Original Research Article

Antidiarrheal and antimotility activities of stem bark extracts of *Annona reticulata* Linn. in mice model

Abstract

The study was aimed to evaluate the phytochemical screening, *in vivo* evaluation of anti-diarrheal activity, and GI motility of methanolic extract as well as different organic solvent soluble fractions of barks of *Annona reticulata* Linn. The powdered bark of the plant was treated with methanol using cold extraction method and fractionated with solvent-solvent partitioning using organic solvents including n-hexane, chloroform and ethyl acetate. Phytochemical screening revealed the presence of alkaloids, flavonoids, phenolic compounds, diterpenes, carbohydrate, saponins, phenols, tannins and glycosides. The different organic solvent soluble fractions of bark were evaluated at a concentration of 200 mg/kgbw in castor oil induced diarrheal mice model. The aqueous soluble fractions of bark *Annona reticulata* showed highest percentage of inhibition of diarrhea (64.91 ± 1.37%), whereas methanol, n-hexane, chloroform and ethyl acetate soluble fraction showed 26.99 ± 1.79%, 34.85 ± 1.66%, 52.71 ± 1.42% and 45.45 ± 1.54% of diarrheal inhibition, respectively. At the same time, the reference standard Loperamide (5 mg/kg) exhibited 73.21 ± 2.06% inhibition of diarrhea. In GI motility test by charcoal plug method, the 200 mg/kgbw of aqueous soluble fraction showed highest antimotility activity (68.71 ± 3.98%), whereas methanol, n-hexane, chloroform and ethyl acetate soluble fractions showed 66.84 ± 3.38%, 52.01 ± 1.25%, 59.75 ± 3.56% and 54.70 ± 2.12% anti-motility activity, respectively. The standard Loperamide (5mg/kg) revealed 72.41 ± 1.33% inhibition of GI motility, whereas distilled water as control demonstrated 34.06 ± 1.09% of inhibition. This result indicates that the plant extracts have a significant inhibition of GI motility.

Keywords: *Annona reticulata*, Diarrhea, GI motility, Bark extract, Phytochemical.

1. Introduction

Peoples of third world countries are very much prone to some common infectious disease like dysentery, diarrhea due to their unhygienic livelihood, scarcity of pure water, and poor sanitation systems [1]. The World Health Organization (WHO) reported the diarrhea as a second most reason of death of children under age of five [2]. In General, during diarrheal disease, normal bowel movement is changed, which results in increase of water volume in bowel, as well as increase the frequency of stools [3]. There are several reasons of having diarrhea, but common causes are various types of bacterial, viral and parasite infection. The unhygienic food, impure drinking water, poor sanitation system and unhealthy environments are the major causes of such infectious diseases. Besides, several pathological conditions such as increase of luminal osmolarity, electrolyte secretion, decrease of electrolyte absorption, and acceleration of intestinal motility are responsible of causing of diarrhea [4]. The international organization like world health organization (WHO), Centers for Disease Control and Prevention (CDC) are very much aware of control of spread of disease. However, the incidence of diarrhea still high due to lack of awareness of personal hygiene as well as antibiotic resistant developed by diarrhea causing bacterial strain [5,6]. Besides, current therapy with antidiarrheal medicine provides adverse reaction and untoward effects to the patient [7]. Thus, the search for new molecular entity for diarrhea treatment still going on and the medicinal plants are the major sources of them. Plants have long been a very important source of medicinal constituents and many plant species have been screened for the phytochemical compound for using in diarrhea [8]. Due to low cost and least side effects, many international organizations are encouraging to use traditional medicine for the treatment of infectious disease [9,10,11]. Still now, almost 25% of drugs are isolated from plant sources and numerous evidences are available of using the isolated drug in the treatment of disease such as in malaria, diarrhea, dysentery, skin diseases etc [12,13].

Annona reticulata Linn. (Family-Annonaceae, synonym- Bullock's heart, Ramphal, and custard apple) is a traditionally important plant that is used for the treatment of lots of infectious diseases [14,15,16]. There are about 119 different species of Annonaceae has been identified, whereas most of them are shrubs and trees. Various plant part extracts of these families are reported to use in the treatment of diarrhea, dysentery, parasite and worm infection, bacterial infection, dysuria, fever, ulcer, and as insecticides [13,16,17]. The plant extractives of leaves, bark, root, stem bark, seeds are reported to have different pharmacological activities such as antipyretic, anthelmintic, antihyperglycemic, analgesic and anti-inflammatory, antiproliferative, antioxidant, antimicrobial, and wound healing activities (jamkhandi) [18]. However the plant extracts are use in diarrhea and dysentery as traditional medicine, there is no specific report of bark extracts on antidiarrheal effect. For this reason, this study was aimed to evaluate the antidiarrheal activity of different solvent soluble fractions of bark of *Annona reticulata.* Additionally, as the plant extracts of medicinal plants containing alkaloids, flavonoids, tannins, carbohydrates and saponins are reported to exert antidiarrheal activities, the presence of these phytochemical constituents was also evaluated in this study [19].

