Deciphering the Pharmacological Potentials of *Callicarpa longifolia* Lam. by *In vitro* and *In vivo* Approaches

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ABSTRACT: *Callicarpa longifolia* Lam., an important medicinal plant of Bangladesh has been subjected for pharmacological investigations to justify the traditional uses of this plant species. The crude methanol extract of aerial parts of *C. longifolia* and its different Kupchan fractions were evaluated for *in vitro* antimicrobial, anti-thrombotic, membrane stabilizing, cytotoxic, and *in vivo* antidiarrheal activities in mice model. All the test samples exhibited significant antimicrobial efficacy, evidenced by the zone of inhibition ranging from 9.0-13.0 mm against the test organisms in disc diffusion technique. The various solvent fractions of *C. longifolia* showed anti-thrombotic activities in a range of $10.47 \pm 0.37\%$ to $29.94 \pm 0.85\%$. Among all, the chloroform fraction showed significant anti-inflammatory property in both heat - and hypotonic solution induced hemolysis. During cytotoxicity test, the plant samples showed a concentration-dependent lethality against live brine shrimp nauplii as compared to standard vincristine sulfate. At the 3rd hour of study, a significant 76.0% reduction in castor oil-induced diarrhea ($p < 0.05$) was observed following an oral administration of the crude methanol extract at a dose of 400 mg/kg. The observed pharmacological potentials of this plant necessitate comprehensive investigation to isolate and characterize its chemical constituents, as well as to elucidate their mechanistic pathways to delineate therapeutic efficacy.

Key words: *Callicarpa longifolia*, Antimicrobial, anti-thrombotic, cytotoxic, anti-diarrheal, membrane stabilizing.

INTRODUCTION

Medicinal plants have been used in the treatments of various ailments since the dawn of human civilization. Approximately 35% of pharmaceuticals are derived from natural products, comprising 25% from plants, 13% from microorganisms, and about 3% from animals.[1-4] According to WHO, nearly 80% of the world's population relies on traditional medicine, predominantly derived from plant sources, highlighting the necessity and importance of these natural remedies in healthcare systems.[5] However, approximately 6% of botanical species have been studied pharmacologically so far.[6] As a substantial portion of botanical species remains to be investigated, there exists a vast potential for discovering new therapeutic agents that could enhance health outcomes globally.

Callicarpa longifolia Lam. (Family: Lamiaceae) is an evergreen shrub found in China, India, Bangladesh, Bhutan, Pakistan, and Southeast Asia, as well as New Guinea and Australia.[7] The plant is also known as Beautyberry. In Bangladesh, the plant grows in the hill tracts region of Chittagong, Bandarban, and Rangamati districts and also native to Sylhet. The indigenous people in India uses Sangkahero (*C. longifolia*) as medicine for colds and inflammation. The plant has been used traditionally in the treatment of a variety of diseases such as diabetes, pain, diarrhea, acne, swelling, wound, cough and cold by people of several ethnic communities around the world.[8-10] Several studies[8-10] have reported the diverse biological activities and significant medicinal properties of *C. longifolia*

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extract. In previous studies, the leaves of this plant exhibited antibacterial activity against *Salmonella typhi* and *Staphylococcus epidermis*.[9] The ethanol extract of *C. longifolia* promoted wound healing in animal models[10], while also showing anti-atherosclerotic effects by improving lipid profiles in hyperlipidemic rats.[11] The ethanolic leaf extract of the plant has been reported to exhibit analgesic and antidiarrheal activities in murine models.[12,13]

Phytochemical screening tests revealed the presence of important compounds such as glycosides, flavonoids, alkaloids, tannins, saponins, and steroids in the ethanol extract of *C. longifolia*, contributing to its therapeutic potential.[14] Furthermore, phytochemical investigations led to the isolation of various phytocompounds from the leaf extract of this plant including terpenoids such as calliterpenone, calliterpenone monoacetate, and ursolic acid, as well as flavonoids such as apigenin and luteolin, along with their respective glucuronides.[15]

A review of the existing literature indicates a lack of comprehensive studies on evaluating the pharmacological potential of *C. longifolia* grown in Bangladesh. Therefore, in this study we conducted an investigation to assess its antimicrobial, anti-thrombotic, membrane stabilizing, cytotoxic, and anti-diarrheal properties to validate its traditional uses.

