Original Research Article

Ascertainment of in vivo antidiarrheal and in vitro thrombolytic effect of ethanolic extract of leaves of *Amomum dealbatum*

ABSTRACT

Aims: The present study aimed to investigate antidiarrheal and thrombolytic effect of ethanolic extract of leaves of *A. dealbatum* in mice.

Study design: Antidiarrheal effect was evaluated by castor oil-induced diarrhea method at two different concentrations in mice and in vitro thrombolytic activity was analyzed with clot lysis assay of human blood.

Place and duration of study: Department of Pharmacy, International Islamic University Chittagong, Kumira, Chittagong-4318, Bangladesh, between December 2018 and February 2019.

Methodology: The male Swiss mice's were divided into four groups (n = 5). First group were orally treated with 1% Tween-80 (10 ml/kg) and second group was orally treated with loperamide (5 mg/kg). Third and fourth group were orally treated with ethanolic extract of leaves of A. dealbatum at 200 and 400 mg/kg accordingly. Human RBCs were collected for conducting thrombolytic assay. During this study, 1.5 ml of venous blood was drawn from healthy volunteers (n = 10) and Streptokinase was employed as positive control and distilled water was employed as negative control.

Results: In castor oil induced diarrhea model, ethanolic extract of leaves of A. dealbatum at 200, 400 mg/kg and loperamide (5 mg/kg) significantly reduced the number of feces and increase percent of inhibition of defecations compared to negative control. The extract showed percent of inhibition of defecation of 16.67 and 37.50 for 200 and 400 mg/ml respectively where the positive control loperamide showed 66.67%. Percentage of clot disruptions were 4.51 (p<.001), 75.69 (p<.001) and 26.07 (p<.001) for water, streptokinase and 10 mg/ml extract respectively.

Conclusion: Based on the results from in vivo and in vitro activities, the leaves of *A. dealbatum* were found to be a potential source of new antidiarrheal and thrombolytic agents.

Keywords: *Amomum dealbatum*, anti-diarrheal, castor oil, thrombolytic, clot disruptions.

1 INTRODUCTION

Plants are known to be the source of many chemical compounds were used by people of ancient cultures without knowledge of their active ingredients. World Health Organization (WHO) has provided a definition of medicinal plants, that is "A medicinal plant is any plant which, in one or more of its organs, contains substances that can be used for therapeutic purposes or which are precursors for

synthesis of useful drug" [1]. In the Plant Kingdom, medicinal plants form the largest single grouping of plants. It is estimated that 30,000 species worldwide fall in this group, of which around 33% are trees. In last few years, there has been great focus on the possible health benefits of natural substances with antidiarrheal, thrombolytic, antioxidant, antimicrobial, analgesic, antipyretic, sedative, antidepressant, antipsychotic, anticancer, anti-diabetic and others activities [2]. Therefore, it is necessary to establish scientific evidences for therapeutic use of such traditional medicinal plants. Zingiberaceae**,** the ginger family of flowering plants, the largest family of the order Zingiberales, containing 52 genera with a total of about 1600 known species [3]. The family is chiefly distributed throughout tropical and subtropical regions of Africa, Asia, China, Nepal, India, Thailand, Indonesia, Malaysia, Singapore, Brunei, Philippines, Papua New Guinea and the Americas [4]. *Amomum dealbatum* known locally as "Alachengay" is a robust perennial herb, which belongs to a family called Zingiberaceae. This plant is widely found in Bangladesh, Assam, China South-Central, East Himalaya, Laos, Indonesia, Myanmar, Nepal, Thailand, Vietnam [5]. In Bangladesh they distributed in forests and shady places of Chittagong, Chittagong Hill Tracts and Sylhet [6]. Diarrhea is characterized by the passage of abnormally liquid or watery fecal matter associated with increased frequency of defecation (three or more times in a day) and abdominal pain [7, 8]. It is the world's third highest killer disease and about 70% people are affected by diarrhea [9, 10]. The conditions of diarrhoea are particularly dangerous in infants and young children because of the rapidity with which serious dehydration occur [11]. This diseases account for one in nine child deaths worldwide and around 760,000 children death every year [12]. So, many works have been carried out in order to discover new antidiarrheal compounds from natural sources for their diverse pharmacological and biological properties [13]. Thrombosis is a lethal disease which is characterized by the formation of blood clots (thrombus) in the circulatory system because of the imbalance of homeostatic system of physiological procedures [14]. This is connected with acute coronary disorders such as pulmonary emboli, deep vein thrombosis, strokes, heart attacks, and venous thromboembolic disorders that account for sudden morbidity and mortality [15]. Thrombosis leads to vascular blockade and while recovering it causes fatal consequences, such as cerebral or myocardial infarction and even death. Thrombolytic agents including tissue plasminogen activator (t-PA), alteplase, anistreplase, urokinase (UK), and streptokinase and recombinant t-PA therapies have been used as effective treatment for thrombolysis. UK and SK are widely used in India, Bangladesh and other developing countries due to lower cost [16] as compared to other thrombolytic drugs but the use is associated with high risk of anaphylactic reaction, systemic fibrinolysis, hemorrhage, slow reperfusion rate and frequent early reclusions and lacks specificity [17]. Moreover, these drugs are not used in patients who have undergone surgery or those with a history of nervous lesions, gastrointestinal bleeding or hypertension [18]. For that reason, alternatives options as traditional and herbal drugs are highly necessitated and numbers of plants have already been reported to show very emerging and potential thrombolytic agents. This study deals with the pharmacological actions namely antidiarrheal and thrombolytic effects of a newer source of indigenous medicinal plant *Amomum dealbatum*.

