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MODULATORY ANTI-DIARRHEAL EFFECTS OF ASCORBIC ACID IN *SWISS* ALBINO MICE

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Abstract:
Ascorbic acid (AA) has been reported for the management of diarrhea. The anti-diarrheal potential and modulatory activities of AA on some commonly used anti-diarrheal drugs were investigated. For this purpose, the activities of AA on castor oil-induced diarrhea in *Swiss* mice were examined. As standard anti-diarrheal agents, we used prazosin, propranolol, loperamide, and nifedipine with or without AA. The results revealed that AA at 25 mg/kg (i.p.) and all other standard drugs exhibited significant ($p < 0.05$) diarrheal attenuating activities in mice. However, the impact was more pronounced in the loperamide and propranolol groups. AA administrated with prazosin and propranolol had a higher rate of latent periods and a lower rate of diarrheic secretion during the study period (4 h) than that of the other single or mixed groups. Furthermore, a molecular docking study illustrated that AA displayed good binding affinities with (α1) (–5.2 Kcal/mol), α2b (–5.4 Kcal/mol), α2c (-5.6 Kcal/mol), β1(–5.3 Kcal/mol) and β2(–6.4 Kcal/mol) adrenoceptors. Of note, AA exerted a significant anti-diarrheal effect and it was seen to modulate the anti-diarrheal effects of α- and β-adrenergic receptor blocking agents in *Swiss* mice.

Keywords: Ascorbic acid; Castor oil; Diarrhea; *Mus musculus*; Prazosin

Introduction

The global burden of morbidity and mortality associated with diarrhea remains a major concern all over the world (Guandalini and Vaziri, 2007). Many broad types of diarrhea which depend on severity, source or origin, epidemiological observations, causative organisms, duration of episode and fatality have been made. However, diarrhea has been generally and most acceptably classified into three categories depending on the number of days it lasts. It is termed acute if it lasts up to 14 days, persistent if it lasts between 14 and 29 days, and chronic if it lasts up to 30 days or more (DuPont et al., 2014). While the clinical manifestations of the disease vary in attendant severity and duration, the burden and economic toil it takes on affected individuals can not be overemphasized (Zimmermann et al., 2019). Some clinical manifestations of diarrhea include abdominal cramps, nausea and vomiting, which often make patients live a low-quality sedentary lifestyle (Cavalcanti et al., 2019).

While contaminated food and drinks are the most renowned cause of diarrhea in humans, the effect includes an imbalance in the body electrolytes which results in hyper-secretory feedback and induction of intestinal contraction. It should be noted that many therapeutics act by inhibiting gut motility as well as preventing electrolyte discharge (Shah et al., 2010). Commonly used anti-diarrheal

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agents such as loperamide, nifedipine, prazosin, and propranolol are without some mild or acute side effects such as abdominal discomfort, altered pulsation, nausea, dizziness, stomach disorder, constipation, drowsiness, and tiredness (Guandalini and Vaziri, 2007).

Ascorbic acid (AA), otherwise called vitamin C, is renowned for its antioxidant potential and other therapeutic applications in humans (Knight et al., 2016). In a study, pre-treatment of AA (150 mg/kg/day) has been seen to prevent gastrointestinal damage induced by radiation in mice (Yamamoto et al., 2010). However, a reduced concentration (5 mg/day) of AA has been suggested as a tolerable dose for Charcot-Marie-Tooth type 1A patients (Toth, 2009). An earlier report suggests that AA at one table (1000 mg) three times/day after the third week significantly lowers the number of diarrheic frequency in neonatal calves (Seifi et al., 1996). Due to the multi-therapeutic nature of the AA, with recognized relevance and application in the management of stress-related conditions linked to inflammation of the immune system, it has been used in combination therapy with other therapeutic agents (Sorice et al., 2014).

