ORIGINAL ARTICLE

Antidiarrheal and thrombolytic effects of methanol extract of *Wikstroemia indica* (L.) C. A. Mey leaves

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Context: Medicinal plants contribute as potential sources of therapeutic uses. *Wikstroemia indica*, a traditional medicinal plant, has long been used as anti-inflammatory, antiviral, antimalarial, anti-mitotic, antitumor, and anti-HIV in different parts of the world. **Aims:** The aim was to investigate the antidiarrheal and thrombolytic effect of *W. indica* leaf extract. Settings and Design: Sample collection, identification, solvent extraction, and crude extract preparations were led to evaluate the antidiarrheal effect in *in vivo* model and the thrombolytic effect in *in vitro* model. **Materials and Methods:** Castor oil-induced diarrhea and enteropooling assays and gastrointestinal motility tests were used to examine the *in vivo* antidiarrheal activity in Wistar albino rat. *In vitro* clot lysis model was undertaken to investigate the thrombolytic action of the extract. Data were analyzed using statistical software (Statistical Package for Social Science, SPSS, version 19.0, SPSS Inc., USA). **Results:** The diarrheal episode was inhibited by 18.64% and 28.96% for the methanol extract at the doses of 200 and 400 mg/kg, respectively. The extract significantly ($P < 0.05$) reduced the intestinal volume and intestinal transit in comparison to control. The extract also reduced the rate of defecation, accumulation of fluid, and transit of charcoal oil. The extract showed a moderate thrombolytic effect compared to the reference control. **Conclusion:** Methanol extract *of W. indica* might be triggered the premonition of novel drug discovery in the future due to its antidiarrheal effect in the animal model.

Key words: Antidiarrheal, castor oil, streptokinase, thrombolytic, *Wikstroemia indica*

INTRODUCTION

Diarrhea is one of the major life-threatening diseases for the children of third world country. According to World Health Organization (WHO), Bangladesh is one of the vulnerable countries for children-diarrhea while 17% of Bangladeshi children under five admitted in the pediatrics ward dies of this disease.[1,2] To treat diarrhea, multifaceted lines of treatments have been adopted since long although the synthetic drugs were taken as the most worthy option. But many of the synthetic antidiarrheal drugs have several adverse effects. People, therefore, highly prefer complementary and alternative treatment with herbal drugs. WHO also emphasized and endorsed the traditional medicinal practice as a part of special diarrheal disease control program to combat diarrheal incidences.[3]

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Blood clotting is one of the major problems for circulating the blood through blood vessel. Blood vessel can be blocked in the presence of emboli and thrombi which block the blood flow causing the tissues deprived from the oxygen and nutrition. This ultimately leads to infarction of tissue or death of tissues. In the presence of tissue plasminogen activator (t-PA) and urokinase, plasminogen is converted into the active plasmin. Fibrinolytic agents work through infusion of analogs of t-PA.[4] Fibrinolytic therapy with recombinant t-PA treatment is limited by a fairly slow reperfusion rate and frequent early reocclusions. Moreover, the platelet-rich thrombi are highly resistant to lysis by t-PA.[5] However, plant-derived drugs are safer and dependable options in these cases due to their incredible pharmacological activities, economic viability, and less side effects in different healthcare management system.[6] Their tremendous effects as antiplatelet,[7,8] anticoagulant,[9,10] antithrombotic,[11] and thrombolytic agents have also been directed towards the discovery, development, and use of plants sources products in thrombus degeneration.

Wikstroemia indica is a small shrub belonging to *Thymelaeaceae* family. It is a traditional Chinese herbal and used in several diseases as an abortifacient in clinical practice.[12] Number of bioactive compounds have been

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isolated from *W. indica*.[13-18] Researchers have reported the anti-inflammatory, antiviral, antimalarial, antimitotic, antitumor, anti-HIV and antifungal, toxicological effects of different parts of *W. indica*. However, antidiarrheal and thrombolytic effects are yet to be studied. This study investigated the antidiarrheal effect in the animal model and thrombolytic effect in clot lysis model.[18-23]

MATERIALS AND METHODS

Plant Material

Wikstroemia indica (*Thymelaeaceae*) fresh leaves were collected from Ispahani Hill, Chittagong district, Bangladesh in the month of September, 2011. The plant was taxonomically identified and authenticated from "Bangladesh Forest Research Institution" at Chittagong. A sample specimen is preserved in BFRI as authentication no. 5515.

