 mg/kg dosages of EECI orally for five days. At the 6th day animals are treated with 20% v/v solution of CCl_4 in olive oil (0.5 ml/kg) intraperitoneally. After 24th hour of CCl_4 injection animals were sacrificed under CHCl3.Bloods were collected from artery and heparinized then it was centrifuged for 15 minutes at 3000rpm. The serum was collected and stored at -20ºC for further analysis. Then serum liver biomarkers as AST, ALP, total bilirubin and albumin were assessed according to the manufacturer's protocol and previously reported methods using Biochemical analyzer. The creatinine levels from sera were also estimated to analyze nephroprotective activity of the studied plant.

Statistical Analysis

The findings of all experiments were presented as mean ± SEM. The studied groups were compared with each other by one-way analysis of variance (ANOVA) using SPSS 20 software. A p value <0.05, < 0.001 were counted statistically considerable.

Results

Effect of EECI on Castor Oil-Induced Diarrhea

The outcomes showed that EECI noticeably ($p<0.05$) reduced the frequency of defecation as compared to control group. The extract treated groups significantly reduced the number of feces at 1 to 4 hours as to control and standard group. EECI showed statistically significant reduction of defecation with 57.14% inhibition at the dose of 200 mg/kg body weight. At 100mg/kg dose EECI showed 42.86% of diarrhea inhibition whereas the standard drug loperamide showed 78.57% of inhibition at the dose of 5mg/kg.

Table 1: Effects of the EECI on the castor oil-induced diarrhea in mice

Group	Total number of feces	Number of diarrhea feces	Inhibition of diarrhea (%)
Control	7 ± 0.942	3.5 ± 0.65	
Standard (Loperamide 5 mg/kg)	3.75 ± 1.59	0.75 ± 0.55	78.57
Extract (100 mg/kg)	4.5 ± 1	2 ± 0.47	42.86
Extract (200 mg/kg)	4.25 ± 0.29	1.5 ± 0.33**	57.14

All values are expressed as mean ± SEM (n = 4); significance at ** P < 0.05 as compared to control. The statistical analysis is done by ONE-WAY-ANNOVA Dunnett test.