2. Materials and Methods

2.1. Plant Materials and Extract Preparation

The stem bark of *Annona reticulata* was collected from Noakhali region of Bangladesh on February, 2016 and the plant sample was subjected to National Herbarium, Dhaka for identification. The experience taxonomist identified the plant sample and provided a identification number (accession number: DACB-44872). The collected bark was separated from undesirable materials or plants parts. They were sundried for one week and subjected to grinding to make coarse powder. About 600 gm of powdered material was taken in clean desiccators and soaked in 2300 ml of methanol. The container with its content was kept for a period of 12 days accompanying occasional shaking and stirring. The whole mixture then underwent a coarse filtration by a piece of clean, white cotton and final filtration by Whatman filter paper (Bibby RE200, Sterilin Ltd., UK). The filtrate was evaporated by using rotary evaporator and then kept under ceiling fan for several days. It rendered a gummy concentrate of brownish black color. The gummy concentrate was designated as crude extract of methanol and the extract was kept at 4 °C for further analysis.

2.2. Solvent-Solvent Partitioning

The solvent-solvent partitioning of methanolic crude extract of plant part was performed by modified Kupchan method [20]. The 5 gm of crude methanol extract was triturated in 90 ml of methanol containing 10 ml of distilled water. The crude methanol extract was dissolved completely in the methanol-water solvent system and the solution was taken in a separating funnel having 100 ml of n-hexane. The mixture was shaken, then kept undisturbed and the organic portion was collected. The process was repeated thrice and the n-hexane fractions were collected and evaporated under ceiling fan for seven days. The 12.5 ml of distilled water was added in remaining solution of n-hexane wash and mixed properly. Then the solution was taken in a separating funnel and extracted with chloroform (100 ml × 3). The chloroform fraction was evaporated under fume hood and preserved at 4 °C. The solution that left after washing with n-hexane and chloroform was mixed uniformly with 16 ml of distilled water. Then the solution was taken in a separating funnel and extracted with ethyl acetate for three times (100 ml × 3). The ethyl acetate soluble fraction was evaporated and the remaining fraction was preserved as aqueous fraction.

2.3. Phytochemical Screening

The preliminary phytochemical screening was performed according to studied protocol [21]. Testing of different chemical group such as alkaloid, flavonoid, tannin, terpene, steroid, glycoside, protein, etc present in plant extract was performed with 10 ml of crude methanolic extract with specific reagent. The details of the test procedure, observations and decisions are given in table 1.

2.4. Experimental Animals

The Swiss albino mice of both (male and female) sex weighing 20–30 g and aged 6–8 weeks were purchased from the animal house of the Department of Pharmacy, Jahangirnagar University, Dhaka-Bangladesh. All of the animals were kept in plastic cages at room temperature and on a 12 h light-dark cycle. The animal had free access to standard pet diet (pellet food) and water *ad libitum*. The experiment was done in the Physiology Laboratory of the Department of Pharmacy at Noakhali Science and Technology University. The mice were acclimatized to laboratory environment for 1 week prior to the experiment. Standard pet diet was withdrawn 18 h prior to

the beginning of all the experiments. The care and handling was according to international guidelines for the use and maintenance of experimental animals [22,23].