Preparation of Extract

The leaves were washed through clean water, chopped, and air-dried at room temperature 26°C ± 1°C for 7–10 days then pulverized in the electric grinder. The powder (325 g) obtained was successively extracted in methanol (55–60°C). Powder was dissolved in methanol for 1-week at room temperature with occasional shaking then filtered through a cotton plug followed by Whatman filter paper No 1. The solvent evaporated at room temperature turned into semisolid, preserved in the sample tube at 4°C until further use.

Animals

Six-seven week old Wistar albino rats (120–150 g) of either sex collected from the animal house of International Centre for Diarrheal Diseases and Research (ICDDR, B) were used for this research. They were acclimatized in well cross-ventilated room at 25°C ± 2°C temperature and 55% humidity for 1-week before and during the experiment. The animals were fed standard pellet diet with a continuous supply of fresh water *ad libitum*. Animals were taken care according to the National Institute of Health Guide for Care and Use of Laboratory Animal (Pub. No. 85–23 revised 1985).

Drugs and Chemicals

Reference drug Loperamide (Square Pharmaceuticals Ltd., Bangladesh), castor oil (WELL's Heath Care, Spain), normal saline solution (0.9% NaCl), and charcoal meal (10% activated charcoal in 5% gum acacia) were used in antidiarrheal test. Streptokinase (SK) vial (Polamin Werk GmbH, Herdecke, Germany) was used in thrombolytic activity test.

Acute Toxicity Test

Wistar albino rats maintained under standard laboratory condition were used for acute toxicity study. A total of five animals received a single oral dose (0.5, 1.0, 1.5, and 2.0 g/kg bw) of the extract. Animals were kept over-night fasting prior to administration. After administration of the extract, food was withheld for further 3–4 h. Animals were observed individually once during the first 30 min after dosing, periodically during the first 24 h (with special attention during the first 4 h) and daily thereafter for a period of 14 days for delayed toxicity. Once daily cage side observation including changes in skin and fur, eyes and mucous membrane, respiratory and circulatory rate, autonomic and central nervous system changes were observed. The effective therapeutic dose was taken as one tenth of the median lethal dose (LD_{50} > 2.0 g/kg).[24]

Antidiarreal Activity

Castor Oil Induced Diarrhea

Diarrhea was induced by Castor oil using the established method as stated by Awouters *et al*. with little modification.[25] The experimental animals were fasted for 18 h before commencement of the experiment. Animals were divided into four groups five in each group. Group I was treated as control giving only normal saline (2 mL/kg) while the Group II received loperamide (5 mg/kg). Groups III-IV received methanol extract of *W. indica* leaves (200 and 400 mg/kg bw i.p.). One hour later, all groups received castor oil 1 mL/animal orally. Then all rats were put in cages lined with adsorbent papers and observed for 4 h for the presence of characteristic diarrheal droppings. The total number of diarrheal feces of the control group was considered 100%. Diarrheal inhibition measured from the total score and activity of each group and expressed as % inhibition of diarrhea. The percent (%) inhibition of defecation was measured using the following formula.[25]

% Inhibition of defecation = [(A − B)/A] × 100
A = Mean number of defecation caused by castor oil.
B = Mean number of defecation caused by drug or extract.

Castor Oil-Induced Enteropooling

The experimental animals were divided into four groups five in each. All the animals fasted for 18 h prior to experiment. Group I was treated as control (saline 2 mL/kg bw orally), Group II received standard drug (loperamide 5 mg/kg bw i.p.) and Groups III-IV received methanol extract (200 and 400 mg/kg bw i.p.) 1 h before the administration of castor oil to induce diarrhea. After 2 h, all the animals were sacrificed, and the small intestine from the pylorus to the cecum was isolated. Then the intestinal content was collected by milking into a graduated tube, and the volume was measured.[26]

Gastrointestinal Motility Test

Gastrointestinal motility test was carried out according to the modified method described by Rahman *et al*.[27] All the

tested animals fasted for 18 h before the experiment, and they were divided into four groups five in each group. All the rats received castor oil to produce diarrhea. One hour later, Group I received saline water (saline 2 mL/kg bw orally), Group II received standard drug (loperamide 5 mg/kg bw i.p.), and Groups III-IV received methanol extract (200 and 400 mg/kg bw i.p.). After 1-h of treatments (i.p. administration), all animals received 1 mL of charcoal meal (10% charcoal suspension in 5% gum acacia) orally. One hour later, all animals were sacrificed and the distance traveled by the charcoal meal in the intestine, from the pylorus to the cecum was measured and expressed as a percentage of distance moved.[27]