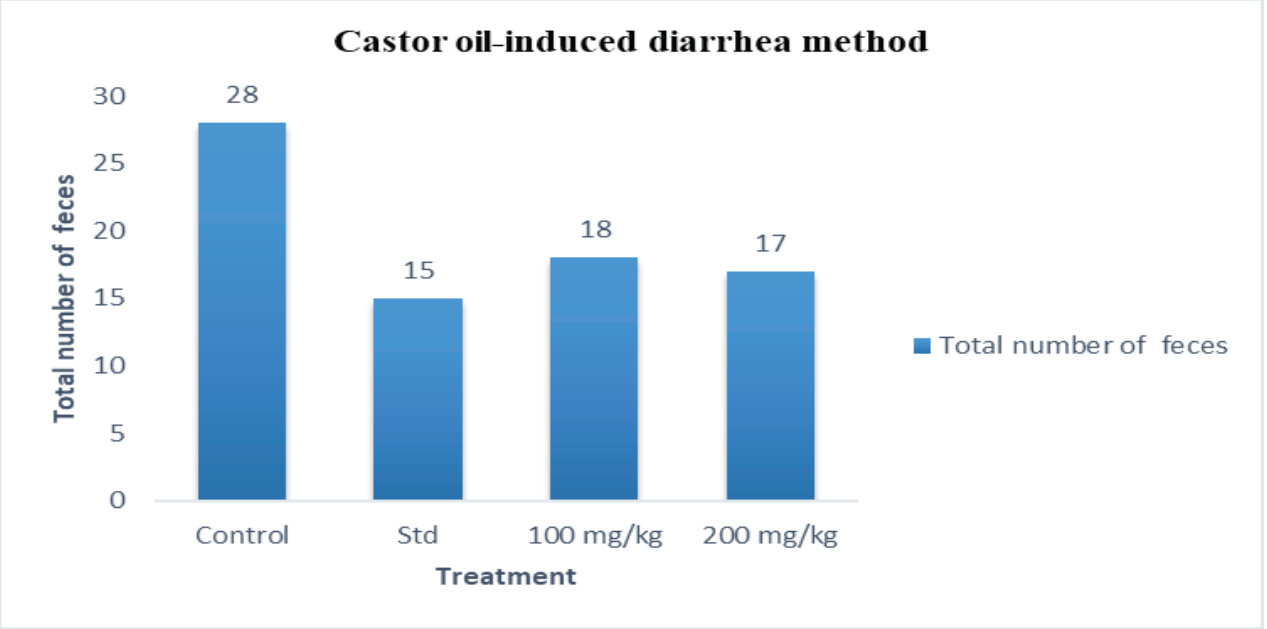

Figure 1: The bar chart of total number of feces in four hours

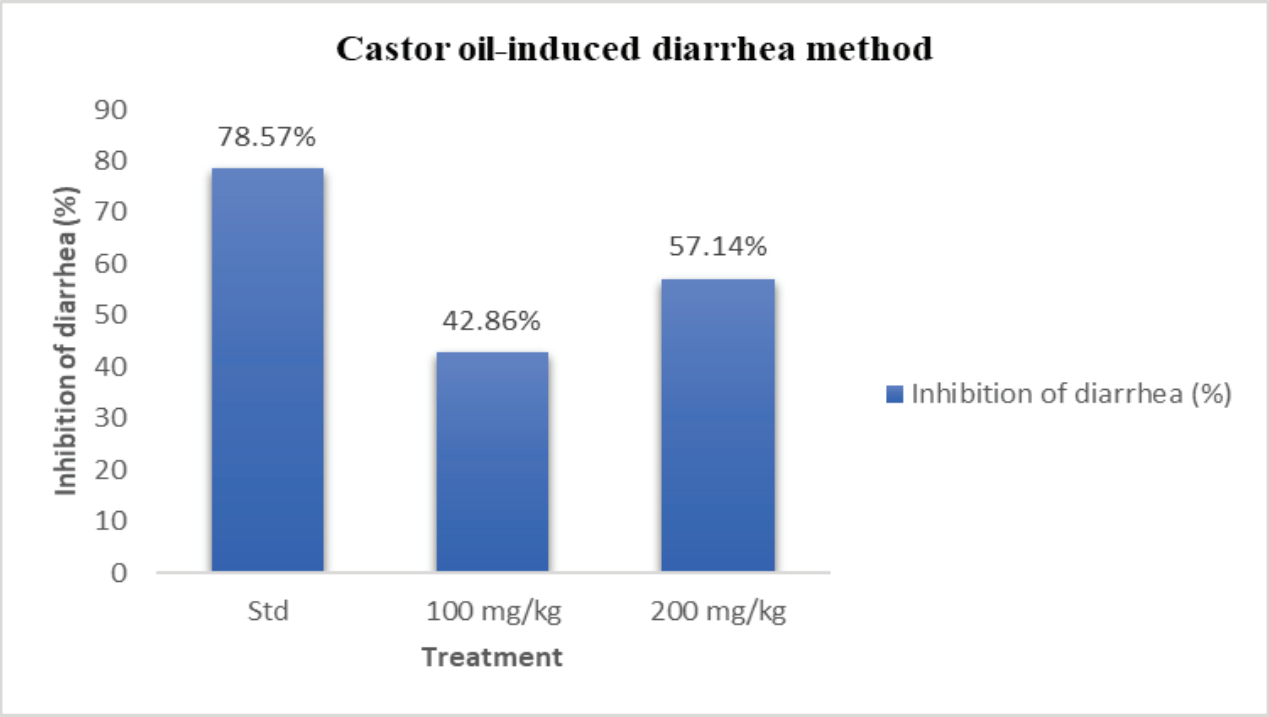

Figure 2: The bar chart of inhibition of diarrhea feces in four hours

Effect of EECI in Brewer's Yeast Induced Pyrexia

Brewer's Yeast induced Pyrexia test was done for the evaluation of analgesic potential of EECI. The EECI at 100 and 200 mg/kg doses pointedly ($P < 0.001$) reduce the temperature of mice after 24 hours which was compared to standard aspirin (Table 2). The antipyretic potential of EECI was not dose dependent but pyrexia reduction was faster than aspirin.

