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Antidiarrheal, cytotoxic and thrombolytic activities of methanolic extract of *Hedychium coccineum* leaves

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ABSTRACT: The study reports the *in vivo* antidiarrheal and *in vitro* cytotoxic and thrombolytic activities of methanolic extract of *Hedychium coccineum* leaves (MEHCL). The antidiarrheal activity was evaluated by castor oil-induced diarrhea, whereas the intestinal motility by charcoal marker. In addition, brine shrimp lethality bioassay and human blood clot lysis were used to evaluate the cytotoxic and thrombolytic activities, respectively. In antidiarrheal study, castor oil-induced diarrhea and gastrointestinal motility exhibited a significant dose dependent reduction in diarrhea and defecation and an extremely significant ($P < 0.0001$) inhibition in intestinal motility and peristalsis index by 200 and 400 mg/kg of MEHCL. The brine shrimp lethality bioassay revealed a considerable cytotoxic effect of MEHCL (LC_{50}= 81.59 µg/mL; $R^2 = 0.927$) while in thrombolytic a significant percentage of clot lysis (17.36%, $P < 0.01$) demonstrated. The findings suggest that *H. coccineum* leaves could be potential sources for biological activity.

KEYWORDS: *Hedychium coccineum*, Zingiberaceae, antidiarrheal, thrombolytic.

INTRODUCTION

Diarrhea is one of the major infectious diseases in third world countries for the child [1], which caused by disturbances in secretion and absorption of the intestine, causing increased volume rate of feces [2]. As indicated by the World Health Organization (WHO), Bangladesh is one of the susceptible to children-diarrhea, while 17% of Bangladeshi children (<5 years) admitted in the pediatric ward [1, 3]. Acute and chronic type diarrhea may occur where acute diarrhea caused due to epidemiological reasons such as traveling. The chronic type of diarrhea lasting more than four weeks [4, 5]. Around 88% of deaths identified with diarrheal are because of insufficient sanitation and poor cleanliness while the primary causative agent for diarrhea is *S. flexneri*, *S. aureus*, *E. coli* and *S. typhi* [6]. Diarrhea remains a concern in developing countries despite development in public health and economic wealth. Presently, the drugs used in the treatment of diarrhea are associated with adverse effects such as GIT disturbances, skin rash, fever, eosinophilia synonym, dry mouth, etc. [7]. The World Health Organization (WHO) has presented a program to prevent diarrheal disease with traditional herbal medicines [8]. Medicinal plants exhibited antidiarrheal properties by controlling the gastrointestinal delay travel, suppress gut motility, increase water adsorption, or decrease electrolyte discharge [9]. Plants offer therapeutic effects because of the presence of substances like alkaloids, tannins, and essential oils which act by producing physiological activity on the human body [10]. *Artemia nauplii* or Brine shrimp used for the LC_{50} study of medicinal plants, which is a preliminary test for toxicity measurement [11, 12].

Hedychium coccineum (Zingiberaceae) is commonly known as the scarlet ginger lily, which used as an ornamental plant in native Asia. It is locally known as

Aichhia and Mansila [13]. *H. coccineum* roots used in treating headaches and flowers pulped used in swollen body parts [14]. The Indian tribal people believe that wearing the flower behind the ear could be effective against the evil eye and disease [15]. It also reported being used as antipyretics and anti-inflammatory [13]. According to the reports, some essential oil compounds of *H. coccineum* found in Mauritius (East Africa) in rhizome part which is 44.4% of (E)-nerolidol, 24.2% of trans-sesquisabinene hydrate , α –Terpineol (0.6 %), α -fenchyl acetate (0.2 %) β-pinene (1.8%) and 2.4% of α-pinene [16]. There are no scientific report of traditional and pharmacological uses of *H. coccineum.* But a similar species named *Hedychium coronarium* available which used in various traditional uses such as inflammation, skin diseases, headache and rheumatic pain in Vietnam and China [17, 18].

Hence, the biological activity and phytochemical analysis not yet evaluated. So, the present study aimed to evaluate the *in vivo* antidiarrheal activity by Swiss albino mice and *in vitro* cytotoxicity by brine shrimp lethality assay and thrombolytic activity of methanolic extract of *H. coccineum* leaves.