2 Materials and Methods

2.1 Drugs and chemicals

All chemicals and reagents used in this study were of analytical grade. Ethanol (Merck, Germany) was used as a solvent during extraction. Standard streptokinase were purchased from Popular Pharmaceuticals Limited, Bangladesh. Loperamide (Square Pharmaceuticals Limited), castor oil (WELL's Heath Care, Spain) and Tween 80 (HiMedia Laboratories Pvt. Limited, Mumbai, India) were also used in this research.

2.2 Plant materials

Amomum dealbatum was collected from kaptai shitapahar, Chittagong, Bangladesh on end of December 2017 and was identified by National Herbarium Institute, Mirpur, Dhaka, Bangladesh.

2.3 Extraction

After collection of whole plants of *A. dealbatum* was thoroughly washed with water. Then the selected plant part (leaves) was dried and powdered. About 520 g of the powdered materials of plant was taken separately in a clean, flat bottomed glass container and soaked in 2500 ml of ethanol at room temperature for two weeks accompanying occasional shaking and stirring. Then the solution was filtered using filter cloth and Whatman filter paper (Bibby RE200, Sterilin Ltd., UK) and concentrated with a rotary evaporator (RE-EV311-V, LabTeck S.R.L, Italy). It rendered a gummy concentrate of deep green color. The gummy concentrate was designated as crude ethanolic extract.

2.4 Experimental animals

All animal procedures and experimental protocols were approved by the Research Ethics Committee of the institution and were carried out in accordance with the Guide for the Care and use of Laboratory Animals [19]. Swiss albino mice, weighing about 25–30 gram, were collected from Jahangir Nagar University, Savar, Bangladesh. The animals were provided with standard laboratory food and distilled water ad libitum and maintained at natural day-night cycle having proper ventilation in the room. All the experiments were conducted in an isolated and noiseless condition. The study protocol was approved by the P&D Committee, Department of Pharmacy, International Islamic University Chittagong, Bangladesh (Pharm-P&D-37/07'12). The animals were acclimatized to laboratory condition for 10 days prior to experimentation.

2.5 Effect on Castor oil induced diarrhea

The male Swiss mice's were divided into four groups (n = 5). First group were orally treated with 1% Tween-80 (10 ml/kg) and second group was orally treated with loperamide (5 mg/kg). Third and fourth group were orally treated with ethanolic extract of leaves of *A. dealbatum* at 200 and 400 mg/kg accordingly. Castor oil (0.5 ml/animal) was administered after 60 minutes. Immediately after administering castor oil, each animal was kept in an individual cage with a floor lined with blotting paper. The characteristic diarrheal droppings (wet & dry feces) were noted and observed for 4 hours study for each mouse. 100% was considered as the total number of feces of control group [20]. At the beginning of each hour old papers were replaced with the new ones. Percentage of inhibition of defecation was calculated relative to the control using the following relationship-

$$\text{Inhibition of defecation (\%)} = \frac{A-B}{A} \times 100$$

Where, A is mean number of defecation feces of the control group and B is mean number of defecation caused by standard or plant extracts.