Table 2: Effects of the EECI on Brewer's Yeast Induced Pyrexia Test

Drug	Temperature (F°)						
	0 Hour	18 Hour	19 Hour	20 Hour	21 Hour	22 Hour	23 Hour
Blank	99.53 ± 0.03	100.23 ± 0.09	101.8 ± 0.16	102.25 ± 0.97	103.35 ± 0.74	102.58 ± 0.88	103.1 ± 0.86
Aspirin 300 mg/kg	99.5 ± 0.03	101.75 ± 0.28**	95.92 ± 0.14***	94 ± 0.41***	95.52 ± 0.55***	97.37 ± 0.39**	99.25 ± 0.15**
EECI 100 mg/ kg	99.3 ± 0.10	100.53 ± 0.27	93.5 ± 0.61***	91.83 ± 0.19***	93.35 ± 0.71***	94.7 ± 1.14***	96.43 ± 1.35**
EECI 200 mg/kg	99.32 ± 0.11	100.75 ± 0.33	94.27 ±0 .24***	92.47 ± 0.43***	95.35 ± 0.55***	96 ± 1.48***	96.13 ± 1.75**

All values are expressed as mean ± SEM (n = 4); significance at ** $P < 0.05$, ***$P < 0.001$ as compared to blank. The statistical analysis is done by ONE-WAY-ANNOVA Dunnett test.

