

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

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6
7 **ABSTRACT**

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

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

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

15 **Methodology:** The male Swiss mice's were divided into four groups (n = 5). First group was orally
16 treated with 1% Tween-80 (10 ml/kg) and second group was orally treated with loperamide (5 mg/kg).

17 Third and fourth group were orally treated with ethanolic extract of leaves of *A. dealbatum* at 200 and
18 400 mg/kg accordingly. Human RBCs were collected for conducting thrombolytic assay. During this
19 study, 1.5 ml of venous blood was drawn from healthy volunteers (n = 10) and Streptokinase was
20 employed as positive control and distilled water was employed as negative control.

21 **Results:** In castor oil induced diarrhea model, ethanolic extract of leaves of *A. dealbatum* at 200, 400
22 mg/kg and loperamide (5 mg/kg) significantly reduced the number of feces and increase percent of
23 inhibition of defecations compared to negative control. The extract showed percent of inhibition of
24 defecation of 16.67 and 37.50 for 200 and 400 mg/ml respectively where the positive control
25 loperamide showed 66.67%. Percentage of clot disruptions were 4.51 ($p < .001$), 75.69 ($p < .001$) and
26 26.07 ($p < .001$) for water, streptokinase and 10 mg/ml extract respectively.

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

29
30 **Keywords:** *Amomum dealbatum*, anti-diarrheal, castor oil, thrombolytic, clot disruptions.

31
32 **1 INTRODUCTION**

33
34 Plants are known to be the source of many chemical compounds was used by people of ancient
35 cultures without knowledge of their active ingredients. World Health Organization (WHO) has provided
36 a definition of medicinal plants, that is "A medicinal plant is any plant which, in one or more of its
37 organs, contains substances that can be used for therapeutic purposes or which are precursors for

38 synthesis of useful drug" [1]. In the Plant Kingdom, medicinal plants form the largest single grouping
39 of plants. It is estimated that 30,000 species worldwide fall in this group, of which around 33% are
40 trees [2]. In last few years, there has been great focus on the possible health benefits of natural
41 substances with antidiarrheal, thrombolytic, antioxidant, antimicrobial, analgesic, antipyretic, sedative,
42 antidepressant, antipsychotic, anticancer, anti-diabetic and others activities [3]. Therefore, it is
43 necessary to establish scientific evidences for therapeutic use of such traditional medicinal plants.
44 Zingiberaceae, the ginger family of flowering plants, the largest family of the order Zingiberales,
45 containing 52 genera with a total of about 1600 known species [4]. The family is chiefly distributed
46 throughout tropical and subtropical regions of Africa, Asia, China, Nepal, India, Thailand, Indonesia,
47 Malaysia, Singapore, Brunei, Philippines, Papua New Guinea and the Americas [5]. *Amomum*
48 *dealbatum* known locally as "Alachengay" which belongs to a family called Zingiberaceae. This plant
49 is a robust perennial herb, growing up to 3 meters tall with a thick rhizome. Leaves oblong-lanceolate,
50 pubescent beneath. Spikes oblong, peduncle as long as the spike. Corolla-tube cylindric; segments
51 obtuse, half as long as the tube; lip deflexed, ligulate, red-yellow. Fruit ovoid, strongly ribbed [6]. *A.*
52 *dealbatum* is widely found in Bangladesh, Assam, China South-Central, East Himalaya, Laos,
53 Indonesia, Myanmar, Nepal, Thailand, Vietnam [6]. In Bangladesh they distributed in forests and
54 shady places of Chittagong, Chittagong Hill Tracts and Sylhet [7]. Diarrhea is characterized by the
55 passage of abnormally liquid or watery fecal matter associated with increased frequency of defecation
56 (three or more times in a day) and abdominal pain [8, 9]. It is the world's third highest killer disease
57 and about 70% people are affected by diarrhea [10, 11]. The conditions of diarrhea are particularly
58 dangerous in infants and young children because of the rapidity with which serious dehydration occur
59 [12]. This diseases account for one in nine child deaths worldwide and around 760,000 children death
60 every year [13]. So, many works have been carried out in order to discover new antidiarrheal
61 compounds from natural sources for their diverse pharmacological and biological properties [14].
62 Thrombosis is a lethal disease which is characterized by the formation of blood clots (thrombus) in the
63 circulatory system because of the imbalance of homeostatic system of physiological procedures [15].
64 This is connected with acute coronary disorders such as pulmonary emboli, deep vein thrombosis,
65 strokes, heart attacks, and venous thromboembolic disorders that account for sudden morbidity and
66 mortality [16]. Thrombosis leads to vascular blockade and while recovering it causes fatal
67 consequences, such as cerebral or myocardial infarction and even death. Thrombolytic agents
68 including tissue plasminogen activator (t-PA), alteplase, anistreplase, urokinase (UK), and
69 streptokinase and recombinant t-PA therapies have been used as effective treatment for
70 thrombolysis. UK and SK are widely used in India, Bangladesh and other developing countries due to
71 lower cost [17] as compared to other thrombolytic drugs but the use is associated with high risk of
72 anaphylactic reaction, systemic fibrinolysis, hemorrhage, slow reperfusion rate and frequent early
73 reclosures and lacks specificity [18]. Moreover, these drugs are not used in patients who have
74 undergone surgery or those with a history of nervous lesions, gastrointestinal bleeding or
75 hypertension [19]. For that reason, alternatives options as traditional and herbal drugs are highly
76 necessitated and numbers of plants have already been reported to show very emerging and potential

