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IN VIVO AND IN VITRO EVALUATION OF PHARMACOLOGICAL ACTIVITIES OF *Hedychium coccineum* RHIZOMES EXTRACT

Sadia Islam Nishi[1†], Niloy Barua[1,2†], Mohammed Aktar Sayeed[1], Abu Montakim Tareq[1], Sahnaj Begum Mina[1], Talha Bin Emran[3*], Kuldeep Dhama[4*]

[1]Department of Pharmacy, International Islamic University Chittagong, Chittagong-4318, Bangladesh
[2]Drug Discovery, GUSTO A Research Group, Chittagong 4203, Bangladesh
[3]Department of Pharmacy, BGC Trust University Bangladesh, Chittagong-4381, Bangladesh
[4]Division of Pathology, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly-243 122, Uttar Pradesh, India
[†] These authors contributed equally to this work.

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ABSTRACT

The study reports the *in vivo* antidiarrheal and *in vitro* anthelmintic, cytotoxic, and thrombolytic activity of methanol extract of *Hedychium coccineum* rhizomes (MEHC). The antidiarrheal activity was determined using Castor oil-induced diarrhea and Gastrointestinal motility test in mice at the doses of 200 and 400 mg/kg body weight, whereas an aquarium worm, *Tubifex tubifex*, was used to determine the anthelmintic activity. The cytotoxic and thrombolytic activity of MEHC was performed by Brine shrimp lethality bioassay and clot lysis method respectively. In antidiarrheal, castor oil-induced diarrhea and gastrointestinal motility exhibited a significant reduction in diarrhea and defecation and an extremely significant inhibition in intestinal motility and peristalsis index by 200 and 400 mg/kg of MEHC. The MEHC (5, 10, and 20 mg/mL) showed a significant dose-dependent manner paralysis time and times to death in multiple comparisons to the different levamisole concentrations (0.5, 0.8, and 1 mg/mL) at *in vitro* anthelmintic activity. The brine shrimp lethality bioassay exhibited a weak LC_{50} (681.95 µg/mL; R^2 = 0.951) while in thrombolytic a significant percentage of clot lysis (32.70%, $P < 0.05$) demonstrated. The findings demonstrate that *H. coccineum* rhizomes could be potential sources for biological activity.

* Corresponding author
E-mail: kdhama@rediffmail.com (Kuldeep Dhama);
talhabmb@bgctub.ac.bd (Talha Bin Emran)

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1 Introduction

Helminthiases are parasitic worm infections that cause morbidity and mortality to the host (Ferrer-Font et al., 2020), whereas the human and animal infected in their lifespan. In developed and under-developing nations, infection of helminths is a severe problem due to the constant contamination of eggs and larvae with the environment (Nalule et al., 2013). Enteric helminths are among the most common parasites of humankind, yet just very negligible information exists about their impacts on the gastrointestinal tract in hosts and particularly which worms responsible for diarrhea. The literature review recommends that five helminthic parasites related to the human diarrheal disease are *Trichinella spiralis* (infection in early periods), *Trichuris trichiura*, *Strongyloides stercoralis*, *Capillaria philippinensis*, and *Schistosoma mansoni*. Every one of these parasites has an invasive stage during which mature worms, their eggs, or larvae in contact with the host's intestinal mucosa and cause local inflammation, which led to change in the gut by structurally and functionally. But, intraluminal worms don't usually intervene with their host's intestinal formation and function to an extent enough to provoke diarrhea (Caner et al., 2020; Săsăran et al., 2020). In recent years, blood circulation problems have been reported due to the formation of a clot in blood by the infection of a parasite. The blood vessel is blocked due to the formation of a thrombus which causes improper blood flow (Diamond, 2016; Emran et al., 2015a).