MATERIALS AND METHODS

Chemicals and reagents

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

Collection and preparation of extract

Leaves of *H. coccineum* collected from Fatikchari Upzilla in Chittagong (Chittagong Hill tracts Area) in February 2019 with the help of local guide Mr. Abul Kashem. The plant identified by botanist and taxonomist Dr. Shiekh Bokhtear Uddin, Professor, Department of Botany, University of Chittagong, Bangladesh. Freshly collected leaves cut into small pieces to make them suitable for grinding purposes. The leaves dried for ten days under shade and ground and finally dried in an oven at 45 °C for 24 hours. The materials were ground into a coarse powder with the help of grinder and macerated in 1.5 L methanol for 96 hours at room temperature with occasional shaking and stirring. Then the filtered through a cotton plug followed by Whatman filter paper [19]. The solvent was evaporated with water both at 40°C temperature to get viscous mass. The percentage of the yield of methanol extract *H. coccineum* leaves was 4.76%.

Experimental animals

Mice weighing range about 28-32 gm procured from the animal house of the Department of Pharmacy, Jahangirnagar University, Savar, Dhaka, Bangladesh. All the animals familiarized themselves with the new environment for one week. During the experiment period, the animals kept in a well-ventilated animal house at 25 °C temperature. They supplied with standard pellets and fresh potable water. All the mice were kept within the cage in the animal house and maintained with a natural 12hrs light and dark cycle. The experimental animal handled according to international guidelines for the use and maintenance of experimental animals under the reference of Pharm/PND/161/31-2019 [20].

Antidiarrheal activity (*In vivo*)

Castor oil-induced diarrhea

Mice were fasted for 18 hours before the test with free access to water and divided into four groups (n=5). The mice were screened initially by giving 0.4 mL of castor oil and only those showing diarrhea selected for the experiment. The control group received vehicles only (distilled water containing 1% Tween-80), positive control received standard anti motility drug loperamide (5 mg/kg body weight) as oral suspension, test group received suspension of methanolic leaves extract of *H. coccineum* at the oral dose of 200 and 400 mg/kg body weight, respectively. After 1 hour of treatment, 0.4 mL of castor oil administered by oral gavage and placed in separate cages having adsorbent paper (blotting paper) at the bottom. The characteristics of diarrheal droppings (wet & dry faces) were noted every hour in four hours of study for each mouse. At the beginning of each hour, the old paper replaced with the new one [21].

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.

Gastrointestinal motility test by charcoal marker

Mice have treated same as the previously described method of Castor oil-induced diarrhea. After one hour of the oral administration, 1 mL of charcoal solution (10% charcoal, 5% gum acacia) given orally. Later, one hour's mice sacrificed with a high dose of chloroform anesthesia. Measured the total length of the small intestine and the distance traveled by charcoal from the pylorus to cecum was measured [22].

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

Brine shrimp lethality bioassay (*In vitro*)

Brine shrimp lethality bioassay of cytotoxicity evaluated by using the using *Artemia salina* (shrimp eggs). In the artificial seawater (3.8% NaCl solution/l, w/v), the shrimp eggs hatched for 48 hours for maturing the shrimp called nauplii. The extract was dissolved in DMSO (50 µL in 5 mL solution) to prepare the test sample with artificial seawater (3.8% NaCl/L in tap water) to obtain the serially diluted concentrations of 25, 50, 100, 200 and 400 µg/mL. Vincristine sulfate used as a positive control as the preceding method in a serial concentration dilution 0.125, 0.25, 0.5, 1, 5 and 10 µg/mL. Each concentration contains ten nauplii. After 24 hours, all concentration inspected by an amplifying glass and the number of living and dead nauplii in each concentration was observed and recorded [23, 24].

$$\% \text{ of mortality} = (N_1/N_0) \times 100$$

Where, N_0= the number of nauplii taken; N_1= the number of nauplii dead.