77 thrombolytic agents. This study deals with the pharmacological actions namely antidiarrheal and
78 thrombolytic effects of a newer source of indigenous medicinal plant *Amomum dealbatum*.

79

80 **2 Materials and Methods**

81

82 **2.1 Drugs and chemicals**

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

88 **2.2 Plant materials**

89 *Amomum dealbatum* was collected from kaptai shitapahar, Chittagong, Bangladesh on end of
90 December 2017 and was identified by National Herbarium Institute, Mirpur, Dhaka, Bangladesh
91 (Accession number: DACB-43725).

92 **2.3 Extraction**

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

100 **2.4 Experimental animals**

101 All animal procedures and experimental protocols were approved by the Research Ethics Committee
102 of the institution and were carried out in accordance with the Guide for the Care and use of
103 Laboratory Animals [20]. Swiss albino mice, weighing about 25–30 gram, were collected from
104 Jahangirnagar University, Savar, Bangladesh. The animals were provided with standard laboratory
105 food and distilled water ad libitum and maintained at natural day-night cycle having proper ventilation
106 in the room. All the experiments were conducted in an isolated and noiseless condition. The study
107 protocol was approved by the P&D Committee, Department of Pharmacy, International Islamic
108 University Chittagong, Bangladesh (Pharm-P&D-37/07'12). The animals were acclimatized to
109 laboratory condition for 10 days prior to experimentation.

110 **2.5 Effect on Castor oil induced diarrhea**

111 Castor oil induced diarrhea method described by Franca 2008 [21] was followed for this study. Four
112 groups of five mice were selected for the final experiment. Group I received 1% Tween-80 (10 ml/kg),
113 second group received loperamide (5 mg/kg) and other groups received ethanol extract 200 and 400
114 mg/kg accordingly. Castor oil (0.5 ml/animal) was administered after 60 minutes. Immediately after
115 administering castor oil, each animal was kept in an individual cage with a floor lined with blotting

116 paper. The characteristic diarrheal droppings (wet & dry feces) were noted and observed for 4 hours
117 study for each mouse. 100% was considered as the total number of feces of control group [22]. At the
118 beginning of each hour old papers were replaced with the new ones. Percentage of inhibition of
119 defecation was calculated relative to the control using the following relationship-

$$120 \text{ Inhibition of defecation (\%)} = \frac{A-B}{A} \times 100$$

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

123 **2.6 Thrombolytic activity**

124 The thrombolytic activity of plant extracts was evaluated by the method developed by Prasad et al.
125 [23] with modification to use streptokinase as standard[18, 24].

126 **2.6.1 Red blood cells (RBC) collection**

127 Human RBCs were collected for conducting thrombolytic assay. Male volunteers- weighing average
128 65 and free from diseases were selected to collect RBCs (using a protocol approved by Institutional
129 Ethics Committee).

130 **2.6.2 Specimen**

131 100 mg *A. dealbatum* ethanolic extract was suspended in 10 ml distilled water and the suspension
132 was shaken vigorously on a vortex mixer. The suspension was kept overnight and decanted to
133 remove the soluble supernatant, which was filtered through a 0.22-micron syringe filter. 100 µl of the
134 plant extract was added to the eppendorf tube which contained the clots to check thrombolytic activity
135 [23, 24]. Streptokinase was employed as positive control and distilled water was employed as
136 negative control.