MATERIALS AND METHODS

Collection and identification of plant materials. Aerial parts (leaves and stems) of *C. longifolia* were collected from Lati Tila, Juri Upazila, Sylhet, Bangladesh in July, 2023. An official taxonomist from Bangladesh National Herbarium identified (Accession number: DACB 90640) and verified the plant.

Extraction of plant samples. About 2.0 kg of powdered plant material was macerated in distilled methanol in clean, amber-colored bottles for two weeks, with regular stirring and shaking. Following maceration, the mixture was filtered using cotton and an EYELA rotary evaporator was used to concentrate the filtrate. The resulting semi-solid residue of plant extract was collected and dried at room temperature, producing a crude extract (about 68.0 gm) with a yield of 3.4%. The crude extract was then partitioned into *n*-hexane, chloroform, ethyl acetate and aqueous fractions following the modified Kupchan partitioning method.[16]

Table 1. Test samples of *C. longifolia* used in pharmacological investigations.

Test Sample	Sample code	Amount (gm)
Crude methanol extract of aerial parts of *C. longifolia*	MECL	68.0
n-Hexane soluble fraction of methanol extract of *C. longifolia*	HFCL	12.0
Chloroform soluble fraction of methanol extract of *C. longifolia*	CFCL	31.0
Ethyl acetate soluble fraction of methanol extract of *C. longifolia*	EFCL	8.0
Aqueous soluble fraction of methanol extract of *C. longifolia*	AFCL	9.4

Test Animals. Swiss albino mice (4-5 weeks old) of both sexes, weighing 20-25 g, were procured from the animal house at Jahangirnagar University. The *in vivo* anti-diarrheal activity of the plant's crude methanol extract was conducted at the Institute of Nutrition and Food Science (INFS) animal house, University of Dhaka. Five groups of randomly selected mice (n=6) were used for the investigation. Mice were kept in cages made of polypropylene in a standard laboratory environment, including a controlled dark-light cycle, relative humidity of 56-60% and a temperature of 25 ± 2°C. They were fed pelletized feed from ICDDR,B and provided with *ad libitum* access to water. The Ethical Review Committee of the University of Dhaka's Faculty of Pharmacy granted ethical clearance prior to the commencement of any animal research, with reference number Fa. Ph. E/026/23. Prior to the main study, the acute toxicity of the crude extract was assessed in mice in accordance with OECD chemical

testing guidelines.[17] A single 800 mg/kg oral dose was administered to a group of six mice, with toxicological signs monitored up to 24 hours. No mortality or toxicity was observed at the administered dose. Based on these findings, safe doses of 100, 200 and 400 mg/kg/day were selected for further study.

Antimicrobial activity. The most common disc diffusion method[18] was adopted to evaluate the antimicrobial activity of the plant samples. Here, antibacterial drug kanamycin and fluconazole were used as the controlling agents.

Anti-thrombotic activity. The anti-thrombotic activity was determined by method reported in Prasad *et al.* (2007).[19] About 4.0 ml of whole blood was obtained from healthy human volunteer and was distributed into seven previously weighed marked Eppendorf tube (0.5 ml/tube). All of the tubes then underwent incubation at 37^0C for 45 min for the formation of clots. After the clot had formed, the serum was entirely withdrawn without breaking the clot layer, and clot weight was determined by weighing each tube containing the clot (Clot weight = Weight of tube with clot - Weight of only tube). 100 µl of aqueous solution of crude extract and different Kupchan fractions (10 mg/ml) was separately taken to each pre-weighed tube containing clot. Similarly, to serve as positive and negative controls, Streptokinase (30,000 I.U) and distilled water, both 100 µl were added to the corresponding tubes, respectively. Following a 90-min incubation period at 37°C, the clot lysis of each tube was monitored. The discharged liquid was removed from all tubes and they were reweighed to determine the weight difference after clot lysis. The anti-thrombotic activity of the extracts was expressed as percent clot lysis using the following equation:

$$\% \text{ Clot lysis} = \frac{\text{weight of the lysed clot}}{\text{weight of clot before lysis}} \times 100$$

The study was replicated three times using blood specimens of three different individuals.

Membrane stabilizing assay. The test materials were subjected to evaluate the membrane-stabilizing activity against heat- and hypotonic solution-induced hemolysis of human blood.[20]

Cytotoxic activity. The cytotoxic activities of the crude methanol extract and Kupchan fractions (n-hexane, chloroform, ethyl acetate and aqueous) of *C. longifolia* at the conc. ranging from 400.0 to 0.781 µg/ml were evaluated using the brine shrimp lethality bioassay.[21]

Antidiarrheal activity. Castor oil induced anti-diarrheal efficacy of crude methanol extract of *C. longifolia* was conducted by methodology reported in Arise *et al*., (2016).[22] With some modifications. Group I and group II acted as the control groups and received 1% tween 80 solution (10 ml/Kg, p.o.) and loperamide hydrochloride (3 mg/kg, p.o.), respectively. Group III, IV and V were the test groups and orally received 100, 200, 400 mg/kg doses of the plant extract. 30 min after the administration of the doses, 1 ml of highly refined analytical-grade castor oil was orally given to each test animal to induce diarrhea. They were put in separate cages with adsorbent paper beneath. An hour following the castor oil administration, number of feces excreted by each mouse was counted and recorded at each hour during the study. Then, the percent inhibition of diarrhea (%I) were calculated using the equation:

$$\% \text{ Reduction of diarrhea} = \frac{D_{Control} - D_{Test}}{D_{Control}} \times 100\%$$

Where, D represent the mean number of feces in the respective groups.

Statistical analysis. Observed data were analyzed with SPSS 27.0. The findings from the experiment were reported as mean ± SEM. The statistical significance was determined using ANOVA (analysis of variance) and the significance was tested at different P value (*p <0.05, **p <0.01, ***p <0.001) against control.

RESULTS AND DISCUSSION

C. longifolia has been utilized for years to treat and prevent a variety of ailments in traditional medicine. In this study, *C. longifolia* was subjected for antimicrobial, anti-thrombotic, membrane stabilizing, cytotoxic and anti-diarrheal efficacy. The

findings were summarized in Tables 2-4 and Figures 1-4.

In the disc diffusion method for antimicrobial screening, the methanol extract of aerial part of *C. longifolia* and its various solvent fractions were subjected to investigate the antimicrobial activity against a series of microorganisms (Table 2). The antimicrobial activity was expressed as the zone of inhibition (mm) ranging from 9.0-13.0 mm against the test microbial species compared to the standard kanamycin. The results revealed that all plant extracts, at the dose of 4.0 mg/ml, exhibited notable antimicrobial activity, with varying degrees of efficacy. Among all, the crude extract showed activity against all the test strains with maximum growth inhibitory effect against *Escherichia coli* (13.0 mm) and *Aspergillus niger* (13.0 mm). For the *n*-hexane fraction at 400 µg/disc, significant antibacterial activity was observed with zones of inhibitions of 10.0, 10.0 and 12.0 mm (Table 2) against *S. lutea*, *V. mimicus* and *E. coli* respectively while other test strains were observed as resistant to the n-hexane fraction. Like the crude methanol extract, the chloroform and the ethyl acetate fraction at the dose of 4.0 mg/ml represented a significant antimicrobial activity against all the test strains. For the chloroform fraction, maximum activity was observed against *E. coli* (12.0 mm) and *V. mimicus* (12.0 mm). The aqueous fraction was found to be active against *S. lutea* (12.0 mm), *E. coli* (11.0 mm), S. *paratyphii* (9.0 mm), *Sh. dysenteriae* (9.0 mm), *V. mimicus* (9.0 mm) and fungal strains *A. niger* (10.0 mm) and *C. albicans* (12.0 mm). The observed antimicrobial activity correlates with the previous study (Susilawati et al., 2018) which explained the antibacterial and wound healing activity of the ethanol extract of *C. longifolia* leaf. Numerous studies have proposed that the antimicrobial constituents of plant extracts, such as phenolic compounds, alkaloids, and terpenoids, exert their effects through multiple mechanisms. These include interactions with microbial cell membrane proteins and enzymes, leading to membrane disruption; damage to the cell wall; penetration into the intracellular environment; and coagulation of cellular contents, which ultimately contribute to microbial cell death.[23,24]