Thrombolytic activity (*In vitro*)

Thrombolytic activity test performed using the method described by Prasad et al. [25, 26]. As a stock solution, lyophilized streptokinase vial (1500000 IU) mixed adequately with 5 mL (30,000 IU) sterile distilled water. This suspension used as a stock from which 100 µL (30,000 IU) used for *in vitro* thrombolysis. A 3 mL of blood withdrawn from healthy volunteers (n=5, age: 20-23 years) without a history of anticoagulant therapy or an oral contraceptive. 0.5 mL per tube blood distributed to each previously weight eppendorf tubes and incubated at 37 °C for 45 minutes to form the clot. After the formation of the blood clot, removed the serum without disturbing the clot, and each eppendorf tube reweighed for calculating the clot weight. 100 µL extract (100 mg/10 mL) added to each tube having the pre-weighed clot. In similar manner, previously suspended streptokinase (100 µL) and 100 µL distilled water were added separately to each eppendorf while the streptokinase and distilled water used as the positive and negative control group. Incubation was done for 90 minutes at 37 °C and observed clot lysis. The released fluid was removed and reweighed the tube to calculate the difference in weight after clot disruption.

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

Statistical analysis

The experimental results analysed by GraphPad Prism (version 7) software. Results represented in Mean ± Standard error mean (SEM) and statistical analysis carried by unpaired t-test of one-way ANOVA where $P < 0.05$ considered as statistically significant.

RESULTS

Antidiarrheal activity

Castor oil-induced diarrhea

The castor-oil induced diarrhea assay by methanolic extract of *H. coccineum* leaves (MEHCL) observed for four hours whereas a significant dose-dependent manner activity depicted (Table 1). Diarrheal episodes predominantly reduced by the positive control loperamide (5 mg/kg) in an extremely significant ($P < 0.0001$) manner (65.63%) while the MEHCL exhibited 42.71% and 53.12% by 200 and 400 mg/kg dose. In defecation phase, 400 mg/kg exhibited maximum inhibition (54.31%, $P < 0.001$) while the positive control loperamide (65.63%, $P < 0.0001$).

Table 1. The effect of methanolic extract of *H. coccineum* leaves 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 [d]	65.63
MEHCL 200	10.00 ± 2.73	31.51	3.67 ± 0.88 [a]	42.71
MEHCL 400	6.67 ± 1.26 [c]	54.31	3.00 ± 0.38 [c]	53.12

Results represented in Mean ± SEM (n=5). [a] $P < 0.05$, [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).

Castor oil-induced intestinal motility test (Charcoal marker)

The intestinal motility by castor oil-induced followed by charcoal marker exhibited an extremely significant ($P < 0.0001$) reduction in peristalsis movement for all doses of MEHCL when compared with the negative control. A maximum percentage of inhibition (41.98%, $P < 0.0001$) observed by 400 mg/kg dose followed by 23.67% in 200

mg/kg while the standard drug loperamide 48.09% as shown in Table 2.

Table 2. The effect of *H. coccineum* leaves extract with reference drug 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 (%)
Control	50.33 ± 0.33	43.66 ± 2.91	86.69 ± 5.23	-
Loperamide (5)	52.66 ± 0.33 [c]	22.66 ± 1.45 [c]	43.04 ± 2.79 [d]	48.09
MEHCL 200	56 ± 1.15 [c]	33.33 ± 1.76 [b]	59.52 ± 2.76 [c]	23.67
MEHCL 400	60 ± 1.00 [d]	25.33 ± 1.33 [c]	42.22 ± 3.00 [d]	41.98

Results represented in Mean ± SEM (n=5) [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).

Cytotoxic Assay

Brine Shrimp Lethality Bioassay

MEHCL showed different mortality rate at randomly selected concentration (25, 50, 100, 200 and 400 µg/mL). The mortality rate of brine shrimp nauplii found to be increasing with the increase of the concentration of the MEHCL. The LC_{50} values of MEHCL)81.59 µg/mL, R^2 =0.927) whereas the LC_{50} values of Vincristine sulfate was 1.63 µg/mL (R^2 = 0.8779). The results presented in Figure 1.