2.6 Thrombolytic activity

The thrombolytic activity of plant extracts was evaluated by the method developed by Prasad et al. [21] with modification to use streptokinase as standard[17, 22].

2.6.1 Red blood cells (RBC) collection

Human RBCs were collected for conducting thrombolytic assay. Male volunteers- weighing average 65 and free from diseases were selected to collect RBCs (using a protocol approved by Institutional Ethics Committee).

2.6.2 Specimen

100 mg *A. dealbatum* ethanolic extract was suspended in 10 ml distilled water and the suspension was shaken vigorously on a vortex mixer. The suspension was kept overnight and decanted to remove the soluble supernatant, which was filtered through a 0.22-micron syringe filter. 100 µl of the plant extract was added to the eppendorf tube which contained the clots to check thrombolytic activity [21, 22]. Streptokinase was employed as positive control and distilled water was employed as negative control.

2.6.3 Thrombolytic assay

During this study, 1.5 ml of venous blood was drawn from healthy volunteers (n = 10) and transferred to three different pre-weighed sterilized eppendorf tube (0.5 ml/tube). The eppendorf tubes were incubated at 37 ºC for 45 minutes. After formation of a clot, serum was completely discarded from the tubes (carried out without disturbing the clot formed). Each eppendorf tube was weighed to determine weight of the clot. Each eppendorf tube was appropriately labeled and 100 µl of the plant extract (10 mg/ml) was added to the tubes. 100 µl of streptokinase and 100 µl of water were distinctly added to the control tubes numbered. The tubes were incubated again at 37 ºC for 90 minutes and observed for clot lysis. After the following incubation, the obtained fluid was discarded from the tubes. They were again weighed to observe the weight of released clot [21, 22]. Every test samples were examined in triplicate. Finally, the result was expressed as percentage of clot lysis which is calculated by the following equation-

$$\text{\% of clot lysis} = \frac{\text{Weight of released clot}}{\text{Clot weight}} \times 100\%$$

2.7 Statistical analysis

The data from antidiarrheal and thrombolytic assay were expressed as Mean ± Standard Error Mean (SEM) and analyzed by one-way analysis of variance (ANOVA) followed by Dunnett 't' test using SPSS software of 20 version. $p < 0.05$ was considered statistically significant.

3 RESULTS

3.1 Effect on castor oil- induced diarrhea

We evaluated the effect of ethanolic extract of *A. dealbatum* leaves on castor oil induced diarrhea. The trend in number of feces was also observed for control (14.40, $p<.01$), standard (4.80, $p<.01$), extracts 200 mg/kg (12.00, $p=.05$) and 400 mg/kg (9.00, $p<.01$) of plant sample (Table 1). When calculating percentage of inhibition of defecation , it was observed that the inhibition of defecation (%) in the dose of 200 mg/kg and 400 mg/kg are 16.67% and 37.50% respectively while standard loperamide (5 mg/kg) showed 66.67% (Figure 1).

Table 1: Effects of ethanolic extract of leaves of *A. dealbatum* on diarrhea induced by castor oil in mice.

Groups	Dose	No. of feces	% of inhibition of defecation
Control	10 ml/kg	14.40±0.87##	
Standard	5 mg/kg	4.80±0.20**	66.67
EE	200 mg/kg	12.00±1.38#	16.67
	400 mg/kg	9.00±0.55**,##	37.50

Here, EE stands for ethanolic extract and Data are presented as mean ± S.E.M. ANOVA was employed, followed by Dunnett's test and significant differences were represented by *$p=.05$, **$p<.01$, ***$p<.001$ vs control group treated with . Tween 80 was employed as negative control and loperamide was employed as standard. $^{\#}p=.05$, $^{\#\#}p<0.01$ and $^{\#\#\#}p<.001$ in relation to the loperamide.