137 **2.6.3 Thrombolytic assay**

138 During this study, 1.5 ml of venous blood was drawn from healthy volunteers (n = 10) and transferred
139 to three different pre-weighed sterilized Eppendorf Tube (0.5 ml/tube). The Eppendorf Tubes were
140 incubated at 37 °C for 45 minutes. After formation of a clot, serum was completely discarded from the
141 tubes (carried out without disturbing the clot formed). Each Eppendorf Tube was weighed to
142 determine weight of the clot. Each Eppendorf Tube was appropriately labeled and 100 µl of the plant
143 extract (10 mg/ml) was added to the tubes. 100 µl of streptokinase and 100 µl of water were distinctly
144 added to the control tubes numbered. The tubes were incubated again at 37 °C for 90 minutes and
145 observed for clot lysis. After the following incubation, the obtained fluid was discarded from the tubes.
146 They were again weighed to observe the weight of released clot [23, 24]. Every test samples were
147 examined in triplicate. Finally, the result was expressed as percentage of clot lysis which is calculated
148 by the following equation-

$$149 \text{ \% of clot lysis} = \frac{\text{Weight of released clot}}{\text{Clot weight}} \times 100\%$$

150 **2.7 Statistical analysis**

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

154

155 3 RESULTS

156

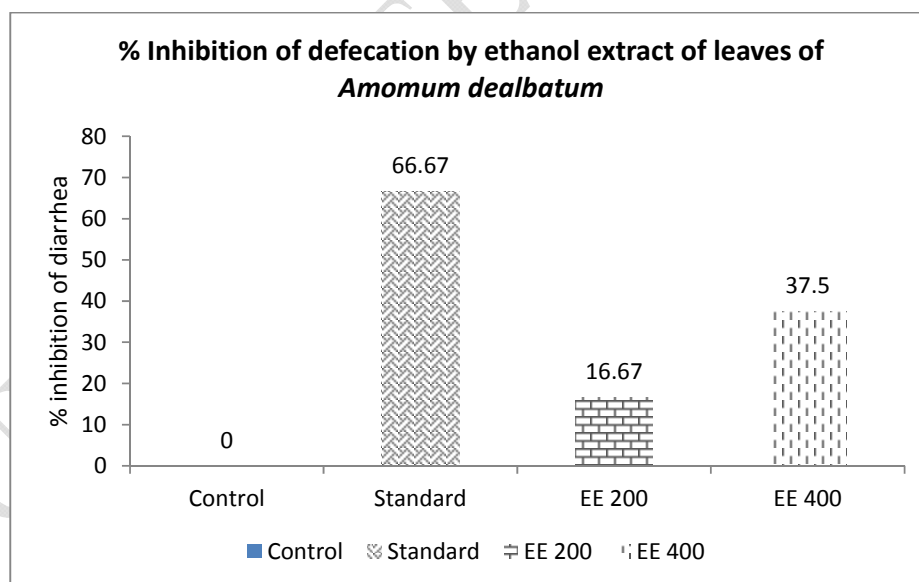
157 3.1 Effect on castor oil- induced diarrhea

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

164 **Table 1: Effects of ethanolic extract of leaves of *A. dealbatum* on diarrhea induced by castor**
165 **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

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



170

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

173 3.2 Thrombolytic activity

174 The effects of ethanolic extract of leaves of *A. dealbatum* on in-vitro clot lysis are showed in Table 2. It
175 is evident that percentage of clot lysis was 75.69% ($p<.001$) when 100 µl of streptokinase (1,50,000

176 I.U.) was used as a positive control, while in the case of water (negative control) the percentage of
 177 clot lysis was negligible (4.51%, $p < .001$) and the extract (10 mg/kg) showed moderate potentiality
 178 (26.07%, $p < .001$) compared with streptokinase.