Opioid receptors have three pharmacological subtypes, μ, δ and ϰ (Connor and Christie, 1999; Dietis et al., 2011). The conventional opioid receptors are found in the vas deferens, knee joint, gastrointestinal, heart, and immune system, among many other places, across the central nervous system and, to a lesser degree, across the periphery (Stein et al., 2003). The opioid receptor is a class of receptor with many pharmacological importances, such as analgesia, anxiety, alleviating depression symptoms (Ide et al., 2010), diarrhea, irritable bowel syndrome (Maltz and Fidler, 2017), increasing gastrointestinal motility and some other biological effects.

In both health and sickness, the adrenergic system is critical for maintaining bodily homeostasis. Adrenergic receptor, belong to G-protein couple receptor family (Pierce et al., 2002; Philipp and Hein, 2004), has two major classes of receptor: α- adrenoceptor (aα1A, α1B, α1D, α2A, α2B,and α2C) and β-adrenoceptors (β_1-, β_2 , and β_3-) (Molinoff, 1984; Philip and Hein, 2004). They are found in the heart, brain, and adipose tissue and regulate several effects, including pain sensation (Diatchenko et al., 2006), vasodilation, decrease blood pressure (Rokosh and Simpson, 2002).), breakdown glycogen, diarrhea (Bricker et al., 2001) etc.

This study evaluates the anti-diarrheal potential of AA in a castor oil-induced diarrheal mouse model. Additionally, the interaction capacity of AA with the commonly used anti-diarrheal agents such as loperamide, prazosin, propranolol, and nifedipine has also been investigated.

Materials and Methods

Animal (in vivo) study

Reagents and Chemicals

Square Pharmaceuticals Ltd provided loperamide (LOP) and prazosin (PRA) while castor oil was acquired from a local market in Bangladesh. ACI Ltd. and Drug International Ltd. Bangladesh graciously contributed propranol (PRO) and nifedipine (NIF), respectively. Merck, India, produced ascorbic acid (AA) and tween 80.

Experimental mice

The animal resource section of Jahangir Nagar University (JU) in Dhaka provided the mature albino mice utilized in the experiments (weighing 22 - 30 g). The mice were housed in sterilized polypropylene cages with sterile rice husk as bedding under conventional climatic conditions (temperature: 25 ± 2 ºC, humidity: 50 ± 5%, and 12 hour light/dark cycles). All the housed mice with unrestricted access to pellets as their basal diet and water *ad libitum* were naturalised for approximately seven days before commencement of study. The mice, experimental and control groups were randomly assigned to have their food withdrawn 12 hours before the experimental hours. The test protocol was approved by the Department of Pharmacy, BSMRSTU, Gopalganj, Bangladesh (Approval number # 20150109004).

Groups and Treatments (Castor Oil-Induced Diarrhea in Mice)

The standard approach, which was based on the Awouters et al (1978) method with minor changes, was used. Briefly, 30 minutes following the sample (Group-II) and control (Group-I & Group-VI) treatments, each mouse was given 0.5 mL of castor oil. Similarly, AA (Group-II) was co-treated for 15 minutes with Group-III to Group-VI (Group-VII to Group-X) (Table 1). The animals (n = 5) were then observed for latency and total defecation for up to 4 hours in each group.

Table 1. Treatment given the overnight-fasted mice

Treatment groups	Dose
Gr-I: VEH (i.p.)	10 mL/kg
Gr-II: AA (i.p.)	25 mg/kg
Gr-III: LOP (p.o.)	3 mg/kg
Gr-IV: PRA (i.p.)	1 mg/kg
Gr-V: PRO (i.p.)	10 mg/kg
Gr-VI: NIF (i.p.)	2.5 mg/kg
Gr-VII: (Gr-II + Gr-III)	AA25+LOP3
Gr-VIII: (Gr-II + Gr-IV)	AA25+PRA1
Gr-IX: (Gr-II + Gr-V)	AA25+PRO10
Gr-X: (Gr-II + Gr-VI)	AA25+NIF2.5

VEH (Vehicle): 0.05% Tween 80 dissolved in 0.9% NaCl solution, i.p.: intra-peritoneal, p.o.: Per oral.