Medicinal plants have been useful in folk medicine since 4000–5000 B.C. (Hosseinzadeh et al., 2015). Uses of the medicinal plant are well accepted in traditional log-term use and scientific research due to their safety and efficacy. As a result of their ease of access, particularly contrasted with present-day drugs, plants have turned into a significant part of human healthcare (Thaipitakwong et al., 2018). *Hedychium coccineum* or scarlet ginger lily belongs to the Zingiberaceae family which is used as an ornamental plant in native Asia. It is locally known as Aichhia and Mansila (Tushar et al., 2010; Shifah et al., 2020). *H. coccineum* roots are used in treating headaches and flowers used in swollen body parts (Quattrocchi, 2012; Shifah et al., 2020). The Indian tribal people believe that wearing of the flower behind the ear could be effective against evil eye and disease (Shifah et al., 2020). It is also reported to be used as antipyretics and anti-inflammatory (Tushar et al., 2010; Shifah et al., 2020). According to report, some essential oil compounds of *H. coccineum* are found in Mauritius (East Africa) which is 44.4% of (E)-nerolidol, 24.2% of trans-sesquisabinene hydrate, 27% of linalool, and 14% of α-pinene (Gurib-Fakim et al., 2002; Shifah et al., 2020). The present study has evaluated the *in vivo* antidiarrheal and *in vitro* anthelminthic activity by methanol extract of *H. coccineum* rhizomes using Swiss albino mice and helminths parasites.

2 Materials and Methods

2.1 Chemicals and reagents

Loperamide (Square Pharmaceuticals Ltd. Dhaka, Bangladesh), levamisole (ACI Limited (Dhaka, Bangladesh), castor oil (WELL's Health Care, Madrid, Spain), methanol (Merck, Darmstadt, Germany) were purchased from the cited sources. Streptokinase (Beacon Pharmaceutical Ltd, Mymensingh, Bangladesh), sea-salt non-ionized NaCl, vincristine sulphate (Sigma-Aldrich Co.) were used in this study. All drugs and chemicals were of analytical grade.

2.2 Experimental animals

Swiss albino mice weighing about 25 – 35gm of both sexes are used for the experiment. The mice were obtained from the university animal house. All the animals were adjusted to the new environment for one week under room temperature (25±2 ºC) and access to food and water. The experimental animal was handled according to international guidelines for the use and maintenance of experimental animals. At the end of the observation period, all the mice were slaughtered by diethyl ether anesthesia (Du Sert et al., 2020).

2.3 Collection and preparation of plant extract

Rhizome of the plant *H. coccineum* was collected in March 2019 from Fatikchari Hill tract area, Chattagram, Bangladesh. It was identified and authenticated by a taxonomist. The rhizomes were cut into small pieces and air-dried for 7 days. After grinding, about 800 gm coarse powders were soaked in 2400mL methanol in a glass bottle for 7 days at room temperature with vigorous shaking. It was filtered by Whitman filter paper #1 number. Here, 600mL solvent was obtained after filtering. The obtained filtrate evaporated in a water bath at 40℃ to yield viscous mass. The prepared dried extract was kept in a vial at 4℃ for further pharmacological screening.

2.4 Antidiarrheal activity (*In vivo*)

2.4.1 Castor oil-induced diarrhea

Experimental animals (Swiss albino) are separated into four groups and each group contains three mice. All mice fasted for 12 hours for this experiment. Experimental mice were received 1% tween 80 as a negative control, loperamide (5 mg/kg body weight, p.o.) as a positive control, and test groups of methanol rhizome extract of *H. coccineum* (200 and 400 mg/kg body weight, p.o.), respectively. One hour later, 0.5mL castor oil was induced to all mice by oral gavage. Then they were placed in a cage with blotting paper. It should be changed after one-hour intervals during an observation period of 4 hours. The total number of feces (wet & dry diarrheal feces) were counted and noted (Tareq et al., 2020).

Inhibition (%) = [(A- B)/A] × 100

Where A = mean number of diarrheal feces of the control group; B = mean number of diarrheal feces of the treated group.