Table 2. Antimicrobial activities of different partitionates of *C. longifolia*.

Test microorganisms	Zone of inhibition (mm)					
	MECL	HFCL	CFCL	EFCL	AFCL	Standard
Bacteria				**Kanamycin**		
Bacillus subtilis	11.0	--	9.0	13.0	--	27.0
Sarcina lutea	12.0	10.0	10.0	11.0	12.0	28.0
Staphylococcus aureus	9.0	--	9.0	10.0	--	27.0
Escherichia coli	13.0	12.0	12.0	12.0	11.0	28.0
Salmonella paratyphi	10.0	--	11.0	9.0	9.0	27.0
Shigella dysenteriae	12.0	--	10.0	11.0	9.0	27.0
Pseudomonas aeruginosa	9.0	--	10.0	10.0	-	27.0
Vibrio mimicus	12.0	10.0	12.0	9.0	9.0	28.0
Fungi						**Fluconazole**
Aspergillus niger	13.0	--	9.0	9.0	10.0	32.0
Candida albicans	11.0	--	11.0	10.0	12.0	33.0

Here, MECL = Methanol crude extract of *C. longifolia*, HFCL = n-Hexane soluble fraction, CFCL =Chloroform soluble fraction, EFCL = Ethyl acetate soluble fraction, AFCL = Aqueous soluble fraction of *C. longifolia*.

Thrombosis is a life-threatening condition characterized by the accumulation of blood clots, or thrombi, within the circulatory system. It becomes particularly critical when associated with arterial

complications, as these can lead to acute coronary syndromes with potentially fatal outcomes. Commercially available thrombolytic agents, such as alteplase, urokinase, anistreplase, and streptokinase, are commonly used worldwide to treat thromboembolic disorders.[24,25] Considering the limitations of most synthetic antithrombotic medicines, there is a growing demand for the development of alternative therapeutic options. In this study, various fractions of *C. longifolia* were tested for their thrombolytic potential by assessing their efficacy in breaking down blood clots.

According to this study, the thrombolytic activities of the different *C. longifolia* fractions ranged from 10.47 ± 0.37% to 29.94 ± 0.85% (Figure 1). The highest thrombolytic activity (29.94 ± 0.85%) was exerted by MECL, followed by CFCL (14.93 ± 0.24%), AFCL (14.12 ± 0.14%) and EFCL (11.30 ± 0.112%). The positive control, streptokinase, induced the highest clot lysis of 64.589 ± 0.226% ($p<0.05$), compared to the negative control that showed 3.15 ± 0.23% clot lysis.