Statistical Analysis

This study's data were submitted for a one-way analysis of variance (ANOVA), and the findings were presented as the mean standard deviation (SD). GraphPadPrism® - GraphPad Software, Inc. (Version: 6.0) was used for statistical analysis, and the Newman-Keuls post hoc test was used; differences at $p < 0.05$ were judged significant at the 95% confidence interval.

Molecular docking study

For the molecular studies, therapeutically relevant proteins which include α1, α2b, α2c, β1 and β2 adrenoceptors were used for the ligand-protein interaction evaluation. Models of proteins were obtained from the protein data bank (PDB) as homology models of the proteins (Table 4) were built on Modeller 9.19. The docking grid of 40 × 40 × 40, 0.8 Å (enclosing the active binding sites) generated on the ProBiS server was used. The ligands, AA, were docked into the predicted binding pocket of selected proteins utilizing Auto dock Vina.

Results

Animal study

In comparison to the VEH group, AA and the standards (LOP, PRA, PRO, NIF) dramatically ($p< 0.05$) enhance latent durations in diarrheal mice. The latency duration was longer in the LOP (24.2 1.6 min) and PRO (21.8 2.4 min) groups than in the AA, PRA, and NIF groups. However, AA when co-administered with the standards resulted PRA group (26.8 3.3 min) led to the greatest increase in latent time, followed by AA + PRO, AA + LOP, and AA + NIF groups, respectively. AA co-treated with the LOP, significantly increased the latency period than the AA group, but remained unchanged compared to the LOP group. Furthermore, AA co-treated with NIF decreased the latency period compared to the individually treated groups, AA and NIF (Table 2).

Table 2. The latent duration of the treatment groups was determined in castor oil-induced diarrheal mice

Therapeutic groups	Dose (route of administration)	Latency (min)
Gr-I: VEH	10 ml/kg (i.p.)	9.2 ± 2.8
Gr-II: AA	25 mg/kg (i.p.)	18.2 ± 1.9*
Gr-III: LOP	3 mg/kg (p.o.)	24.2 ± 1.6$^{*\#}$
Gr-IV: PRA	1 mg/kg (i.p.)	16.8 ± 1.8*
Gr-V: PRO	10 mg/kg (i.p.)	21.8 ± 2.4^{*b}
Gr-VI: NIF	2.5 mg/kg (i.p.)	16.8 ± 3.9*
Gr-VII: (AA + LOP)	25 + 3 mg/kg	24.3 ± 2.8$^{*\#bcd}$
Gr-VIII: (AA + PRA)	25 + 1 mg/kg	26.8 ± 3.3$^{*\#bcd}$
Gr-IX: (AA + PRO)	25 + 10 mg/kg	25.2 ± 2.1$^{*\#bcd}$
Gr-X: (AA + NIF)	25 + 2.5 mg/kg	14.8 ± 1.8*

Values are mean ± SD (n = 5); anova one way followed by newman-keuls *post hoc* test; $p < 0.05$ when compared to the *Gr-I, $^{\#}$Gr-II, ^{a}Gr-III, ^{b}Gr-IV, ^{c}Gr-V, ^{d}Gr-VI in respective hour; VEH: 0.05% Tween-80 dissolved in 0.9% NaCl solution; AA: Ascorbic acid; LOP: Loperamide; PRA: Prazosin; PRO: Propranolol; NIF: Nifedipine

Similarly, findings in Table 3 show that AA at 25 mg/kg lowers the quantity of diarrheal discharges substantially ($p < 0.05$) when compared to the VEH group. On the 4th hour, AA showed the greatest decrease in diarrheal secretions (3.6 ± 2.8); the LOP and PRO groups showed the greatest attenuation in diarrheal secretions. In this case, the activities of AA piqued the PRA group's interest. In addition, in the AA + LOP, AA + PRA, and AA + PRO groups, AA co-treated with the standards substantially decreased diarrheal secretions. In comparison to the LOP group, AA co-administered with PRA and PRO was more successful in decreasing diarrheal secretions. AA co-treated with NIF resulted in augmented diarrheic secretions when compared to the NIF group animals (Table 3).