2.4.2 Gastrointestinal motility test by charcoal meal

All mice have fasted for 18 hours. Experimental animals (Swiss albino) are separated into four groups and each group contains three mice. One hour after administration of treatment group as described in Castor oil-induced diarrhea method, 1 mL charcoal meal (10% charcoal in 5% gum acacia) administrated to mice. After one hour, the abdomen was opened and removed the small intestine. Measured the total length of the small intestine and the distance traveled by charcoal from the pylorus to the cecum was measured (Barua et al., 2020).

Inhibition (%) = [(A- B)/A] × 100

Where A = Distance travel by the charcoal control group (cm); B = Distance travel by the charcoal test groups group (cm).

Peristalsis index = (Distance travel by the charcoal meal / Total length of the small intestine) × 100

2.5 Anthelmintic activity (*In-vitro*)

In this anthelmintic experiment, an aquarium worm of *Tubifex tubifex* was used and testing was performed in a triplicate manner. Petri dishes were taken for the test sample and mark them as different concentration-wise. The test sample of different concentrations (5, 10, and 20 mg/mL) were placed into Petri dishes. As a positive control, levamisole (0.5, 0.8, and 1 mg/mL) and as a negative control, distilled water was used. The standard drug levamisole was prepared freshly in double distilled water (Adnan et al., 2019). Here,7-10 worms were put in each petri dish. Two different stages 'Time of paralysis' and 'Time of death' was observed. Time for paralysis was noted when no movement could be observed except when the worms were shaken with perforce. Time for death was noted when the worms lost their motility followed by fading away from their body colors.

2.6 Cytotoxic activity

Brine shrimp lethality bioassay was performed by the standard method with slight modification (Meyer et al., 1982; Bristy et al., 2020). Brine shrimp eggs (*Artemia salina*) were collected from an aquarium shop (Dhaka, Bangladesh). These were hatched in artificial seawater (3.8% NaCl) for 48 h. After 48 hours of incubation at room temperature (25-29°C), ten living nauplii for each test tube were collected by a capillary tube. The extract (10mg) was made by dissolving them in DMSO (100µml) and 10mL seawater to get serially diluted concentrations of 62.5, 125, 250, 500, and 1000 µg/mL. Vincristine sulfate was used as a positive control in a serial concentration dilution of 0.25, 0.50, 1, 5, and 10 µg/mL. After 24 hours, by using a magnifying glass, the number of alive nauplii was counted and the percentage of lethality was calculated by the following equation:

$$\% \text{ Mortality} = \frac{N0-N1}{N0} x100$$

Where, N_0 = Number of nauplii taken, N_1 = Number of nauplii dead.

2.7 Thrombolytic activity

This test was performed according to the standard method with slight modification (Bristy et al., 2020). Streptokinase (SK) vial of 15, 00,000 I.U. was used as a standard drug which was purchased from Beacon Pharmaceutical Ltd, Mymensing, Bangladesh. 5mL sterile distilled water was added with it and mixed properly. This suspension was used as a stock solution from which 100µL (0.1mL) was used for in vitro thrombolysis. Whole blood (1.5 mL) was withdrawn from healthy human volunteers (n=6) without a history of oral contraceptive or anticoagulant therapy. Distribute 0.5mL of blood in each previously weighed sterile eppendorf and incubated them for 45 min at 37 °C to form the clot. After completing incubation, remove the serum from each eppendorf tube without disturbing the clot. Measure the weight of each eppendorf without serum to determine the clot weight.100µL (10mg/mL) of methanol extract was added to the test sample marked. As a positive control, 100µL of streptokinase and as a negative control, 100µL of saline were separately added to the control tube marked. All the test eppendorf were then incubated again for 90 min at 37 °C and observed clot lysis. After incubation blood serum was removed again (if clot lysis forms). Eppendorf was again weighed to observe the difference in weight after clot disruption. Difference obtained in weight taken before and after clot lysis was expressed as the percentage of clot lysis.

% of clot lysis = (weight of clot after remove of fluid/clot weight) × 100

2.8 Statistical analysis

The experimental results were analyzed by GraphPad Prism (version 7.0) software. Results represented in Mean ± SEM (standard error mean) and statistical analysis carried by unpaired t-test of one-way ANOVA (analysis of variance) where $P < 0.05$ was considered as statistically significant.