2.5. Castor Oil-Induced Diarrhea in Mice

The evaluation of anti-diarrheal activities of different solvent soluble fractions of plant extract was performed in castor oil induced diarrheal model. The experimental procedure was performed according to studied protocol with a slide modification [24,25,26]. Mice were randomly divided into control, positive standard and test group each containing six mice. Before starting of any treatment, each mouse was weighed properly and the doses of the test samples and control material (distilled water) were adjusted accordingly. The tail of each mouse was marked by a permanent marker to identify the mouse from each other and marked as M1= mice 1 (having 1 dot on its tail), M2= mice 2 (having 2 dots on its tail), M3 = mice 3 (having 3 dots on its tail), and so on. Each mouse was fed with 1ml of highly pure analytical grade castor oil which would induce diarrhea. The control group received vehicle (plain distilled water) at dose of 10 ml/kgbw (PO). The positive standard group received loperamide at the dose of 5 mg/kgbw orally (PO). The test group received different extractives at the doses of 200 mg/kgbw. Each animal was placed in an individual jar of which the floor surface was covered with absorbent tissue paper. The weight of individual tissue paper was taken before using them. The floor covering was changed at every hour and their weights with feces were taken. After 60 minutes of administration of test samples the mice of all groups were orally treated with 0.5 ml of castor oil. The 60 minutes interval between the administration of test samples and castor oil was given to ensure proper absorption of the administered samples**.** After that, the mice were placed in transparent plastic cages to observe the consistency of fecal matter and frequency was detected in each 5 hours. Wet feces were read at the end of the experiment by lifting the paper placed in the transparent beaker. The percentage of defecation was measured afterwards and percentage of inhibition of defecation was measured.

2.6. Data Collection and Calculation

The total number of defecation for each mouse was noted up to for 5 h and the data was evaluated statistically to find significant value. The observation was performed for each mouse of all groups and the consistency of fecal matter and frequency of defecation was recorded. The percentage of inhibition of defecation was calculated using following formula-

$$\%\ inhibition\ of\ Defecation = \frac{(1 - \mathrm{B})}{\mathrm{A}} \times 100$$

Where ‘A’ indicates mean number of defecation by castor oil, ‘B’ is mean number of defecation by drug extracts.

2.7. Gastrointestinal Motility Assay

Gastrointestinal motility assay was done by charcoal plug method or charcoal induced GI motility test method following reported protocol with slide modification [27,28]. Loperamide was used as standard constipating agent while activated charcoal and methyl cellulose was used as motility inducer. In experimental design, mice were randomly divided into seven groups, each containing six mice. The weight of each mouse was recorded and marked with a permanent marker in their tail. The seven group of mice consists of control, positive standard, and test groups (different extractives and concentration) containing six mice in each group. At first, 1 ml of castor oil was given orally in every mice of each group to produce diarrhea. Control group received vehicle (plain distilled water) at dose 10 ml/kgbw (PO). The positive standard received loperamide at a dose of 5 mg/kgbw (PO). The test group received different extractives at the doses of 200 mg/kgbw. After 1 h of plant extractive dose, all mice received 1mL of charcoal

meal (10% charcoal suspension in 5% gum acacia) orally. After 1 h of charcoal meal administration, all mice were sacrificed and dissect the intestine. The distance travelled by charcoal meal in intestine (from pylorus to caecum) was measured and reported as percentage of distance travelled [29,30].

2.8. Statistical Analysis

The results were presented as mean ± standard error of mean (SEM). The one-way ANOVA test with Dunnett's post hoc test was used to analyze and compare the data using GraphPad Prism ver. 5 (GraphPad Software, San Diego California USA)., while $p < 0.05$–0.001 were considered as statistically significant.

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3. Results and Discussion

3.1. Phytochemical Screening

The phytochemical analysis conducted on methanolic bark extract of *Annona reticulata* Linn. revealed the presence of tannins, flavonoids, saponins, proteins, diterpenes, phenols, cardiac glycosides, carbohydrate and alkaloids. Tannins are known to be useful in the treatment of inflamed or ulcerative tissues and they have noticeable activity in cancer prevention [31,32]. Thus, the bark of *Annona reticulata* containing tannins may serve as a potential source of bioactive compounds in the treatment of cancer. Flavonoids are reported to exhibit membrane permeability activities and inhibit membrane-bound enzymes such as ATPase and phospholipase A2 [33]. This property of plant extracts of *Annona reticulata* may explain the mechanisms of antioxidant activities. Flavonoids also serve as health promoting compound by anionic radicals presence on its [33]. Thus, the flavonoids present in *Annona reticulata* may support the usefulness of this plant in folklore remedies in the treatment of stress-related ailments as well as dressings for wounds, bruises, cuts and sores. Additionally, the plant extract was revealed to contain saponins which produces anti-inflammatory effects and are major ingredients of most of the biological effects [34]. The presence of phenols in plant extract may be useful in the preparation of several antimicrobial compounds such as dettol and cresol [35].