Table 3. Diarrheal secretions of mice in different treatment groups at 1st, 2nd, 3rd and 4th hours (stool number count)

Treatment groups	1st h	2nd h	3rd h	4th h
GR-I: VEH	15.2 ± 2.3	10.8 ± 2.6	9.8 ± 1.8	7.8 ± 2.8
GR-II: AA	10.0 ± 2.8*	6.8 ± 2.4*	6.4 ± 1.8*	3.6 ± 2.8*
GR-III: LOP	6.2 ± 2.6$^{*\#}$	4.8 ± 2.5$^{*\#}$	3.8 ± 1.3$^{*\#}$	1.8 ± 1.6$^{*\#}$
GR-IV: PRA	8.8 ± 2.4$^{*\#}$	6.6 ± 1.2*	4.2 ± 2.2$^{*\#}$	3.6 ± 2.8*
GR-V: PRO	7.4 ± 3.1$^{*\#}$	5.4 ± 2.2$^{*\#b}$	5.0 ± 1.0$^{*\#}$	4.2 ± 0.8*
GR-VI: NIF	8.2 ± 3.7$^{*\#}$	7.4 ± 1.7*	5.2 ± 2.8$^{*\#}$	2.2 ± 1.8$^{*\#abc}$
GR-VII: (AA + LOP)	6.0 ± 1.2$^{*\#bcd}$	3.0 ± 1.8$^{*\#abcd}$	2.2 ± 1.2$^{*\#bcd}$	1.6 ± 1.4$^{*\#bc}$
GR-VIII: (AA + PRA)	4.6 ± 1.1$^{*\#abcd}$	3.4 ± 1.4$^{*\#bcd}$	1.8 ± 0.8$^{*\#bcd}$	0.4 ± 1.3$^{*\#abcd}$
GR-IX: (AA + PRO)	4.8 ± 2.7$^{*\#c}$	3.4 ± 1.8$^{*\#abcd}$	2.6 ± 1.7$^{*\#bcd}$	1.6 ± 0.9$^{*\#bc}$
GR-X: (AA + NIF)	8.8 ± 1.6$^{*\#}$	7.8 ± 2.9*	5.6 ± 1.9*	3.2 ± 0.8^{*c}

Values are mean ± SD (n = 5); anova one way followed by newman-keuls *post hoc* test; $p < 0.05$ when compared to the *Gr-I, $^{\#}$Gr-II, ^{a}Gr-III, ^{b}Gr-IV, ^{c}Gr-V, ^{d}Gr-VI in respective hour; VEH: 0.05% Tween-80 dissolved in 0.9% NaCl solution; AA: Ascorbic acid; LOP: Loperamide; PRA:Prazosin; PRO: Propranolol; NIF: Nifedipine

In silico study

The results of the molecular docking experiment indicated that AA possessed good to moderate energies towards all the target proteins studied. AA showed the best interaction with (α1) (–5.2 Kcal/mol), α2b (–5.4 Kcal/mol), α2c (-5.6 Kcal/mol), β1 (-5.3 Kcal/mol) and β2 (-6.4 Kcal/mol) (Table 4). The observed ligand-protein interactions at the binding site together with interactions with some amino acid residues are as indicated. Hydrophobic interactions and hydrogen bonding are indicated by the red and green lines respectively. The 2D and 3D structures of non-bond interactions of AA with adrenoceptor subunits are shown in Figure 1.

Table 4. Molecular docking study of AA with alpha and beta adrenoceptors

Target protein	Binding affinity (Kcal/mol)	Interacting amino acids
α1	-5.2	SER90, PRO184, PHE106
α2b	-5.4	GLN68, ASP432
α2c	-5.6	ASN111, TYR427, SER108, CYS202, ASP131
β1	-5.3	ASN329, ASP200
β2	-6.4	SER207, ASN293, TYR316, ASN312, ASP113