3 Results

3.1 Effect of extract inhibit diarrheal consequences in a dose-dependent manner

The castor oil-induced Swiss albino mice for diarrhea followed by a 4-hour observation summarized in Table 1. An extremely

significant (P < 0.0001) reduction was observed in the defecation phase in standard drug loperamide (63.01%) as well as the extract, with the maximum effect observed at 400 mg/kg (52.05%). The oral treatment in different doses exhibited a significant dose-dependent manner inhibition in the diarrhea phase. The maximum inhibitory diarrhea effects observed in 400 mg/kg (42.67%, P <0.05) in comparison to negative control followed by 200 mg/kg (37.5%), while the loperamide exhibited an extremely significant reduction (65.63%, P < 0.001).

3.2 Effect of extract lowers peristalsis movement in gastrointestinal motility test

The charcoal induced as a marker for gastrointestinal motility exhibited an extremely significant (P < 0.0001) reduction in peristalsis movement for all doses of MEHC when compared with negative control. Here, 400 mg/kg exhibited a significant percentage of inhibition (46.24%) followed by 21.48% in 200 mg/kg while the standard drug loperamide 64.49% (Table 2).

Table 1 The effect of methanol extract of *H. coccineum* rhizomes on castor oil induced diarrhea in Swiss albino mice

Treatment (mg/kg)	Total number of feces	Inhibition of defecation (%)	Total number of diarrheal feces	Inhibition of diarrhea (%)
Negative Control (0.1 mL/mice)	14.60 ± 0.46	-	6.40 ± 0.47	-
Loperamide (5)	5.40 ± 0.14 ^{d}	63.01	2.20 ± 0.12 ^{c}	65.63
MEHC 200	9 ± 1.73 ^{a}	38.36	4 ± 1.15	37.5
MEHC 400	7 ± 1.15 ^{c}	52.05	3.67 ± 0.88 ^{a}	42.67

Results represented in Mean ± SEM (n=3); ^{a} P < 0.05, ^{b} P < 0.01, ^{c} P < 0.001 and ^{d} P < 0.0001 are statistically significant in comparison to Tween-80 (Control) followed by unpaired t-test of one-way ANOVA (GraphPad Prism 7)

Table 2 The effect of methanol extract of *H. coccineum* rhizomes with reference to Loperamide on intestinal motility in mice by using charcoal as a marker

Treatment (mg/kg)	Total Length of Intestine (cm)	Distance Travel by Charcoal (cm)	Peristalsis Index (%)	Inhibition (%)
Negative Control (0.1 mL/mice)	55.60 ± 0.85	47.12 ± 0.87	84.75± 0.16	-
Loperamide (5)	55.83 ± 1.30	16.73 ± 0.42 ^{d}	29.96 ± 0.03 ^{d}	64.49
MEHC 200	55 ± 1	37 ± 1.15 ^{c}	67.25 ± 1.22 ^{d}	21.48
MEHC 400	55 ± 0.58	25.33 ± 1.45 ^{d}	46.01 ± 2.16 ^{d}	46.24

Results represented in Mean ± SEM (n=3); ^{c} P < 0.001 and ^{d} P < 0.0001 are statistically significant in comparison to Tween-80 (negative control) followed by unpaired t-test of one-way ANOVA (GraphPad Prism 7).