Table 1: Phytochemical screening of crude methanolic extracts of bark of *Annona reticulata*

Phytochemicals	Name of test	Name of reagents	Observation	Result
Alkaloids	i) Mayer's test	i) 2 ml plant extract, 0.2 ml dil HCl, 1.0 ml Mayer's reagent	i) Yellow precipitation	+
	ii) Wagner's test	ii) 2 ml extract,0.2 ml dil HCl, 1 ml iodine solution	ii) Reddish brown precipitation	
	iii) Hager's test	iii) 2 ml plant extract, 0.2 ml dil HCl, 1 ml picric acid solution	iii) Yellow precipitation	
Carbohydrates	Molisch's test	Filtrates of extract, few drops of alcoholic a-naphthol solution, few drops conc. H_2SO_4	Violet ring at the junction was absent	-
Reducing sugar	i) Benedict's test	i) 0.5 ml aqueous extract of plant, 5 ml benedict's solution, boiled 5 min and cooling	i) No red precipitation	-

	ii) Fehling's test	ii) 2 ml aqueous extract of plant, 1 ml (equal mixture of A and B) fehling's solution, boiled few	ii) No red or brick red precipitation	
Cardiac glycoside	Legal's test	2 ml plant extracts, treated with sodium nitropruside in pyridine and sodium hydroxide	Pink or blood red colour.	+
Flavonoid's	i) Alkaline Reagent test	i) 2 ml extract, 4-5 drops of sodium hydroxide, dil. HCl acid	i) Intense yellow color > to colorless	+
	ii) Lead acetate test	ii) 2 ml plant extract, 4-5 drops lead acetate solution	ii) Yellow precipitation	
Saponins	Foam test	1 ml extract solution diluted to 20 ml water, shaken for 15 min	1 cm layer of foam	+
Gums	Molisch's test	5 ml extract solution, molish reagent and sulpheric acid added	Red violet ring at the junction	+
Phytosterol	Libermann-Burchard test	1 ml extract solution, 2 ml Libermann-Burchard reagent	No reddish-purple color	-
Terpenes	Salkowski's test	Plant extract, choloroform>filtrate> few drops of conc.H2SO4> allowed to	No yellow color	+
	Copper acetate test	Plant extract dissolve in water, added 3-4 drops copper acetate solution	Emerald green color	
Phenols	Ferric chloride test	5 ml extract solution, 1 ml 5% $FeCl_3$ solution	Greenish black precipitation	+
Proteins	Xanthoproteic test	Solution of plant extracts, 4-5 drops of conc. nitric acid	Yellow color was absent	-

192 (+) presence, (-) absence of compound

3.2. Plant Extracts Inhibits Castor Oil Induce Diarrhea

The different plant extracts has been reported to analyze the antidiarrheal activities using standard protocol, castor oil induced diarrhea in mice. The acquired results were found to be comparable to that of standard drug loperamide (5 mg/kg body weight) with retardation to the severity of diarrhea [36]. In the present study, the bark extracts of *Annona reticulata* displayed significant activity against castor oil induced diarrhea. Different fraction of bark extracts of plant showed anti-diarrheal activity in which aqueous fraction showed highest anti-diarrheal activity of 64.91 ± 1.37% diarrhea inhibition at 200 mg/kgbw. The crude methanolic extract showed lowest anti diarrheal activity of 26.99 ± 1.79% diarrheal inhibition at the same concentration. At the same time the reference standard loperamide exhibited 73.21 ± 2.06% diarrhea inhibition at concentration of 5 mg/kgbw. On the other hand, HSF, CHSF and EASF showed 34.85 ± 1.66%, 52.71 ± 1.42% and 45.45 ± 1.54% diarrhea inhibition, respectively.

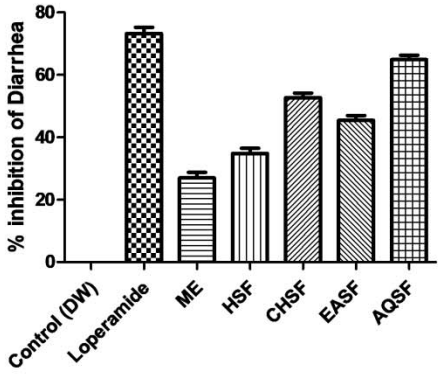

Fig. 1: Antidiarrheal activities of different organic solvent soluble fractions of bark extract of *Annona reticulata.* The Swiss albino mice was treated (PO) with 10 ml/kgbw DW (control), 5 mg/kgbw loperamide and 200 mg/kgbw of various plant extractives (ME = methanol extract, HSF = n-hexane soluble fraction, CHSF = chloroform soluble fraction, EASF = ethyl acetate soluble fraction, and AQSF = aqueous soluble fraction). After 1 hour, castor oil was introduced (0.5 ml) post orally to each mouse and diarrheal activity was evaluated up to 5 hours. The results are expressed in Mean ± SEM.