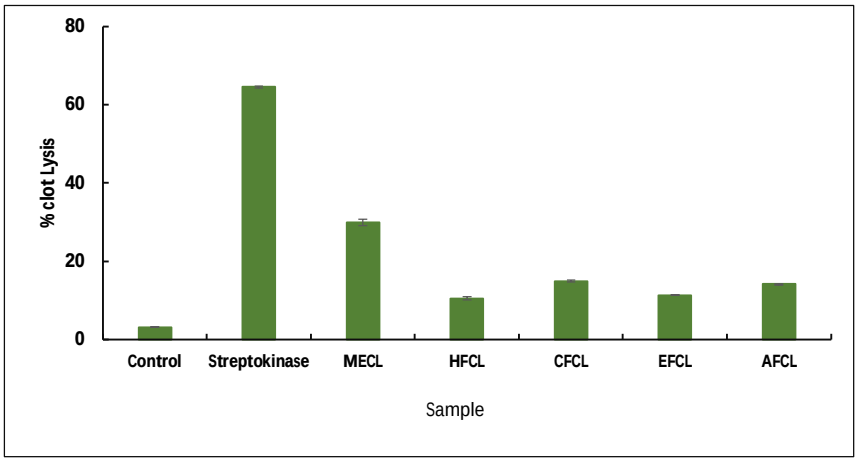

Figure 1. Anti-thrombotic activity of different solvent fractions of *C. longifolia* extract. Here, MECL = Methanol crude extract of *C. longifolia*, HFCL = n-Hexane soluble fraction, CFCL =Chloroform soluble fraction, EFCL = Ethyl acetate soluble fraction, AFCL = Aqueous soluble fraction of *C. longifolia*.

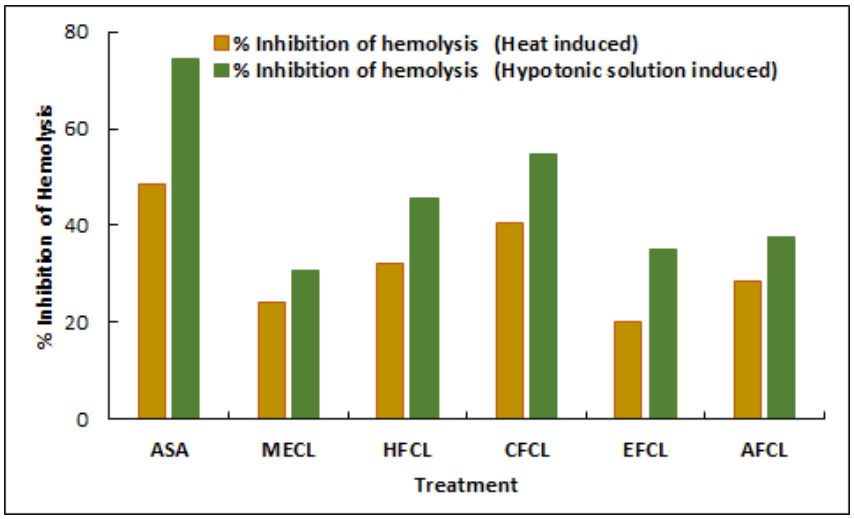

Figure 2. Membrane stabilizing assay of different solvent fractions of *C. longifolia* extract. Here, ASA= Acetyl salicylic acid; MECL = Methanol crude extract of *C. longifolia*, HFCL = n-Hexane soluble fraction, CFCL =Chloroform soluble fraction, EFCL = Ethyl acetate soluble fraction, AFCL = Aqueous soluble fraction of *C. longifolia*.

The comparison between positive and negative controls in the thrombolytic assay clearly showed that water addition did not induce clot lysis. Notably, all extract samples exhibited statistically significant clot lysis ($p < 0.05$) compared to the negative control (distilled water). Literature suggests that terpenoids and flavonoids are likely contributing to the predominant biological activities observed in plants of this genus.[26] It has been reported that secondary plant metabolites including flavonoids, tannins, phenolics may function as anti-thrombotic agents.[20]

The RBC membrane stabilization assay is a method used to assess the in vitro anti-inflammatory activity of a plant extract by measuring its ability to protect red blood cell membranes from lysis under stressful conditions such as heat, hypotonic solution etc. Erythrocyte membranes, or human red blood cell (HRBC) membranes, are commonly used in membrane stabilization tests due to their structural similarity to lysosomal membranes. Compared to the standard acetyl salicylic acid (ASA) at 0.10 mg/ml, the soluble fractions of *C. longifolia* methanol extract at 2.0 mg/ml significantly inhibited heat- and hypotonicity-induced lysis of human erythrocyte membranes (Figure 2).