Table 3 Anthelmintic activity of methanol extract of *H. coccineum* rhizomes

Test sample	Concentration (mg/mL)	Time taken for paralysis (min)	Time taken for death (min)
Control (Water)	--	--	--
MEHC	5	11.35 ± 0.38 $^{\#\,**\,d}$	16.07 ± 0.82 $^{\#\#\,c}$
	10	6.25 ± 0.14 $^{\#\#\,b}$	11.00 ± 0.20 $^{\#\#\,c}$
	20	4.10 ± 0.52 $^{\#\#\,*}$	7.05 ± 0.18 $^{\#\#\,*}$
Standard (Levamisole)	0.5	14.41 ± 0.24	51.32 ± 0.26
	0.8	6.26 ± 0.29	12.21 ± 1.63
	1	3.30 ± 0.40	6.50 ± 0.36

Results represented in Mean ± SEM (n=3); $^{\#}$ P < 0.01, $^{\#\#}$ P < 0.0001 compared to levamisole (0.5 mg/mL); * P < 0.05, ** P < 0.001 compared to levamisole (0.8 mg/mL) and ^{b} P < 0.01, ^{c} P < 0.001 ^{d} P < 0.0001 compared to levamisole (1 mg/mL)followed by unpaired t-test of one-way ANOVA (GraphPad Prism 7)

3.3 Effect of extract on decreases paralysis time *in vitro*

The anthelminthic activity of MEHC was directly proportional to the concentration, which similar to the standard drug levamisole. The MEHC concentration (5, 10, and 20 mg/mL) exhibited a different significant paralysis time and times to death, compared to the different levamisole concentrations (0.5, 0.8, and 1 mg/mL). Among them, 20 mg/mL showed significant paralysis time (4.10 ± 0.52 min), whereas the times to death were 7.05 ± 0.18 min. The standard drug levamisole (1 mg/mL) showed 3.30 ± 0.40 min for paralysis, and the time for death was 6.50 ± 0.36 (Table 3).

3.4 Effect of the extract significantly reduces the mortality of brine shrimp

The lethality of brine shrimp was shown in Figure 1. The degree of lethality was shown by methanol extract was not concentration depended because the 1000µg/mL concentration showed 30% mortality while the 125 µg/mL showed 90% mortality in brine shrimp. Although, the extract showed a weakly toxic LC_{50} (681.95 µg/mL) while the regression equation was [y = -0.0611x + 91.667; R^2 = 0.951]. The positive control vincristine sulfate showed a highly toxic LC_{50} ranging 1.63 µg/mL [y = 6.9658x + 38.665; R^2 = 0.8779].

3.5 Effect of the extract significantly increases clot lysis activity

The thrombolytic activity of methanol extracts of *H. coccineum* rhizomes shown in figure 2. The MEHC extract showed a significant ($P < 0.05$) percentage of clot lysis 32.70 ± 0.77 in comparison with negative control water (3.78 ± 0.49); whereas the standard streptokinase also exhibited an extremely significant ($P < 0.0001$) clot lysis (75.35 ± 5.21).

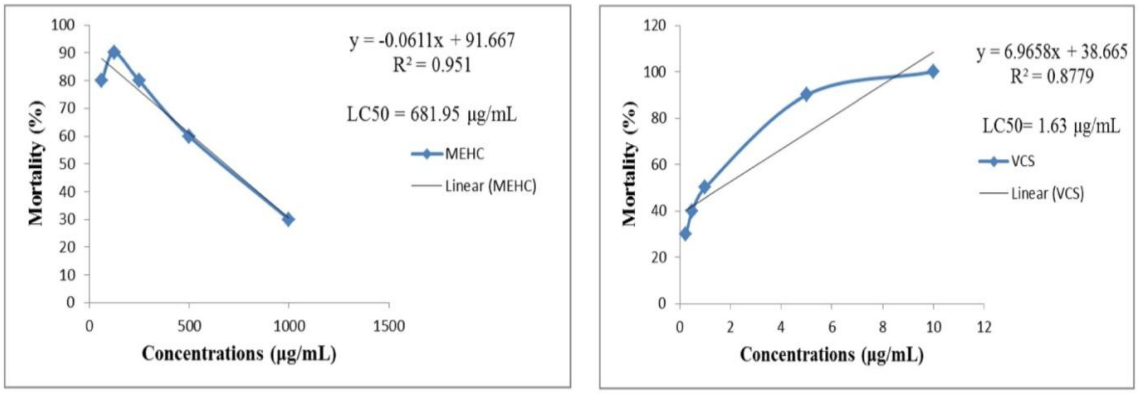

Figure 1 Percentage of mortality of brine shrimp of methanol extract of *H. coccineum* rhizomes (MEHC) and positive control Vincristine sulfate (VCS) at different concentrations.