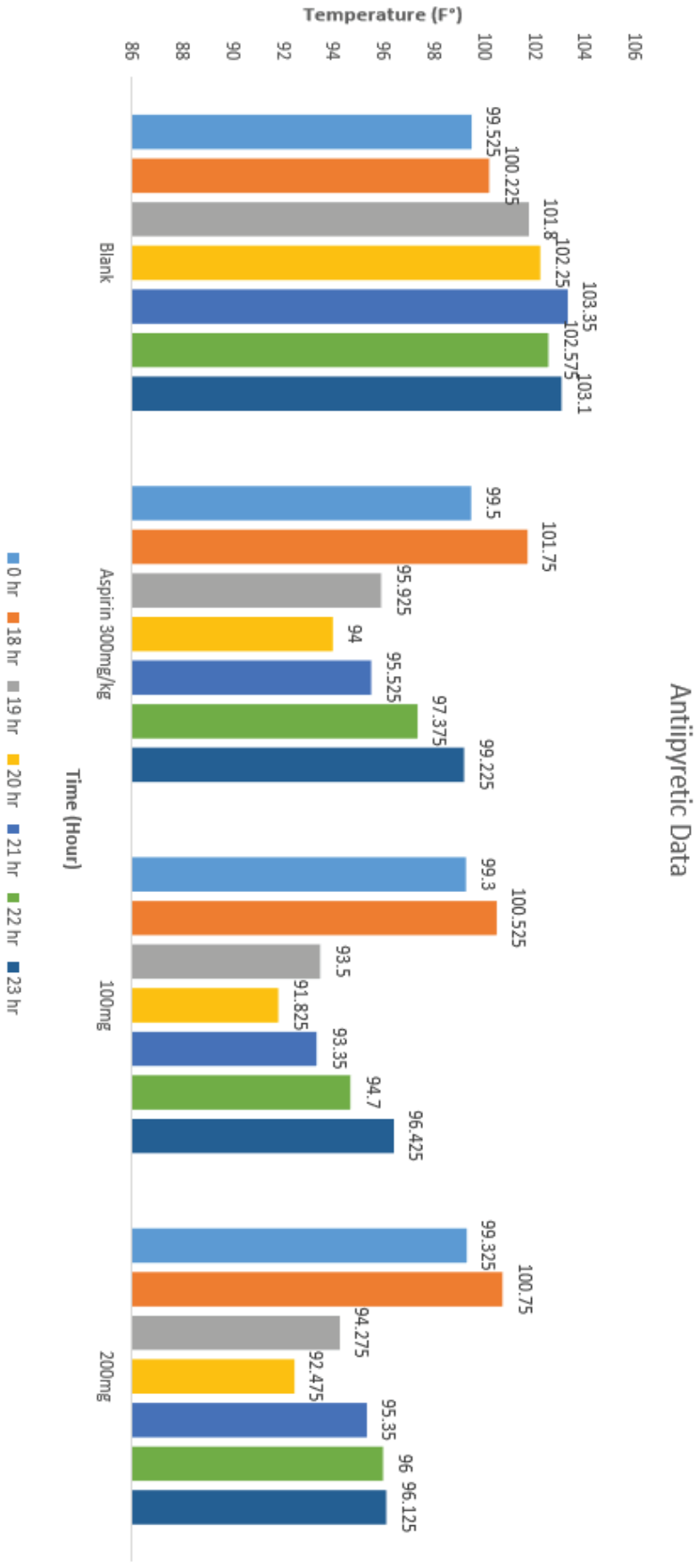

Figure 3: Effect of EECI on Brewer's Yeast induced pyrexia methods

Effect of EECI in CCl_4 induced Hepatotoxicity and Nephrotoxicity

The results of the investigation of the hepato and nephro protective activities of EECI are shown in Table 8. In the CCl_4 control group, substantial hepato and nephro cellular damage was manifested by the elevated levels of ALP, AST, Bilirubin, Creatinine and decreased levels of Albumin when compared with those of controls. But the oral administration of EECI at the doses 100, 200, and 400 mg/kg/day for 5 days significantly ($P < 0.05$) decreased the elevated levels of ALP, AST, Bilirubin, Creatinine and did not increase the reduced levels of Albumin due to toxicity when compared with saline treated mice, and these biochemical parameters were comparable with Silymarin (Table 3)

Table 3: Effect of the EECI on ALP, AST, BL, ALB and CRE in CCl_4-induced liver and kidney damage in mice

Treatment	ALP (mg/dl)	AST (µ/l)	BL (mg/dl)	ALB (mg/dl)	CRE (mg/dl)
Normal control (Saline water)	0.15 ± 0.03	0.3 ± 0.02	0.13 ± 0.01	5703.5 ± 122.01	0.68 ± 0.01
Positive control (CCl_4 induced)	0.23 ± 0.08	0.82 ± 0.07	0.23 ± 0.01	7.23 ± 0.59	1.74 ± 0.04
Standard (Silymarin 200 mg/kg)	0.17 ± 0.07	0.34 ± 0.04**	0.11 ± 0.03**	3251.2 ± 529.5**	1 ± 0.18
EECI 100 mg/kg	0.13 ± 0.003	0.35± 0.05**	0.1 ± 0.03**	5.17 ± 0.43	0.74 ± 0.29**
EECI 200 mg/kg	0.18 ± 0.034	0.1± 0.04**	0.15 ± 0.01	7.05 ± 2.17	0.80± 0.08**
EECI 400 mg/kg	0.21 ± 0.09	0.15± 0.02**	0.20± 0.06	3.02 ± 0.24	0.85 ± 0.26**

All values are expressed as mean ± SEM (n = 4); significance at **$P < 0.05$, as compared to blank. The statistical analysis is done by ONE-WAY-ANNOVA Dunnett test. ALP= Alkaline Phosphatase, AST= Aspartate Aminotransminase, BL= Bilirubin, ALB= Albumin, CRE= Creatinine

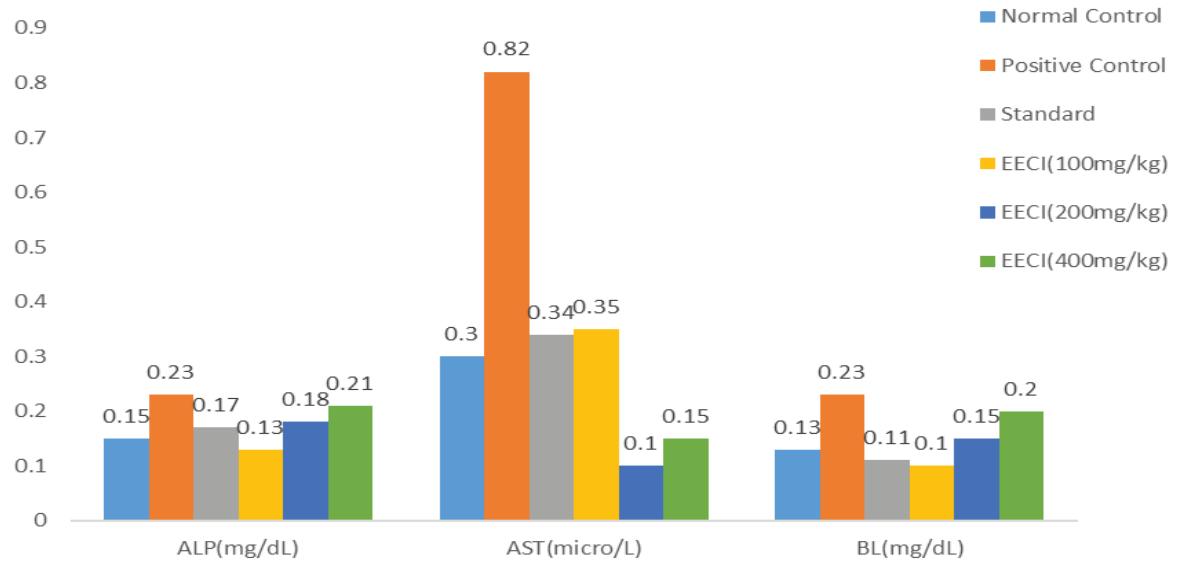

Fig.4 Level of Serum alkaline phosphate , Serum aspartate aminotransminase, Serum bilirubin in different groups of mice