The chloroform fraction (CFCL) of the extract at the dose of 2.0 mg/ml inhibited heat-induced hemolysis of human red blood cells by 40.47%, compared to 48.63% inhibition by standard ASA. All plant fractions exhibited comparatively greater inhibition under hypotonic conditions than in heat-induced hemolysis. In this test, the CFCL also showed maximum 54.56% protection of RBC membrane against hypotonic solution induced hemolysis.

Compounds that stabilize RBC membranes are regarded anti-inflammatory agents, as they inhibit the release of phospholipase enzymes crucial to the inflammatory response.[20,27] By preventing membrane lysis and subsequent enzyme release, these compounds help limit tissue damage and the progression of inflammation.[28] In this study, *C. longifolia* plant samples at 2.0 mg/ml effectively protected human RBC membranes from lysis induced by both heat and hypotonic solution. The notable membrane stabilizing effect observed in *C. longifolia* can be correlated to bioactive secondary metabolites, including flavonoids, tannins, and phenolic compounds in the extract, which are known to inhibit membrane lysis by scavenging free radicals and modulating enzyme activity.[9,14]

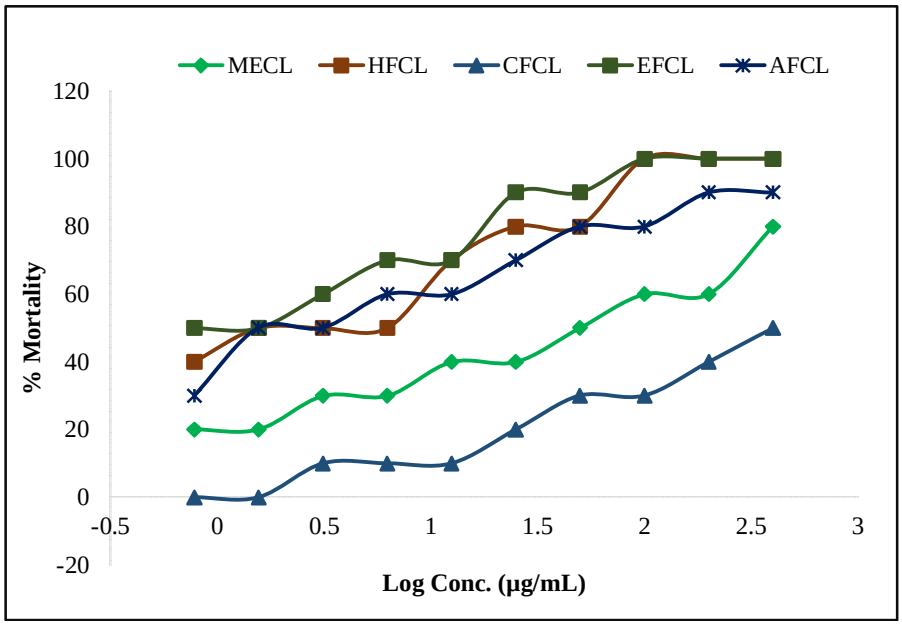

Figure 3. % Mortality of shrimp nauplii after treating with crude methanol extract of *C. longifolia* aerial parts and its Kupchan fractions. Here, MECL = Methanol crude extract of *C. longifolia*, HFCL = n-Hexane soluble fraction, CFCL =Chloroform soluble fraction, EFCL = Ethyl acetate soluble fraction, AFCL = Aqueous soluble fraction of *C. longifolia*.

Table 3. Summary of plant extracts and their LC_{50} against shrimp nauplii.

