

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 were 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 were 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. 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 [2]. 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 [3]. 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 [4]. *Amomum*
48 *dealbatum* known locally as "Alachengay" is a robust perennial herb, which belongs to a family called
49 Zingiberaceae. This plant is widely found in Bangladesh, Assam, China South-Central, East
50 Himalaya, Laos, Indonesia, Myanmar, Nepal, Thailand, Vietnam [5]. In Bangladesh they distributed in
51 forests and shady places of Chittagong, Chittagong Hill Tracts and Sylhet [6]. Diarrhea is
52 characterized by the passage of abnormally liquid or watery fecal matter associated with increased
53 frequency of defecation (three or more times in a day) and abdominal pain [7, 8]. It is the world's third
54 highest killer disease and about 70% people are affected by diarrhea [9, 10]. The conditions of
55 diarrhoea are particularly dangerous in infants and young children because of the rapidity with which
56 serious dehydration occur [11]. This diseases account for one in nine child deaths worldwide and
57 around 760,000 children death every year [12]. So, many works have been carried out in order to
58 discover new antidiarrheal compounds from natural sources for their diverse pharmacological and
59 biological properties [13]. Thrombosis is a lethal disease which is characterized by the formation of
60 blood clots (thrombus) in the circulatory system because of the imbalance of homeostatic system of
61 physiological procedures [14]. This is connected with acute coronary disorders such as pulmonary
62 emboli, deep vein thrombosis, strokes, heart attacks, and venous thromboembolic disorders that
63 account for sudden morbidity and mortality [15]. Thrombosis leads to vascular blockade and while
64 recovering it causes fatal consequences, such as cerebral or myocardial infarction and even death.
65 Thrombolytic agents including tissue plasminogen activator (t-PA), alteplase, anistreplase, urokinase
66 (UK), and streptokinase and recombinant t-PA therapies have been used as effective treatment for
67 thrombolysis. UK and SK are widely used in India, Bangladesh and other developing countries due to
68 lower cost [16] as compared to other thrombolytic drugs but the use is associated with high risk of
69 anaphylactic reaction, systemic fibrinolysis, hemorrhage, slow reperfusion rate and frequent early
70 recusions and lacks specificity [17]. Moreover, these drugs are not used in patients who have
71 undergone surgery or those with a history of nervous lesions, gastrointestinal bleeding or
72 hypertension [18]. For that reason, alternatives options as traditional and herbal drugs are highly
73 necessitated and numbers of plants have already been reported to show very emerging and potential
74 thrombolytic agents. This study deals with the pharmacological actions namely antidiarrheal and
75 thrombolytic effects of a newer source of indigenous medicinal plant *Amomum dealbatum*.

76

77 **2 Materials and Methods**

78

79 **2.1 Drugs and chemicals**

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

85 **2.2 Plant materials**

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

88 **2.3 Extraction**

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

96 **2.4 Experimental animals**

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

106 **2.5 Effect on Castor oil induced diarrhea**

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

116
$$\text{Inhibition of defecation (\%)} = \frac{A-B}{A} \times 100$$

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

119 **2.6 Thrombolytic activity**

120 The thrombolytic activity of plant extracts was evaluated by the method developed by Prasad et al.
121 [21] with modification to use streptokinase as standard[17, 22].

122 **2.6.1 Red blood cells (RBC) collection**

123 Human RBCs were collected for conducting thrombolytic assay. Male volunteers- weighing average
124 65 and free from diseases were selected to collect RBCs (using a protocol approved by Institutional
125 Ethics Committee).

126 **2.6.2 Specimen**

127 100 mg *A. dealbatum* ethanolic extract was suspended in 10 ml distilled water and the suspension
128 was shaken vigorously on a vortex mixer. The suspension was kept overnight and decanted to
129 remove the soluble supernatant, which was filtered through a 0.22-micron syringe filter. 100 µl of the
130 plant extract was added to the eppendorf tube which contained the clots to check thrombolytic activity
131 [21, 22]. Streptokinase was employed as positive control and distilled water was employed as
132 negative control.

133 **2.6.3 Thrombolytic assay**

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

145
$$\% \text{ of clot lysis} = \frac{\text{Weight of released clot}}{\text{Clot weight}} \times 100\%$$

146 **2.7 Statistical analysis**

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

150

151 **3 RESULTS**

152

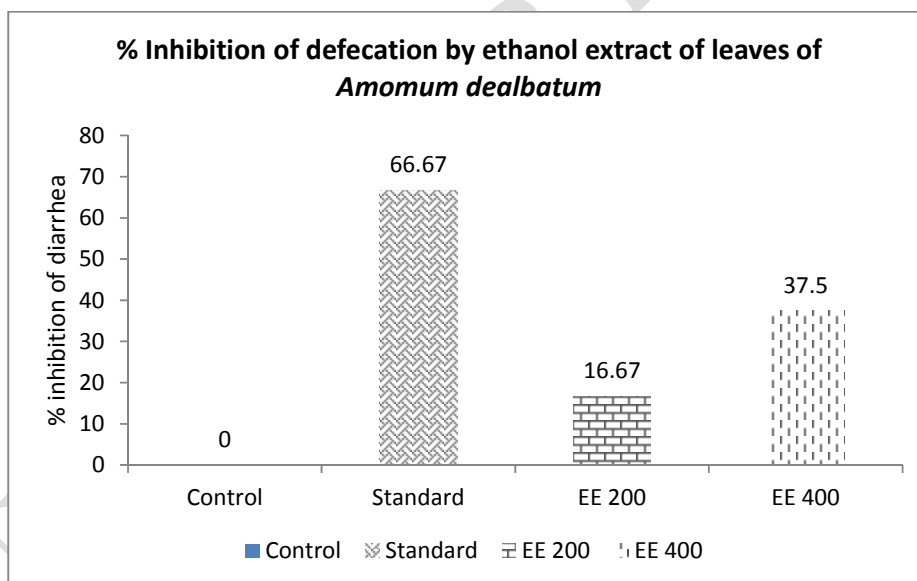
153 **3.1 Effect on castor oil- induced diarrhea**

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

160 **Table 1: Effects of ethanolic extract of leaves of *A. dealbatum* on diarrhea induced by castor**
 161 **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

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



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

169 **3.2 Thrombolytic activity**

170 The effect of ethanolic extract of leaves of *A. dealbatum* on in-vitro clot lysis are showed in Table 2. It
 171 is evident that percentage of clot lysis was 75.69% ($p < .001$) when 100 µl of streptokinase (1,50,000
 172 I.U.) was used as a positive control, while in the case of water (negative control) the percentage of
 173 clot lysis was negligible (4.51%, $p < .001$) and the extract (10 mg/kg) showed moderate potentiality
 174 (26.07%, $p < .001$) compared with streptokinase.

175

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 ^{***,###}

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

181

182 4 DISCUSSION

183

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

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

233

234 **5 CONCLUSION**

235

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

243

244

245 **AVAILABILITY OF DATA AND MATERIALS**

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

248 **COMPETING INTERESRS**

249 The authors declare they have no competing interests.

250

251

252 **CONSENT FOE PUBLICATION**

253 Not applicable

254

255 **ETHICAL APPROVAL**

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

258

259 **COPYRIGHT AND LICENSE**

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

263

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