Thrombolytic Activity

Herbal Preparation

For herbal preparation, the specific amount of weighed extract was dissolved in 10 mL distilled water and shook on a vortex mixture. Then the suspension was kept overnight and decanted to remove the soluble supernatant, which was filtered through a filter paper (Whatman No. 1). The final solution was the desired one for *in vitro* evaluation of clot lysis activity according to the Prasad *et al*.[28]

Streptokinase Solution Preparation

Sterile distilled water (5 mL) was added to commercially available lyophilized SK vial (Polamin Werk GmbH, Herdecke, Germany) 15, 00,000 I.U. and mixed properly. The prepared suspension was used as a stock solution. A 100 µl (30,000 I.U) of stock was used for *in vitro* thrombolysis.

Blood Specimen

Whole blood (4 mL) was drawn from healthy human volunteers (n = 20) without a history of oral contraceptive or anticoagulant therapy using a protocol approved by the Institutional Ethics Committee of Chittagong University, faculty of medicine. An earlier consent, approval number HET-CU2013/3, was taken from the faculty of medicine, University of Chittagong, for collection of blood samples from Human volunteers. Blood collection and preservation were conducted by Dr. M Rafiqur Rahman (Pathologist, Faculty of Medicine, University of Chittagong). A 500 µl of blood was transferred to each of the eight previously weighed microcentrifuge tubes to form clots.

In Vitro Thrombolytic Study

The thrombolytic activity of methanol extract was carried as reported earlier.[28] Briefly, 4 mL venous blood drawn from the healthy volunteers was transferred in preweighed sterile microcentrifuge tube (0.5 mL/tube) and incubated at 37°C for 45 min. After clot formation, serum was completely removed without disturbing the clot and each tube having clot was again weighed to determine the clot weight (clot weight = weight of clot containing tube–weight of tube alone). To each microcentrifuge tube containing preweighed clot, 100 µl of the organic extract was added separately. As a positive control, 100 µl of SK and as a negative nonthrombolytic control, 100 µl of distilled water was separately added to the control tubes numbered. All the tubes were then incubated at 37°C for 90 min and observed for clot lysis. After incubation, amount of released fluid was removed, and tubes were again weighed to observe the difference in weight after clot disruption. Difference observed in weight taken before and after clot lysis was expressed as a percentage of clot lysis. The experiment was repeated with the blood samples of the 20 volunteers.

The percentage of clot lysis was measured through the following formula:

Percent of clot lysis = (Weight of the lysed clot/Weight of clot before lysis) ×100

Statistical Analysis

Data were expressed as mean ± standard deviation. Results were analyzed by one-way ANOVA followed by Bonferroni test using SPSS Data Editor for Windows, Version 19.0 (SPSS Inc., U.S.A.). Values with $P < 0.05$ were considered as statistically significant. The significance between % clot lysis by herbal extract by means of the weight difference was tested by the paired t-test analysis.

RESULTS

Acute Toxicity Test

Acute toxicity assay did not show any toxic effects for the animal within the due course of study.

Effect on Castor Oil Test

Diarrhea was appeared in all tested animals after 1-h of castor oil administration. Both the reference drug (Loperamide) and methanol extract reduced the diarrheal episode in a dose-dependent manner; however, the antidiarrheal effect of loperamide was much greater than that of the extract. Briefly, methanol extract of *W. indica* (200–400 mg/kg, p.o.) significantly ($P < 0.05$), reduced the fecal output produced by castor oil. At doses of 200–400 mg/kg (p.o.), the plant extract significantly ($P < 0.05$) and dose-dependently delayed the onset of diarrhea induced by castor oil when compared with the untreated controls. *W. indica* (200 mg/kg, p.o.) reduced the number of fecal episodes by 18.64% while the dose of 400 mg/kg (p.o.) significantly ($P < 0.001$), reduced the number of animals suffering from diarrhea by reducing defecation by 28.96%. Loperamide (3 mg/kg, p.o.) profoundly ($P < 0.001$), reduced the fecal output produced by castor oil [Table1]. Both doses decreased the intestinal volume 2.28 ± 0.06 and 1.95 ± 0.09 mL by 200 mg/kg and 400 mg/kg, respectively, while loperamide

(standard) decreased 1.57 ± 0.06 mg/kg. The onset of castor oil-induced diarrhea and number of diarrheal episodes were also profoundly prolonged ($P < 0.001$) and reduced ($P < 0.001$), respectively by loperamide. In terms of protection from diarrhea, the 400 mg/kg dose of *W. indica* extract was comparable with the standard drug loperamide.