In the present study, different organic solvent soluble fractions of *Annona reticulata* bark showed significantly reduced amount of feces in castor oil-induced diarrhea on mice. These results suggest that *Annona reticulata* bark contain antidiarrheal components, however the efficacy may vary on extraction procedure by different organic solvent. In the previous report, the phytochemical screening of *Annona reticulata* bark extracts showed the significant presence of phenols and flavonoids [37]. It has been reported that flavonoids and polyphenols were responsible for the antidiarrheal properties [37]. Thus, the significant antidiarrheal activity of the AQSF and CHSF of the bark extracts of *Annona reticulata* could be due to the presence of flavonoids and phenols. Howevr, bioactivity guided isolation of single compound is warranted to evaluate the antidiarrheal activity of those single compound.

3.3. Bark Extracts Showed Significant Inhibition of Gastrointestinal Motility

The effect of plant extracts on GI motility was evaluated by charcoal induced GI motility assay. The presence of charcoal inside the intestine after 30 minutes of feeding proved that the extracts of *Annona reticulata* bark have significent anti-motility activity in comparison with standard drug Loperamide. The percent of inhibition of gastrointestinal motility was found to be highest

in aqueous soluble fraction (68.71 ± 3.98%) followed by methanol (66.84 ± 3.385), chloroform (59.75 ± 3.56), ethyl acetate (54.70 ± 2.12) and n-hexane (52.01 ± 1.25%). Whereas, standard drug Loperamide and distilled water (control) showed 72.41 ± 1.33% and 34.06 ± 1.09% of inhibition of gastrointestinal motility, respectively. Thus, it has been shown that the aqueous soluble fraction possesses higher anti-motility activity compare to other fractions. The anti-motility activity of the extract may be due to the presence of denatured proteins forming protein tannates [38]. The protein tannates makes the mucosa of gastrointestinal tract more resistant and hence reduce secretory diarrhea [39]. This can be due the fact that the bark extract increased the re-absorption of water from the intestinal lumen, decrease intestinal motility in isolated mice ileum [38]. Phytochemical screening revealed the presence of flavonoids, tannins, saponins, cardiac glycosides. Hence, tannins may be responsible for the mechanism of action of reducing effect on GI motility of the selected plant samples [40]. Thus, bioactivity guided isolation can be carried out to separate the bioactive metabolites from the plant.

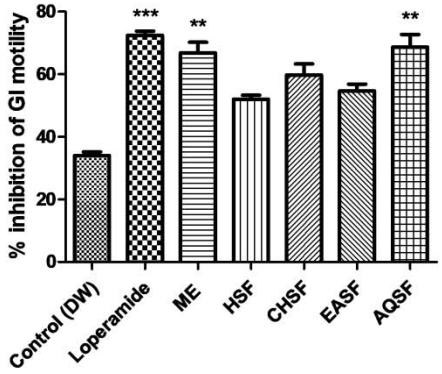

Fig. 2. Inhibition of gastrointestinal motility of different organic solvent soluble fractions of bark extract of *Annona reticulata.* The Swiss albino mice was treated (PO) with 10 ml/kgbw DW (control), 5 mg/kgbw loperamide and 200 mg/kgbw of various plant extractives (ME = methanol extract, HSF = n-hexane soluble fraction, CHSF = chloroform soluble fraction, EASF = ethyl acetate soluble fraction, and AQSF = aqueous soluble fraction). After 1 h, each mouse received 1ml of charcoal meal (10% charcoal suspension in 5% gum acacia) orally. One hour after following the charcoal meal administration, all animals were sacrificed and the distance covered by the charcoal meal in the intestine, from pylorus to caecum was measured and expressed as percentage of distance moved. The results are expressed in Mean ± SEM and *P < 0.05, **P < 0.01, P*** < 0.001; significant difference compared to the control.

4. Conclusion

On the basis of the findings of the present study it can be concluded that the methanolic extracts of bark of *Annona reticulata* Linn. as well as various fractions possess antidiarrheal and anti-GI motility activities. From the in *vivo* test on mice, it has been showed that the extracts possess antidiarrheal activity and significant reduction of GI motility. Finally, this study suggested the isolation of single compound and to evalaute the antidiarrheal and antimotility activities on biological model.

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