Sample	LC_{50} (µg/ml)	Regression equation	R^2
MECL	38.45	y=20.737x + 17.133	R^2 = 0.9428
HFCL	2.32	y=24.965x + 40.858	R^2 = 0.9394
CFCL	800.15	y=18.119x-2.6026	R^2 = 0.944
EFCL	0.91	y=21.743x + 50.877	R^2 = 0.940
AFCL	3.04	y=20.938x + 39.881	R^2 = 0.9528
VS	0.45	y=30.805x + 60.65	R^2 = 0.9731

Here, VS = Vincristine sulfate; MECL = Methanol crude extract of *C. longifolia*, HFCL = n-Hexane soluble fraction, CFCL =Chloroform soluble fraction, EFCL = Ethyl acetate soluble fraction, AFCL = Aqueous soluble fraction of *C. longifolia*.

During cytotoxicity activity test, all the plant samples showed a dose-dependent cytotoxicity against brine shrimp nauplii (Figure 3). Cytotoxic activity of different *C. longifolia* fractions was reported as LC_{50} values, calculated from regression equations derived from plots of percent mortality against the logarithm of sample concentrations (Figure 3 and Table 3). As shown in Table 3, the ethyl acetate fraction exhibited the highest cytotoxicity (LC_{50} = 0.91 µg/ml), closely followed by the n-hexane fraction (LC_{50} = 2.32 µg/ml) and the aqueous fraction (LC_{50} = 3.04 µg/ml). The crude methanol extract demonstrated moderate cytotoxicity with an LC_{50} value of 38.45 µg/ml. Vincristine sulfate used as a positive control, demonstrated the highest efficacy with an LC_{50} value of 0.451 µg/ml, serving as a standard for comparison. After 24 h of observation, no mortality was observed in the negative control group, confirming the reliability and validity of the assay. The findings imply that the *n*-hexane and ethyl acetate fractions possess notable cytotoxic potential against brine shrimp. A similar effect was observed in which the crude methanol extract of *C. macrophylla* bark showed potent activity against brine shrimp nauplii[29], signifying the requirement of further investigation to identify bioactive compounds.

Table 4. Diarrheal episodes after treating the methanol extract of *C. longifolia* aerial parts in mice.

Treatment (n=6)	Dose (mg/kg)	Number of feces (Mean ± SEM)			
		1 hr	2 hr	3 hr	4hr
Control	-	5.75 ± 0.4	6.5 ± 0.53	6.25 ± 0.52	4.25 ± 0.25
Standard	3	2.25 ± 0.21 (60.87***)	1.5 ± 0.24 (76.92***)	0.75 ± 0.21 (88.00***)	0.75 ± 0.25 (82.36***)
MECL	100	4.25 ± 0.21 (26.09)	3.75 ± 0.21 (42.31**)	2.5 ± 0.24 (60.00***)	2.25 ± 0.48 (47.06*)
MECL	200	3.75 ± 0.21 (34.79)	3 ± 0.34 (53.85***)	2 ± 0.34 (68.00***)	1.75 ± 0.63 (58.83**)
MECL	400	3.25 ± 0.4 (43.48**)	2.75 ± 0.62 (57.69***)	1.5 ± 0.24 (76.00***)	1.25 ± 0.25 (70.59***)

*p<0.05, **p<0.01, ***p<0.001 vs control; The percentage reduction in fecal count compared to the control group is provided in parentheses. Standard= Loperamide hydrochloride. MECL = Methanol crude extract of *C. longifolia*.

In the treatment of diarrheal episode, traditional healers commonly use various plants, including *C. longifolia*, as practiced by ethnic groups in Central Kalimantan, Indonesia.[9] However, the efficacy of such remedies remains largely invalidated; thus, the methanol extract of *C. longifolia* aerial parts was evaluated using a castor oil-induced diarrhea model in mice. All treated groups including the standard loperamide hydrochloride (3 mg/kg) and the plant extract (100, 200 and 400 mg/kg) of *C. longifolia* exhibited a statistically significant reduction in number of feces excreted compared to control at 2h, 3h and 4h period of study (Table 4).