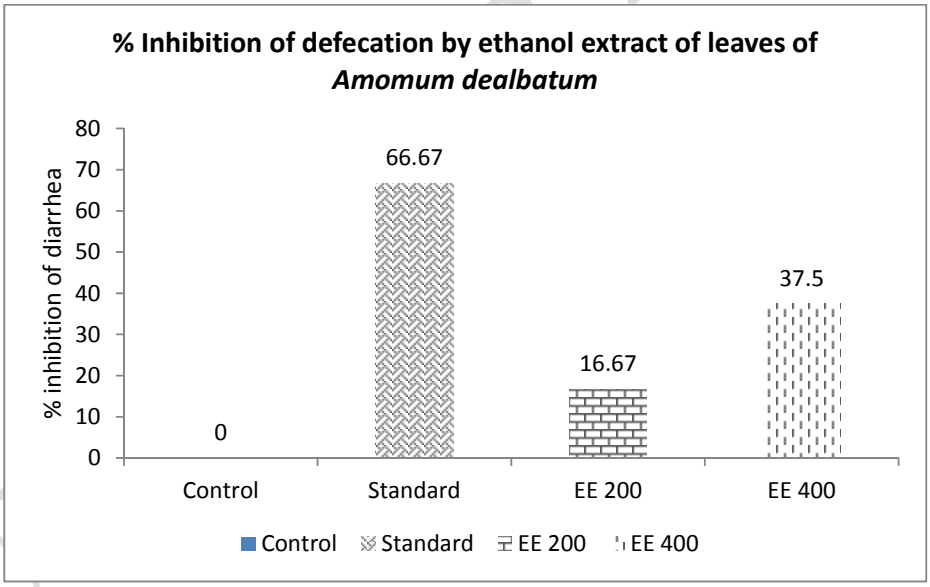

Figure 1: Effect of ethanolic extract of leaves of *A. dealbatum* (200 mg/kg and 400 mg/kg) with positive and negative control on % inhibition of defecation.

3.2 Thrombolytic activity

The effect of ethanolic extract of leaves of *A. dealbatum* on in-vitro clot lysis are showed in Table 2. It is evident that percentage of clot lysis was 75.69% ($p<.001$) when 100 µl of streptokinase (1,50,000 I.U.) was used as a positive control, while in the case of water (negative control) the percentage of clot lysis was negligible (4.51%, $p<.001$) and the extract (10 mg/kg) showed moderate potentiality (26.07%, $p<.001$) compared with streptokinase.

Table 2: Effects of ethanolic extracts of leaves of *A. delbatum* leaves on in-vitro clot lysis.

Treatment		% of clot lysis for human blood
Control		4.51 ± 0.02$^{###}$
Streptokinase	100 µl	75.69 ± 0.54***
EE	10 mg/ml	26.07 ± 0.28$^{***,###}$

Here, EE stands for ethanolic extract and data was presented as mean ± SEM. ANOVA was employed, followed by Dunnett's test and significant differences were represented by *p=.05, **p<.01, ***p<.001 vs control group treated with vehicle. Distilled water was employed as negative control and streptokinase was employed as positive control. $^{\#}p$=.05, $^{\#\#}p$<.01 and $^{\#\#\#}p$<.001 in relation to the Streptokinase.