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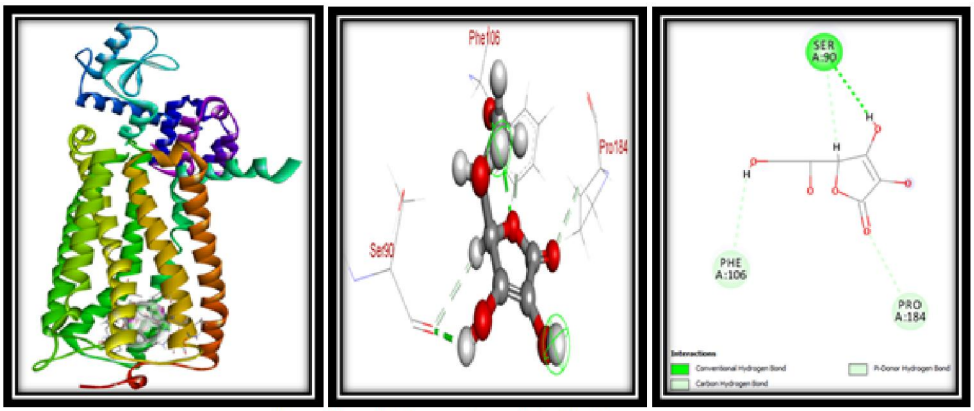
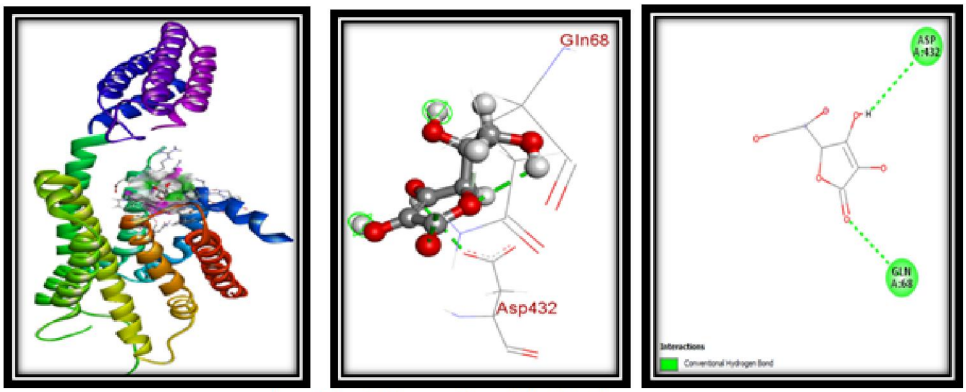
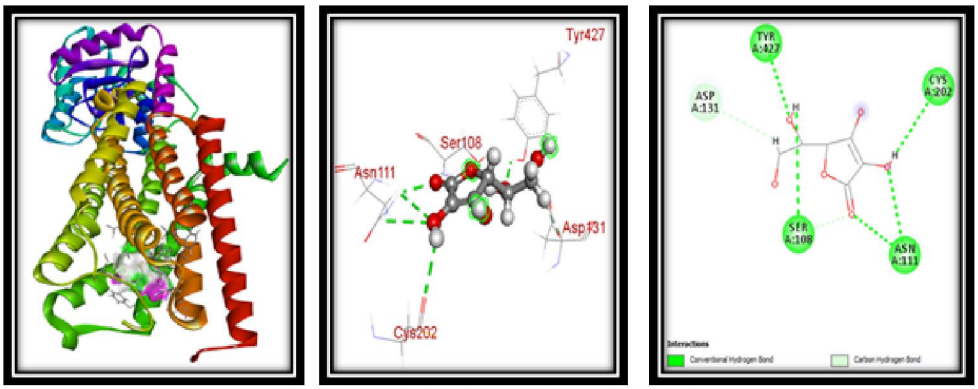