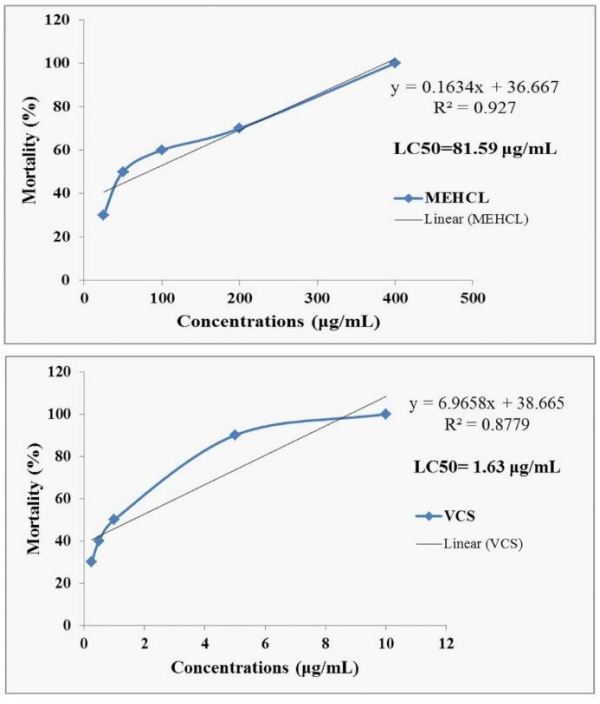

Figure 1. Cytotoxic effect of methanolic extract of *H. coccineum* leaves and positive control vincristine sulphate (VCS) on brine shrimp nauplii at different concentration.

Thrombolytic activity

The addition of 100 µl SK, a positive control (30,000 I.U.) along with 90 minutes incubation at 37 °C, showed 75.35% clot lysis. Clots, when treated with 100 µl sterile 0.9% normal saline as a negative control, showed only negligible clot lysis 3.78%. The MEHCL exhibited a significant percentage of clot lysis (17.36%, $P < 0.01$). The results presented in Figure 2.

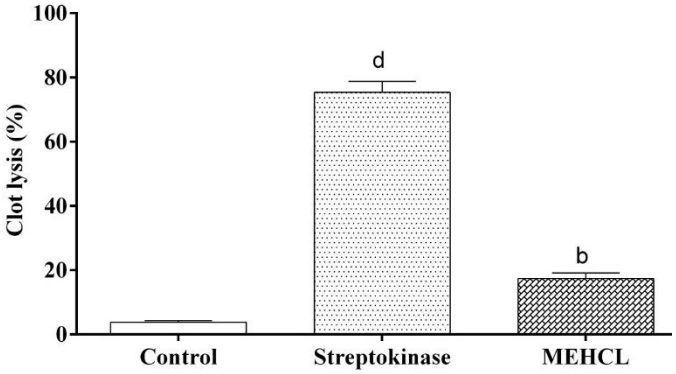

Figure 2. The percentage of clot lysis of methanolic extract of *H. coccineum* leaves standard drug streptokinase (SK). Results represented in Mean ± SEM (n=5). [b] $P < 0.01$, and [d] $P < 0.0001$ are statistically significant in comparison to water (Control) followed by unpaired t-test of one-way ANOVA (GraphPad Prism 7).

DISCUSSION

Diarrhea is generally the outcome of expanded electrolyte emission, altered intestinal motility, expanded luminal osmolarity and reduced electrolyte absorption which occurred by ricinoleic acid, an active component of castor oil [3, 27]. The discharge of ricinoleic acid from castor oil via lipase enzyme induces irritation in the intestinal mucosa. This irritation caused secretion of prostaglandin and nitric oxide, cyclic adenosine monophosphate, platelet-activating factor and tachykinins which are inflammatory mediators. The inflammatory mediators stimulate intestinal motility as well as electrolyte and water increase. This impact could happen as an outcome of enacting the G protein-coupled prostanoid receptor (EP3) on the smooth muscle cell of the intestine by ricinoleic acid [27, 28]. In our study, the MEHCL exhibited a significant reduction in the frequency of diarrhea and thus could be a potential source of phytochemicals that might inhibit the secretion of inflammatory mediators.