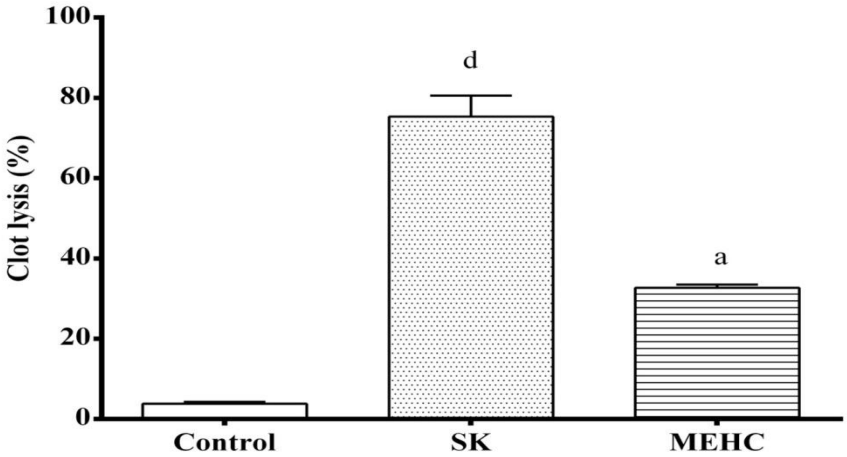

Figure 2 Percentage of clot lysis of human blood by *H. coccineum* rhizomes (MEHC) and standard drug streptokinase (SK). Results represented in Mean ± SEM (n=6).[a] $P < 0.05$ and [d] $P < 0.0001$ are statistically significant in comparison to water (control) followed by unpaired t-test of one-way ANOVA (GraphPad Prism ver. 7).

4. Discussion

Medicinal plants have been utilized for the treatment of different diseases including diarrhea and related gastrointestinal disorders although the efficacy and safety properties of the plant have not been mentioned properly (Komal & Rana, 2013). These days, almost 30% of pharmaceuticals drugs are developed from plants (Sharma et al., 2017). The requirement for more up to date and effective with less expensive and fewer side effects antidiarrheal drugs has become a principal concern (Mishra et al., 2016; Wolfheim et al., 2019). Diarrhea occurs when there is an imbalance between the secretary and absorptive systems of the gastrointestinal tract or potentially a modification of motility of intestinal smooth muscles (Gidudu et al., 2011; Camilleri et al., 2017). Castor oil is used as a diarrhea inducer because of the presence of retinoic acid which has laxative effects. Retinoic acid acts as an active principle of castor oil which changes the permeability of the intestinal membrane and increases the synthesis of prostaglandin and causes diarrhea, not unlike the physiopathology condition that causes diarrhea (Brijesh et al., 2009; Ulla et al., 2015; Patel et al., 2016). According to the literature, castor oil induced diarrhea after administration of 0.1-0.3 mL for mice within 1-2 hours (Kifle et al., 2021). In the current study, a significant diarrheal response was shown after the administration of a higher dose of castor oil (0.5 mL).

The methanol extract of *H. coccineum* rhizomes (200 and 400 mg/kg) significantly ($P<0.001$) inhibits diarrhea and defecation outputs produced by castor oil at a 4hours experiment. At 200 and 400 mg/kg doses, the methanol extract reduced significantly in a dose-dependent manner defecation outputs by 38.36 and 52.05%, respectively. The 400 mg/kg MEHC showed a 42.67% inhibition of diarrhea followed by 37.5% in 200 mg/kg whereas the comparable standard drug loperamide @ 5 mg/kg showed 65.63% inhibition. Here, a dose dependent manner inhibition of diarrhea observed which indicating a higher dose of the methanol extract might show a more promising antidiarrheal effect.