Ascorbic acid interaction with α1 adrenoceptor



Ascorbic acid interaction with α2b adrenoceptor



Ascorbic acid interaction with α2c adrenoceptor

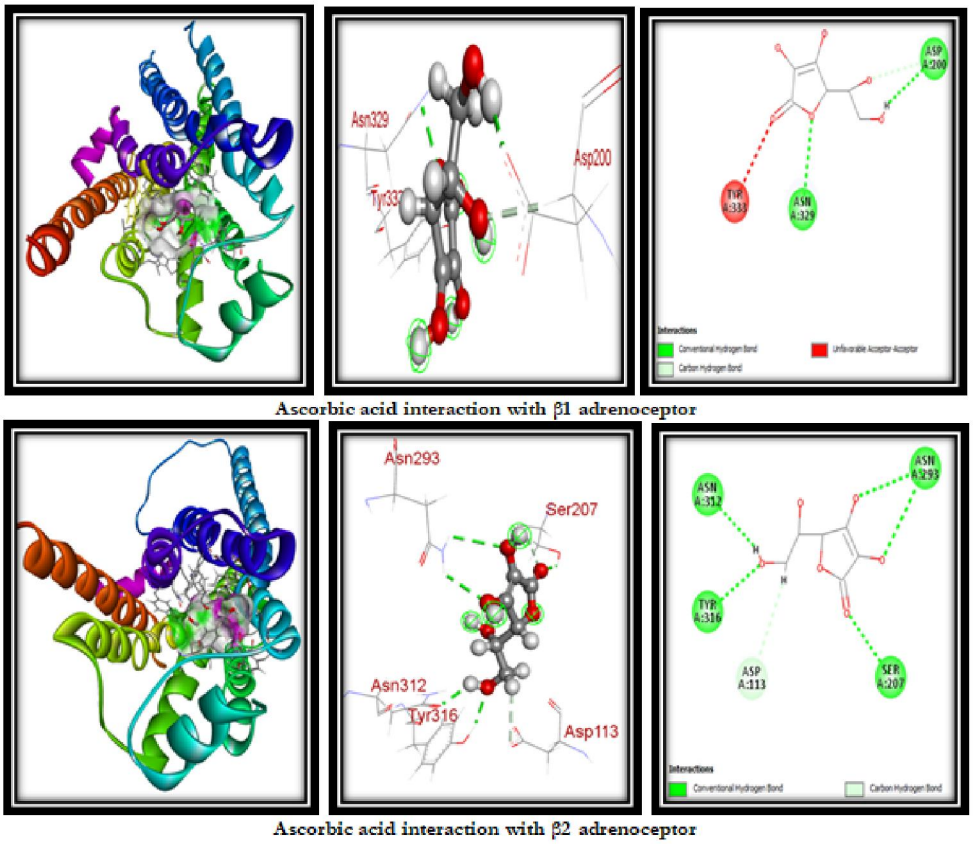


Figure 1. Ascorbic acid interactions with some selected adrenoceptors

Discussion

Diarrhea is one of several intestinal disorders (Chang et al., 1985). Various natural products and synthetic compounds have been reported to possess diarrheal activities. Ricinoleic acid, a primary compound from castor plant seeds and also in the sclerotium of ergot, has been reported to be responsible for the diarrheal effect in animals (Yoshio, 1999). Ricinoleic acid is known to trigger secretion of prostaglandins and platelet-activating factor (Mascolo et al. 1993), thereby inducing mucus release, smooth muscular contraction, and vasodilation in the gastrointestinal track, leading to a condition known as diarrhea (Bello et al. 2016). Other studies have also suggested that ricinoleic acid further stimulates the secretion of nitric oxide (NO) and likewise activates adenylyl cyclase, which causes an increase in cyclic adenosine monophosphate (cAMP) agglomeration. In the intestine, elevation of cAMP congregation reportedly decreases the absorption of sodium and potassium ions, induces peristaltic effect, distorts the membrane viability and reduces Na^+/K^+/ATPase pump activity. The entire process causes electrolyte assembly and H_2O in the gut lumen (Uchida et al. 2000; Rawat et al. 2017). AA is evident to ameliorate ischemia-reperfusion-influenced acute renal damage through a decrease in the renal nitric oxide level in rats (Koul et al. 2015).