EFFECT ON CASTOR OIL INDUCED ENTERPOOLING

Castor oil caused accumulation of water and electrolytes in intestinal loop. Administration of *W. indica* extract produced a significant ($P < 0.05$) reduction compared to the reference antidiarrheal agent loperamide (5 mg/kg) in intestinal weight and volume [Table 2]. Antidiarrheal effects of the extract showed moderate dose-dependent activity, both doses decreased the intestinal volume 2.28 ± 0.06 and1.95 ± 0.09 mL by 200 mg/kg and 400 mg/kg, respectively, while loperamide (standard) decreased 1.57 ± 0.06 mg/kg.

Effect on Intestinal Motility

Both the doses of methanol extract moderately reduced the gastrointestinal distance traveled by charcoal in rats. A dose-dependent response was as well observed in this model. The lower dose 200 mg/kg produced an inhibition of 19.77%, and the higher dose 400 mg/kg produced the inhibition 35.49%. The results are concluded in Table 3.

In Vitro Clot Lysis Study

The positive control 100 µl SK (30,000 I.U.) incubated with the blood clot for 90 min at 37°C, showed 85.25 ± 1.41% clot lysis. On the other hand, the negative control (treated with 100 µl sterile distilled water) showed only negligible clot lysis (4.44 ± 1.15%). The methanol extract of *W. indica* showed 20.09 ± 1.56% clot lysis. Statistical representation of the effective clot lysis percentage by our herbal preparation, positive thrombolytic control (SK), and negative control (sterile distilled water) is summarized in Table 4.

DISCUSSION

Diarrhea is characterized as the abnormally frequent defecation of feces of low consistency which is usually happened due to a disturbance in the transport of water and electrolytes in the intestines. Despite the multiplicity of etiologies, the five major mechanisms responsible for the pathophysiology in water and electrolytes transport are (i) increased luminal osmolarity (osmotic diarrhea), (ii) increased electrolytes secretion (secretory diarrhea), (iii) exudative diarrhea (*Escherichia coli infection*) (iv) rapid movement of food through the intestine (hypermotility) and insufficient time for nutrients to be absorbed; and (v) inflammatory diarrhea (damage to the mucosal lining or brush border, which leads to a passive loss of protein-rich fluids and a decreased ability to absorb these lost fluids).[26]

Table 1: Effect of methanol extract of *W. indica* on castor oil induced diarrhea in rats

Group	Treatment	Total number of feces	Inhibition of defecation (%)	Total number of diarrheal feces	Inhibition of diarrhea (%)
I	Castor oil+Saline (2 mL/kg)	18.18±1.92	-	11.05±1.08	-
II	Castor oil+LPD (5 mg/kg)	7.76±0.66*	57.32	5.00±0.33**	54.75
III	Castor oil+WIEx (200 mg/kg)	13.78±1.11	24.20	8.99±0.32	18.64
IV	Castor oil+WIEx (400 mg/kg)	12.88±1.24*	29.15	7.85±0.32*	28.96

LPD – Loperamide; WIEx – *Wikstroemia indica* extract. Values are expressed as mean±SEM. (n=5). Data were analyzed by statistical software statistical package for social science (SPSS, version 19.0, IBM corporation, New York). Asterisk numericals are statistically significant at *P<0.05, **P<0.01 in comparison to control group

Table 2: Effect of methanol extract of *W. indica* on castor oil induced enterpooling in rats

Group	Treatment	Weight of intestinal content (g)	Volume of intestinal content (mL)	Inhibition (%)
I	Castor oil+Saline (2 mL/kg)	3.22±0.04	2.79±0.18	-
II	Castor oil+LPD (5 mg/kg)	1.84±0.36*	1.57±0.06**	43.73
III	Castor oil+WIEx (200 mg/kg)	2.60±0.05	2.28±0.08	18.27
IV	Castor oil+WIEx (400 mg/kg)	1.32±0.08	1.95±0.09*	30.11

LPD – Loperamide; WIEx – *Wikstroemia indica* extract. Values are expressed as mean±SEM. (n=5). Data were analyzed by statistical software statistical package for social science (SPSS, version 19.0, IBM corporation, New York). Asterisk numericals are statistically significant at *P<0.05, **P<0.01 in comparison to control group