179 **Table 2: Effects of ethanolic extracts of leaves of *A. dealbatum* 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 ^{***,###}

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

185

186 4 DISCUSSION

187

188 Abnormally frequent defecation of feces of low consistency which may be due to a disturbance in the
 189 transport of water and electrolytes in the intestines are called diarrhea. Instead of the multiplicity of
 190 etiologies, (i) increased electrolytes secretion (secretory diarrhea), (ii) increased luminal osmolarity
 191 (osmotic diarrhea), (iii) deranged intestinal motility causing a decreased transit time, and (iv)
 192 decreased electrolytes absorption may be responsible for pathophysiology [25, 26]. Nitric oxide and
 193 ricinoleic acid is the most active component of castor oil which is responsible for diarrhea [26, 27].
 194 Inhibition of intestinal Na⁺ K⁺ ATPase activity, consequently reducing normal fluid absorption,
 195 activation of adenylate cyclase or mucosal cAMP-mediated active secretion [28] and stimulation of
 196 prostaglandin formation and platelet activating factor [29] are several proposed mechanisms to
 197 expound the castor oil induced diarrheal effect [30, 31]. Inflammatory mediators (e.g., prostaglandins
 198 and histamine) are secreted due to irritation and inflammation in the intestinal mucosa in the presence
 199 of ricinoleic acid in the gut. The released prostaglandins commence vasodilatation, smooth muscle
 200 contraction, and mucus secretion in the small intestines. In experimental animals as well as in human
 201 beings, prostaglandins of the E series are envisaged to be strong diarrheagenic agents. Our study
 202 showed that the overall antidiarrheal study reveals the dose dependent activity. All mice from the
 203 control group (treated with vehicle) produced diarrhea after castor oil administration. The decrease in
 204 the severity of the diarrhea was measured by the percent of inhibition of defecation. In our study,
 205 ethanolic extracts of *A. dealbatum* leaves showed moderately reduced amount of feces in castor oil-
 206 induced mice and % inhibition of defecation was 16.67 and 37.50 at 200 and 400mg/kg respectively.
 207 The values were increased as the dose had been increased and showed significant antidiarrheal
 208 effect compared with positive control-loperamide. From these results, it can be enumerated that water
 209 and electrolytes secretion into the small intestine are reduced and may ameliorate electrolyte
 210 absorption from the intestinal lumen consistent with inhibition of hypersecretion [32]. Besides different

211 pathophysiological conditions of diarrhea, hypermotility characterizes diarrhea where the secretory
212 component is not the causative factor [33]. It was reported that some phytochemical compounds like
213 flavonoids and polyphenols were responsible for the antidiarrheal activity properties [34]. However,
214 studies also have shown that flavonoids have ability to inhibit intestinal motility, water and electrolytes
215 secretion [35] and inhibit prostaglandins biosynthesis which are considered to delay castor oil-induced
216 diarrhea [36]. So, the antidiarrheal activity of the ethanolic extract of the leaves of *A. dealbatum* could
217 therefore be due to the presence of flavonoids and phenols. The result was in concord with other
218 species of same family. The damaged regions of the endothelial cell surface or blood vessel are
219 blocked by the deposition of platelets, tissue factor and fibrin is called thrombosis or blood clot
220 formation [37]. In the formation process platelets played the major role and thrombosis is initiated
221 when the activated platelets form platelets to platelets bonds and further bind to the leucocytes and
222 bring them into a complex process of plaque formation and growth [38]. It is the thrombolytic agents
223 which working by disrupting the fibrinogen and fibrin contained in a clot. Plasmin is one of the natural
224 anti-thrombotic agents. After a long process of trial and error several thrombolytic drugs are
225 discovered from various sources. Under this study, we tried to find whether the herbal preparation of
226 *A. dealbatum* leaves possess clot lysis potentiality or not. The percent clot lytic activity was compared
227 with water (negative control) and standard enzyme streptokinase (positive control). The mean % of
228 clot lysis for water and streptokinase was found 4.51% ($p<.001$) and 75.69% ($p<.001$) separately.
229 10 mg/ml extracts of *A. dealbatum* leaves was give 26.07% ($p<.001$) clot lytic activity which is
230 moderate effect compare with the positive and negative control. The result agrees with previous
231 reports where extract of *Amomum subulatum* exhibited fibrinolytic effect [39]. It was narrated that
232 some phytochemicals like saponin, alkaloids and tannin are endangered for thrombolytic activity [40].
233 Therefore the possibility of the presence of these phytochemicals in the leaves extract may be the
234 probable reason of demonstrating the thrombolytic activity.

235

236 5 CONCLUSION

237

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

245

246

247 AVAILABILITY OF DATA AND MATERIALS

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

250 **COMPETING INTERESRS**

251 The authors declare they have no competing interests.

252

253

254 **CONSENT FOE PUBLICATION**

255 Not applicable

256

257 **ETHICAL APPROVAL**

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

260

261 **COPYRIGHT AND LICENSE**

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

265

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