Hemingway, (1991) revealed that AA suppressed diarrhea in neonatal calfs (Hemingway, 1991). Another study has demonstrated that AA (100 mg/kg, p.o.) along with *A. melegueta* (500 mg/kg) possess greater protection against diarrhea (Umukoro and Ashorobi, 2005). Several studies have shown that ascorbic acid reduces prostaglandin concentrations, possibly through inhibition of peroxidation of phospholipids (Stickel et al., 1997; Child et al., 1999). Considering the role of prostaglandin in the regulation of intestinal fluid secretion, these antioxidant nutrients may offer beneficial effects on castor oil–induced diarrhea, whereas prostaglandin induces gastrointestinal motility, which probably induced diarrhea in mice models (Umukoro and Ashorobi, 2005).

Candelario-Jalil et al. (2006) claimed that AA drastically suppresses prostaglandin synthesis (Fiebich et al., 2003; Candelario-Jalil et al., 2006). An excessive amount of prostaglandin may induce gastrointestinal motility and induce diarrhea (Robert et al., 1976; Riviere et al., 1991).

Furthermore, the opioid receptor has antidiarrheal properties with its subtypes including supraspinal μ and δ and peripheral μ, δ and k (Shook et al., 1989) and these are involved in GI motility (Maltz and Fidler, 2017). Other studies revealed that AA blocks GI motility in an experimental model in a dose dependent manner (Umukoro and Ashorobi, 2005). In our study, we found that after administration of AA to a mouse group, it reduced the stool count, possibly via interacting with opioid receptors and blocking GI movement, and increased gut transit time.

In this study, we saw that AA and the standards (LOP, PRA, PRO, NIF) drastically ($p < 0.05$) increased the latency in diarrheal mice when compared to the VEH group. In other words, AA when co-administered with the standards, induced a latent period and a reduction in diarrheic secretions, especially in the AA + PRA and AA + PRO groups.

The alpha-1 (α1*)* adrenergic receptor is a G protein-coupled receptor (GPCR) associated with the Gq heterotrimeric G-protein. Study suggests that adrenoceptor blockade is helpful in reducing the time it takes for food to pass through the intestines and alleviating diarrhea (Bricker et al., 2001). PRA is evident to exert its anti-diarrheal effect through blocking alpha-1 (α1*)* adrenergic receptors. The report suggests that AA has an inhibitory effect on this receptor (Wolfman et al. 1983). Moreover, in a recent clinical trial, AA together with the non-specific beta blocker, PRO, were found effective, when administered previously and continually, in the prophylaxis of fibro dysplasia ossificans progressive flare-ups (Palhares et al., 2019). This study establishes the combined potencies of AA and/or PRA/PRO in diarrheal-induced mice.

K, Na, and Ca ion channel blockers are often given medicine for a range of health issues, including angina pectoris, cardiac arrhythmias, and hypertension, among others. Some studies have also shown that using ascorbic acid with its complex in the therapeutic treatment of coronary heart disease to counteract the negative effects of voltage-gated channel blockers is effective (Ivanov et al. 2016). In this study, AA was seen to augment NIF (a calcium channel blocking agent) mediated decreasing diarrheic secretions, while decreasing the latency period in diarrheal mice.

Molecular docking studies suggested that AA exerts good to moderate binding energies with adrenoceptors, which reveals that AA can regulate adrenoceptor functions. It might induce an antidiarrheal effect. After in vivo study results, we can claim that AA has the ability to suppress diarrhea via regulating several mechanistic pathways like opioids, adrenoceptors, and calcium channels in experimental mice models.

Conclusion

In summary, findings from this investigation suggest that AA at 25 mg/kg displayed remarkable diarrheal-preventing potency in castor oil-induced diarrheal mice as it drastically increased the latency time while lowering diarrheic portions. When used with the conventional medicines PRA and PRO, AA had a higher anti-diarrheal effect. AA had no interference with the anti-diarrheal effect of LOP, but it slightly suppressed the NIF-mediated anti-diarrheal effects in experimental animals. Further studies are required to understand the molecular mechanism (s) behind the combined antidiarrheal effect of AA with PRA or PRO in animal models.

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Ethics approval and consent to participate

As appropriate, this project was approved by the authorizing Department of Pharmacy (Approval No. 20150109004), BSMRSTU, Gopalganj-8100, Bangladesh.

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