The castor oil induced diarrhea for intestinal motility inhibition where the charcoal was used as a marker. The methanol extract inhibits intestinal motility with the increase of the dose. An anti-motility action was observed with the increase of the doses. The maximum peristalsis index and inhibition was exhibited by 400 mg/kg ($P < 0.0001$) dose (46.01, 46.24%, respectively) whereas the standard drug loperamide 29.96, 64.49% ($P < 0.0001$), respectively. The methanol extract's extremely statistically significant intestinal transit reduction was observed by the decrease in GI motility of the charcoal. This result recommends that the crude extracts act on all parts of the small intestine. The availability of substances in the intestine increases by decreasing the motility in gut muscles which increases the absorption time (Ezekwesili et al., 2010; Islam et al., 2013; Kifle et al., 2021).

The aquarium worm *T. tubifex* was used in an anthelmintic study which is a suitable host for the *Myxobolus cerebralis* parasite (Adnan et al., 2019). The results suggested that MEHC showed a dose-dependent manner significant reduction in paralysis and death time of the worm, indicating the possible mechanism of anthelmintic activity. The levamisole is used as a standard drug in different concentrations, which is a nicotinic receptor agonist. It acts by activating the nicotinic acetylcholine receptors on the muscle of the worm resulting in paralysis and death (Jamkhande & Barde, 2014). The different concentrations (5, 10, and 20 mg/mL) of MEHC exhibited a directly proportional anthelminthic activity, whereas the 20 mg/mL showed a similar mechanism like levamisole 1 mg/mL. The current result was suggesting that MEHC could be used or formulated as an anthelminthic agent.

The toxicity of plant substances is an important factor for nowadays researcher (Ahmed et al., 2019). Mostly, mortality and LC_{50} are inversely proportional. The lower the lethal concentration the higher will be the LC_{50} value and vice versa. The value of LC_{50} over 1000 µg/mL is considered to be non-toxic, ranging from 500 - 1000 µg/mL is weakly toxic, moderately toxic for 100 - 500 µg/mL while less than 100 µg/mL is considered as highly toxic (Nguta et al., 2011; Rahman et al., 2020). In the current study, the methanol extract of *H. coccineum* rhizomes exhibited weak toxicity while the vincristine sulfate highly toxic.

Due to the clot formation in blood, the normal oxygen supply in the body was deprived. Plasmin is a fibrinolytic agent which used to lyse blood clot formation by thrombin. Tissue plasminogen activator or tPA are responsible to activate plasmin. The fibrinolytic drug dissolved the thrombin in coronary arteries to reassure the blood flow (Laurence, 1989; Emran et al., 2015b; Mahmud et al., 2015). In the present study, the prevention of clot formation in human blood was found to be significant while in comparison to standard drug streptokinase.

Conclusions

The results of the study revealed that the methanol extract of *H. coccineum* rhizomes showed significant antidiarrheal and anthelmintic effects which is similar to standard drug loperamide and levamisole, respectively while a significant thrombolytic activity with weak cytotoxicity. Further studies are needed to isolate, characterize the compounds responsible for these pharmacological activities. This study may be helpful for further related research works on this plant.

Conflict of interest

The authors report no conflicts of interest. The authors only are answerable for the content and inscription of the paper.

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Author's contribution

This exertion was employed in teamwork of all authors. All the authors have accepted responsibility for the entire content of this submitted manuscript and approved the submission. Authors SIN, NB, AMT, and MAS performed experiments. Authors SIN and NB collected the plants and prepared the extracts and fractions. SIN, AMT, MAS, SBM, and TBE performed statistical analysis. SIN, NB, AMT, MAS, SBM, TBE, and KD conceived the study and designed the experimental procedures. MAS and TBE designed, planned and supervised the experiments. TBE and KD acted for all correspondences. SIN, NB, AMT, MAS, TBEand KD participated in the manuscript draft and has thoroughly checked and revised the manuscript for necessary changes in format, grammar and English standard. All authors read and approved the final version of the manuscript.

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