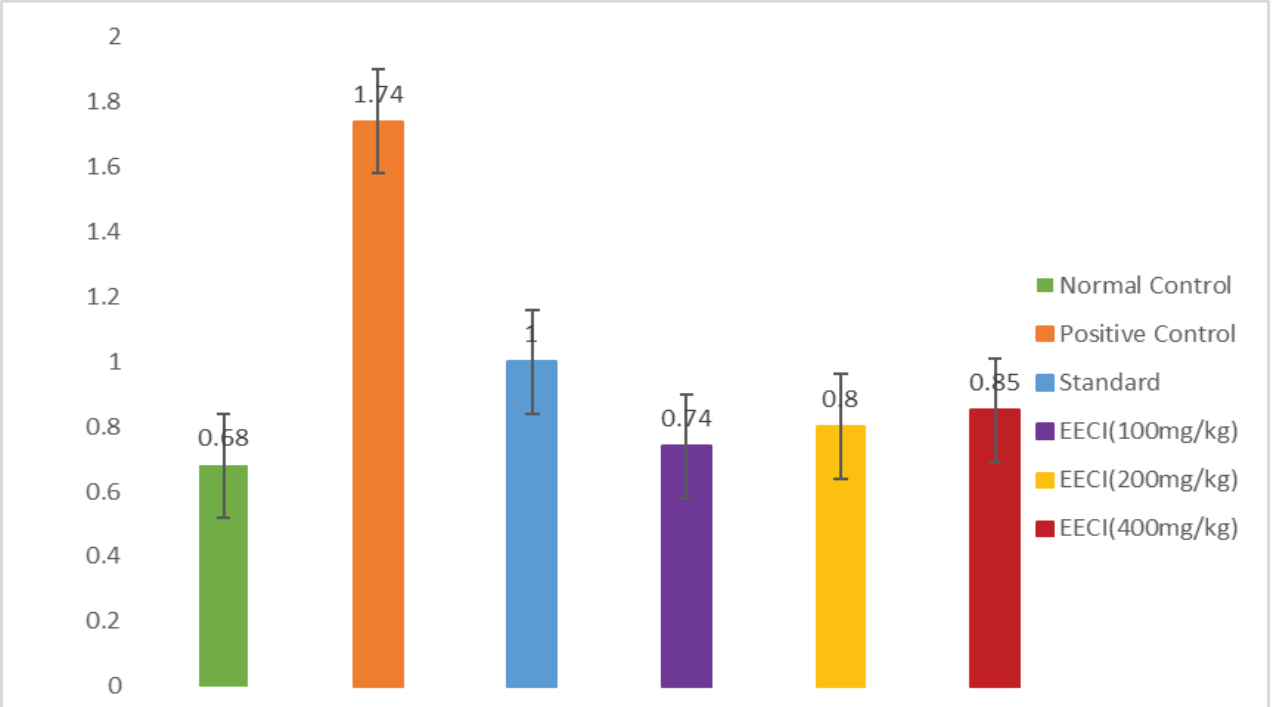

Fig 5. Level of Serum creatinine in different groups of mice

Discussion

EECI have been subjected to Castor oil induced diarrheal method, Brewer Yeast induced Pyrexia test and CCl_4 induced hepato-nephro toxicity test at different doses and significant results were observed. In antidiarrheal potential evaluation, castor oil was used as diarrhea inducer in mice models. Castor oils possess ricinoleic acid that releases prostaglandins. Prostaglandins increase the permeability of mucosal cells causing diarrhea (Mascolo et al., 1993). EECI showed 57.14% inhibition at 200 mg/kg body weight and 42.86% reduction of diarrhea at 100 mg/kg body weight whereas the standard drug loperamide (5mg/kg) showed 78.57% diarrheal inhibitory activity induced by Castor oil. *C. inophyllum* had been reported many times to have tannins, alkaloids, saponins, flavonoids, steroids and/or terpenoids that are responsible for Antidiarrheal potential (Havagiray et al., 2004) by decreasing the release of prostaglandins.