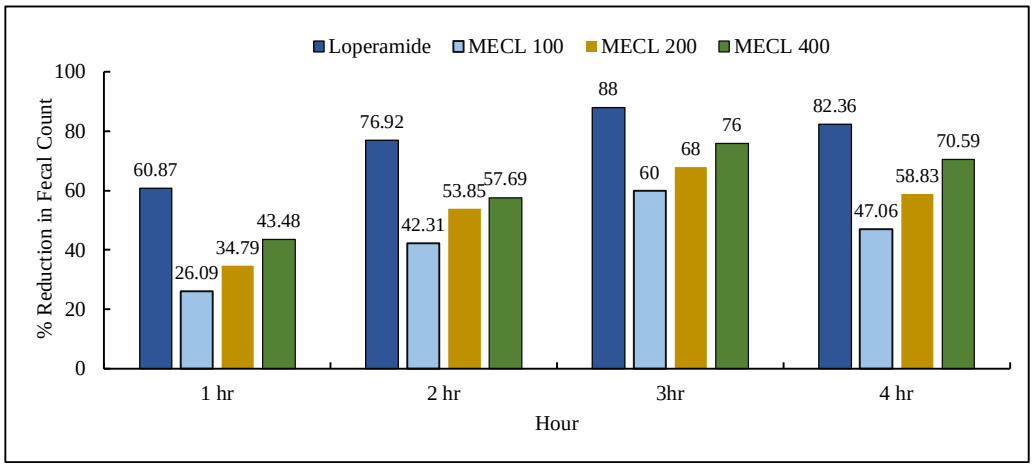

Figure 4. Anti-diarrheal activity of methanol extract of *C. longifolia* aerial parts in mice. MECL = Methanol crude extract of *C. longifolia*.

As shown in Figure 4, all test samples exhibited statistically significant antidiarrheal activity from the 2nd hour onward, with a dose-dependent effect observed across all time points in mice. Among the extract-treated groups, 400 mg/kg dose exhibited the highest antidiarrheal effect, with significant fecal reduction of 43.48% at 1st hour ($p<0.01$), 57.69% at 2nd hour ($p<0.001$), 76.00% at 3rd hour ($p<0.001$) and 70.59% at 4th hour ($p<0.001$). The standard loperamide hydrochloride consistently demonstrated statistically significant ($p<0.001$) decrease in fecal count across the observation period, with percent reductions of 60.87%, 76.92%, 88.00% and 82.36% at 1st, 2nd, 3rd and 4th hours, respectively (Table 4). Medicinally active plant constituents, flavonoids, alkaloids, tannins, terpenoids, saponins, and steroids from serve as vital compounds in the treatment of diarrhea.[30]

CONCLUSION

This study reveals that *C. longifolia* possesses a wide range of pharmacological activities, including antimicrobial, anti-thrombotic, membrane-stabilizing, cytotoxic, and antidiarrheal effects. The crude methanol extract and its various solvent fractions demonstrated significant bioactivity, with the crude extract showing notable antimicrobial efficacy against pathogens such as *E. coli* and *A. niger*. Additionally, the extracts exhibited substantial thrombolytic activity, membrane protection against hemolysis, and concentration-dependent cytotoxicity, with certain fractions showing potent effects. The *in vivo* antidiarrheal activity, observed through a significant reduction in castor oil-induced diarrhea, further confirm the therapeutic potential of this plant. These findings not only validate the traditional uses of *C. longifolia* but also provide a foundation for further investigation into its bioactive compounds. Future studies are needed to isolate, characterize, and understand the mechanisms underlying these effects, which may lead to the development of novel therapeutic agents for treating infections, thrombosis, and gastrointestinal disorders.

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