Table 3: Effect of methanol extract of *W. indica* on small intestinal transit in rats

Group	Treatment	Total length of intestine (cm)	Distance traveled by marker (cm)	Inhibition (%)
I	Castor oil+Saline (2 mL/kg)	107.80±1.93	101.00±2.31	-
II	Castor oil+LPD (5 mg/kg)	103.37±1.36	54.00±0.58**	47.76
III	Castor oil+WIEx (200 mg/kg)	99.13±0.84*	79.53±0.58**	19.77
IV	Castor oil+WIEx (400 mg/kg)	104.33±2.20	67.30±0.78**	35.49

LPD – Loperamide; WIEx – *Wikstroemia indica* extract. Values are expressed as mean±SEM. (n=5). Data were analyzed by statistical software statistical package for social science (SPSS, version 19.0, IBM corporation, New York). Asterisk numericals are statistically significant at *P<0.05, **P<0.01 in comparison to control group

Table 4: Thrombolytic activity of methanol extract of *W. indica*

Treatment Group	% Clot lysis (Mean±SD)	*P* value (two-tailed) when compared to negative control (water)
Control	4.44±1.15	0.001
Streptokinase	85.25±1.41	0.001
WIEx	20.09±1.56	0.001

WIEx – *Wikstroemia indica* extract. Statistical representation of the effective clot lysis percentage by herbal preparations, positive thrombolytic control (Streptokinase) and negative control (sterile distilled water) done by paired t-test analysis; % clot lysis is represented as mean ± SD. and *P* values of all Herbal preparations were < 0.001 was considered as moderate

Medicinal plants have been used in diarrheal disorders due to the established efficacy of different plant-derived preparations with the scientific basis. This study was designed to evaluate the antidiarrheal property of *W. indica* leaf extract in castor oil-induced diarrheal model. Castor oil is an effective laxative. It produces diarrhea due to its active component ricinoleic acid. Liberation of ricinolieic acid from castor oil results in irritation and inflammation of the intestinal mucosa, leading to release of prostaglandins and other inflammatory mediators, which stimulate motility and secretion.[29] The prostaglandins thus released promote vasodilatation, smooth muscle contraction, and mucus secretion in the small intestines. Prostaglandins of the E series are considered to be good diarrheogenic agents in experimental animals as well as in human beings. The results of this study revealed that the leaves extract of *W. indica* produced statistically significant protection against diarrhea and was found to be comparable to loperamide; a drug widely employed against diarrhea disorders which effectively antagonizes diarrhea induced by castor oil, prostaglandin, and cholera toxin.[30]

Anti-diarrheal activity was found in plants possessing tannins, alkaloids, saponins, flavonoids, steroids, and/or terpenoids.[31] Anti-diarrheal activities of flavonoids have been ascribed to their ability to inhibit intestinal motility and hydroelectrolytic secretions which are known to be altered in diarrheic conditions.[32] Tannins present in anti-diarrheal plants denature proteins in the intestinal mucosa by forming protein tannates which may reduce the secretion. Studies on the functional role of tannins also reveal that they could also bring similar functions by reducing the intracellular Ca^{2+} inward current or by activation of the calcium pumping system (which induces the muscle relaxation).[33] Phytochemical screening of *W. indica* leaf extract revealed the presence of alkaloids, tannins, flavonoids, and saponins. These constituents may be responsible for the *in vivo* anti-diarrheal activity of *W. indica*.

Plant materials have been used since long to treat various ailments. In our study, we used methanol extract of *W. indica* to breakdown the thrombus or clot formed after collecting the fresh blood from human volunteers. Although there are many thrombolytic drugs available in the market, some are herbal, and some are modified by recombinant technology in order to make the drug site specific and increase effectivity. But site-specific drugs are found to show various complications like bleeding and sometimes even death in case of some patient.[34-37] In the case of our study, the % of clot lysis by positive and negative control differs moderately as the $P < 0.001$. The thrombolytic activity of methanol extract mild to moderate clot lysis effects (20.09 ± 1.56% clot lysis) compared to SK (85.25 ± 1.41% clot lysis).

The experiment clearly evidences that the methanol extract of *W. indica* leaves possesses moderate antidiarrheal activity and mild to moderate thrombolytic effects. However, these studies are preliminary and indicated the huge scopes for further studies to evaluate the similar effects of the active compounds isolated from the extract.

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