Intestinal motility test by charcoal marker used to determine the peristalsis movement of the intestine. The

ricinoleic acid (bioactive components of castor oil) caused inflammation, irritation in the mucosa level of the intestine, which leads to diarrhea. Inflammation of intestine stimulates the release of prostaglandin resulting in intestinal motility as well as electrolyte and water increase [29]. The α–Terpineol has significant activity in blocking the PGE_2 receptor to exhibit the antidiarrheal effect [30]. This plant contains α–Terpineol in its rhizome which might also present in the leaves as well. In our charcoal marker study, the MEHCL exhibited a significant inhibition in motility by inhibiting the synthesis of prostaglandin.

Brine shrimp lethality bioassay has widely utilized for screening of cytotoxic effects of plant extract [31]. Generally, the smaller the LC_{50}, the higher the toxicity and vice versa. The value of LC_{50} over 1000 µg/mL 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 [32, 33]. The α-pinene and β-pinene reported having a highly toxic cytotoxic activity [34]. In our study, the MEHCL exhibited a toxic LC_{50} (81.59 µg/mL) whereas the vincristine sulfate (1.63 µg/mL). The mortality rate of brine shrimp nauplii found to be increasing with the increase of the concentration of the MEHCL. This plant contains α-pinene and β -pinene as an essential oil in its rhizome which might also present in the leaves as well. The observed cytotoxicity through brine shrimp lethality supports the earlier study on *Hedychium coronarium* which is different plant species of the Zingiberaceae family [35].

Most thrombolytic agents exert their beneficial effect by activating the enzyme plasminogen, which solubilizes the cross-linked fibrin mesh to restore blood flow over blocked blood vessels [36]. The lysis of clots, therefore, is useful for the treatment of clot-related disorders, including myocardial infarction, thromboembolic strokes, deep vein thrombosis, and pulmonary embolism, to clear a blocked artery that prevents permanent damage to the respective tissues [12, 37]. In our study, the MEHCL and streptokinase exhibited a significant percentage of clot lysis in comparison to negative control water. The increase in clot lysis by MEHCL compared to the controls demonstrates its potential use in clot-related disorders.

CONCLUSIONS

In our present study, the methanolic extract of *H. coccineum* leaves exhibited a significant antidiarrheal and thrombolytic activity with significant cytotoxicity (Figure 3). However, further advance study is required to predict the possible mechanism of *H. coccineum* leaves.

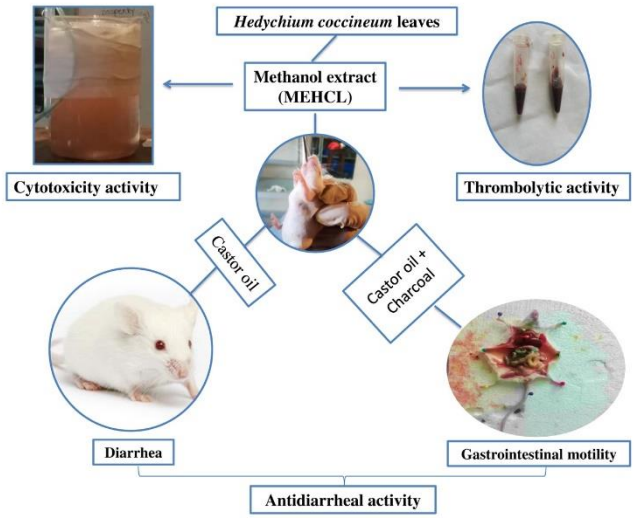

Figure 3. Graphical representation of methanolic extract of H. coccineum leaves on antidiarrheal, thrombolytic and cytotoxic activity.

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CONFLICTS OF INTEREST

Authors declared that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

FS and AMT together planned and designed the research. MAS and MNI arranged the whole facilities for the research and supervised the whole research. MAU, MAM and AU conducted the entire laboratory works with AMT and FS. FS and AMT imparted in study design and interpreted the results putting efforts on statistical analysis with MAS, MNI and TBE. FS, AMT and TBE 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 agreed on the final version of the manuscript.

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