4 DISCUSSION

Diarrhea can be described as the abnormally frequent defecation of feces of low consistency which may be due to a disturbance in the transport of water and electrolytes in the intestines. Instead of the multiplicity of etiologies, (i) increased electrolytes secretion (secretory diarrhea), (ii) increased luminal osmolarity (osmotic diarrhea), (iii) deranged intestinal motility causing a decreased transit time, and (iv) decreased electrolytes absorption may be responsible for pathophysiology [23, 24]. Recent study claims that nitric oxide and ricinoleic acid produces diarrhea through a hypersecretory response which is the most active component of castor oil [24, 25]. There are several mechanisms proposed to explain the diarrheal effect of castor oil including inhibition of intestinal Na+ K+ ATPase activity, consequently reducing normal fluid absorption [26, 27], activation of adenylate cyclase or mucosal cAMP-mediated active secretion [28] and stimulation of prostaglandin formation and platelet activating factor [29]. Usually castor oil is metabolized into ricinoleic acid in the gut, which causes irritation and inflammation in the intestinal mucosa, resulting in the release of inflammatory mediators (e.g., prostaglandins and histamine). The released prostaglandins initiate vasodilatation, smooth muscle contraction, and mucus secretion in the small intestines. In experimental animals as well as in human beings, prostaglandins of the E series are considered to be good diarrheagenic agents. Our study showed that the overall antidiarrheal study reveals the dose dependent activity. All mice from the control group (treated with vehicle) produced diarrhea after castor oil administration. The decrease in the severity of the diarrhea was measured by the percent of inhibition of defecation. In our study, ethanolic extracts of *A. dealbatum* leaves showed moderately reduced amount of feces in castor oil-induced mice and % inhibition of defecation was 16.67 and 37.50 at 200 and 400mg/kg respectively. The values were increased as the dose had been increased and showed significant antidiarrheal effect compared with positive control-loperamide. From these results, it can be predicted that reduction of water and electrolytes secretion into the small intestine may enhance electrolyte absorption from the intestinal lumen consistent with inhibition of hypersecretion [30]. Besides different pathophysiological conditions of diarrhea, hypermotility characterizes diarrhea where the secretory component is not the causative factor [31]. It was reported that flavonoids and polyphenols were

responsible for the antidiarrheal activity properties [32]. However, studies also have shown that flavonoids have ability to inhibit intestinal motility and water and electrolytes secretion [33]. Moreover, Thereby, flavonoids as the inhibitors of prostaglandins biosynthesis are considered to delay castor oil-induced diarrhea [34]. So, the antidiarrheal activity of the ethanolic extract of the leaves of *A. dealbatum* could therefore be due to the presence of flavonoids and phenols. The result was in concord with other species of same family. Thrombosis or blood clot formation is a critical event in which the damaged regions of the endothelial cell surface or blood vessel are blocked by the deposition of platelets, tissue factor and fibrin [35]. In the formation process the major role is played by platelets as the process of thrombosis is initiated when the activated platelets form platelets to platelets bonds. These activated platelets further bind to the leucocytes and bring them into a complex process of plaque formation and growth [36]. It is the thrombolytic agents that lyse clot by disrupting the fibrinogen and fibrin contained in a clot. Plasmin is one of the natural anti-thrombotic agents. After a long process of trial and error the scientists have discovered several thrombolytic drugs from various sources. Under this study, we tried to find whether the herbal preparations of *A. dealbatum* leaves possess clot lysis potentiality or not. The percent clot lytic activity was compared with water (negative control) and standard enzyme streptokinase (positive control). The mean % of clot lysis for water and streptokinase was found 4.51% (p<.001) and 75.69% % (p<.001) separately. Then again the mean percent clot lytic activity of extracts (10 mg/ml) was found 26.07% (p<.001) which is give moderate effect compare with the positive and negative control. The result agrees with previous reports where extract of *Amomum subulatum* exhibited fibrinolytic effect [37]. It was reported that phytochemicals like saponin, alkaloids and tannin are responsible for thrombolytic activity [38]. Therefore the possibility of the presence of these phytochemicals in the leaves extract may be the probable reason of demonstrating the thrombolytic activity.

5 CONCLUSION

To the best of our knowledge, this is the first report about evaluation of *in vivo* antidiarrheal and in vitro thrombolytic activity of ethanolic extract of leaves of *A. dealbatum.* These findings suggest that the plant may be a potential source for the development of new antidiarrheal drug. Also the obtained results confirmed the presence of thrombolytic element in the leaves of *A. dealbatum*. However, further investigations are required to isolate the active constituents responsible for the observed effect and to elucidate the possible mechanisms of action responsible for the anti-diarrheal and thrombolytic activities of this plant.

AVAILABILITY OF DATA AND MATERIALS

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

COMPETING INTERESRS

The authors declare they have no competing interests.

CONSENT FOE PUBLICATION

Not applicable

ETHICAL APPROVAL

The study protocol was approved by the ethical Committee, Department of Pharmacy, International Islamic University Chittagong, Bangladesh (Pharm-P&D-37/07'12).

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The study has not been published before or is not under consideration for publication elsewhere. its publication is permitted by all authors and after accepted for publication it will not be submitted for publication anywhere.

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