In order to investigate antipyretic potential of EECI, Brewer's yeast-stimulated pyrexia method was employed. The present study showed that the EECI showed statistically significant ($p < 0.001$) reduction of yeast induced elevation of body temperature. During the treatment period, both doses of EECI started the reduction of increased temperature from 19 hours to 22 hours and the maximum inhibition observed at 20hrs with 91.83^0F by 100mg/kg dose that was markedly greater than standard aspirin. The findings of the experiment revealed that EECI extracts inhibit the release of prostaglandins from hypothalamus that regulates body temperature (Verma et al., 2008). As 100 and 200 mg/kg doses of EECI immediately and effectively lower the body temperature within one hour of treatment that will add a substantial therapeutic value of the ehnopharmacological use of *C. inophyllum* and responsive chemicals will be investigated in future.

CCl_4, a well-known toxin, is usually used for the investigation of hepatoprotective as well as nephroprotective properties (Johnston & Kroening, 1998). In this experiment CCl_4 caused liver and kidney damage characterized by elevated levels of serum markers (AST, ALP, BL, CRE) and reduce the albumin level. It is bio transformed by the cytochrome P-450 system and produces the trichloro methyl free radical and causes lipid peroxidation, which in turn produces malondialdehyde (MDA) resulting subsequent damage of the membrane. Lipid peroxidative degradation of the bio membrane is one of the major causes of CCl_4 hepatotoxicity. This CCl_4 induced hepatotoxicity is demonstrated by the elevation of thiobarbituric acid reactive substances (TBARS) and a decrease in the activity of the free radical scavenging enzyme (GSH) and the damage of liver cell has been estimated by increased level of AST, ALP. After damaging or injury they are released into blood circulation and raises the level of enzymes in serum (Saraswat et al., 1993). In case of extract administered group (100, 200, 400 mg/kg body weight), the propensity of these enzymes to return in normal level is the probable indicator of antihepatotoxic effects of the extract. All doses significantly reduce the ALP level and 100 mg/kg showed better activity than the standard silymarin group, 200 mg/kg dose also showed same activity like as standard group and 400 mg/kg dose group showed lower activity than the 100 and 200 mg/kg doses.100 mg/kg 200mg/kg 400 mg/kg doses significantly reduce the AST level and 200 mg/kg and 400 mg/kg doses exhibited better activity than the standard silymarin group, and 100 mg/kg dose group showed almost same activity as standard group. The extract administered groups significantly reduce the increased concentration of bilirubin, another indicator of liver destruction where100 mg/kg dose gives better activity than the 200 and 400 mg/kg doses. In this study, EECI did not show any effect on albumin concentration.

Generation of toxic trichloromethyl radical (CCl3•) and trichloromethyl peroxyl radical (CCl3O2•) from CCl_4 produces cytokines like IL-1β, IL-2, and TNFα from leukocytes and renal tubular cell which are responsible for the generation of inflammation resulting acute kidney damage. Inflammation in kidney is also stimulated by NF-κB that further enhances the release of inflammatory cytokines (Ramesh & Reeves, 2004). Creatinine is commonly used as measure of kidney damage and increased concentration level indicates weakened kidney function. CCl_4 induced group increased the creatinine level but extract administered group significantly reduced the creatinine level.100, 200 and 400 mg/kg doses showed better activity than the standard silymarin group.

The overall findings of the aforementioned experiments revealed the protective action against hepato and nephrotoxicity with possible inhibition of inflammatory cytokines that might be potentiated by flavonoids, lactones, carbohydrates, phenolic compounds, sesquiterpene and glycosides present in the ethanolic extract (Pietta, 2000). It can be concluded that EECI (100mg/kg body weight) exhibited the maximum hepato and nephro protective effect that emphasize further investigations for the identification and characterization of responsive active principles.

Conclusion

Calophyllum inophyllum, a valuable gift of nature, which possess potential bioactive chemicals and pharmacological properties that have been reported many times. The current study has proved the abilities with new remarks in some studies like antidiarrheal, antipyretic, hepatoprotective and nephroprotective activities. The overall results of the research concluded that regarding Bangladeshi species *C. inophyllum,* a potential resource of therapeutically active compounds, can be established as a source of medicine regarding Bangladeshi species.

Acknowledgment
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Ethical Approval
The protocol was approved by the Committee on Ethical Compliance in Research of the Department of Pharmacy, Southeast University, Dhaka, Bangladesh.

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