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Identification and biochemical characterization of anti-enteropooling compounds from *Annona senegalensis* root bark

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ABSTRACT

Background and objectives: *Annona senegalensis* root bark is used in the treatment of diarrhea. This study fractionated *Annona senegalensis* root bark to identify the anti-enteropooling compounds and explore the possible mechanism of action of the compounds
Methods: Anti-enteropooling activity of hexane, dichloromethane and aqueous extracts were investigated to determine the most bioactive crude extract. Bioactivity guided fractionation of the most active extract was conducted. The compounds present in the bioactive sub-fraction were identified using GC-MS analysis. The concentration of Na^+, Cl^- and K^+ in the intestinal fluids of rats administered the most active sub-fraction was determined. The effect of the sub-fraction on the small intestine malondialdehyde (MDA) concentration, antioxidant enzymes, Na^+ - K^+ ATPase and cyclooxygenase II activities were evaluated using standard procedures.
Results: Aqueous root bark extract (AR) significantly decreased the weight and volume of intestinal fluids of castor oil induced diarrheal rats. Sub-fraction 1 of dichloromethane fraction of aqueous root bark extract (DFAR1) decreased the weight and volume of intestinal fluids of castor oil-induced diarrheal rats the most. Androstan-3-one and 3-tetradecen-5-yne were found present in DFAR1. The concentration of Na^+ in the intestinal fluid of rats administered DFAR1 significantly decreased when compared with the control. DFAR1 significantly decreased the activities of superoxide dismutase, catalase and cyclooxygenase II. There was no significant difference in MDA concentration and Na^+ - K^+ ATPase activity.
Conclusion: *A. senegalensis* root bark is rich in aldosterone derivative (steroid) and 3-tetradecen-5-yne that prevents enteropooling by stimulating Na^+ absorption and inhibiting cyclooxygenase activity.

Abbreviations: MDA, malondialdehyde; GPx, glutathione peroxidase; SOD, superoxide dismutase; CAT, catalase; COX II, cyclooxygenase II; DR, dichloromethane root bark extract; HR, hexane root bark extract; AR, aqueous root bark extract; HFAR, hexane fraction of aqueous root bark extract; EFAR, ethylacetate fraction of aqueous root bark extract; DFAR, dichloromethane fraction of aqueous root bark extract; AFAR, aqueous fraction of aqueous root bark extract.

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Introduction

Diarrhea is a major health threat to people in the tropical and sub-tropical countries [1]. Diarrhea occurs when there is an imbalance between absorption and secretion of water and electrolytes in the gastrointestinal tract (GIT) [2]. It is an alteration in the movement of electrolytes and water in the intestine [1]. This alteration or imbalance may be due to hypersecretion, reduced absorption, increased intestinal motility, and an increase in luminal osmolarity. Epithelial water absorption occurs as a result of the intraepithelial osmotic coupling of water absorption to solute flow [3]. The entry of solutes into the epithelial cell creates an osmotic difference that energizes the entry of water [3]. Thus, the rate of colonic salt ions (Na^+ and Cl^-) absorption is directly related to water absorption [4]. Therefore, alteration in the movements of electrolytes usually results in fluid accumulation in the digestive tract leading to an enteropooling process [5]. In secretory diarrhea, there exist a net increase in chloride secretion with a resultant decrease in the absorption of sodium and water resulting in watery stool [6]. One of the mechanisms of action of antidiarrheal agents is to restore electrolyte imbalance by either stimulating the re-absorption of sodium and water (anti-enteropooling) or decreasing chloride secretion (anti-secretion). Some plant extracts are capable of stimulating water re-absorption (anti-enteropooling) and/or reducing electrolyte secretion (anti-secretion) [7].

Annona senegalensis belongs to the *Annonaceae* family (commonly called African custard apple). It is used traditionally for the treatment of diarrhea [8]. The root and stem bark mixture are commonly used in the north eastern part of Nigeria to treat diarrhea [9]. Ahmed *et al* [9]. reported that aqueous root bark extract exhibited better anti-enteropooling activity (re-absorbing water) than the aqueous stem bark. Antidiarrheal activity of medicinal plants have been attributed to the presence of phytochemicals such as flavonoids, tannins, steroids, and alkaloids [10,11]. Steroids, tannins, and alkaloids are present in the root and stem barks of *Annona senegalensis* [12–14] but no study has identified the particular compound in *Annona senegalensis* root bark responsible for the aforementioned property. This study, therefore, fractionated the root bark of *Annona senegalensis* in an attempt to identify the bioactive compound(s) responsible for its anti-enteropooling activity and to explore the mechanism of action of the bioactive compound(s).

Materials and Methods

Collection, preparation, and extraction of Annona sengalensis *root bark*

Fresh root barks of *A. senegalensis* were collected and authenticated at the herbarium unit of the University of Ilorin, Ilorin, Nigeria and was assigned a voucher number UILH/001/449. The root barks were washed clean shredded and air-dried under shade to constant weight. They were pulverized separately using mortar and pestle into powder. The powdered root bark was soaked separately in three solvents; hexane, dichloromethane, and water in the ratio 1:10 for 24 hrs at ambient temperature (35°C). The crude extracts were filtered using Whatman No. 1 filter paper. Each of the filtrates were evaporated to dryness at 40 °C under reduced pressure and the dried substance was stored in an airtight bottle until required [15]. When required, dichloromethane and hexane extracts were reconstituted in 20% DMSO while the aqueous extract was reconstituted in 20% Tween.

Experimental animals

Adult albino rats weighing between 130 – 150 g were obtained from the animal breeding unit, department of Biochemistry, University of Ilorin, Ilorin, Nigeria. They were housed in well-ventilated aluminum cages and given standard laboratory diet and water ad libitum. The rats were handled according to the guidelines for the protection and handling of laboratory animals and approved by the University of Ilorin ethical committee and was given an approval number UERC/ASN/2018/1216.

Enteropooling test

Albino rats were fasted for twelve hours and thereafter, received 1 mL castor oil orally to induce diarrhea. One hour after, the rats were randomly divided into seven groups. Groups I and II were administered 1.0 mL 20% tween and 20% DMSO respectively. Group III was administered 3.0 mg/kg b.wt. loperamide while groups IV-VI were administered 100 mg/kg b.wt. hexane root bark (HR), dichloromethane root bark (DR), and aqueous root bark (AR) respectively. After an hour, the rats were sacrificed by ether anesthesia. The edges of the intestine were tied with thread and the intestine carefully removed and weighed. The intestinal content was collected by milking into a graduated tube and the volume measured. The intestine was reweighed and differences between full and empty intestine calculated [16].

Bioactivity-guided fractionation of the most active extract

Solvent-solvent partitioning of the most active extract

The most active extract was subjected to solvent-solvent partitioning with three solvents; hexane, dichloromethane and ethylacetate by the method described by Kupchan *et al* [17]. and modified by Van-Wagener *et al* [18]. All solvent fractions were subjected to anti-enteropooling test (using 50 mg/kg b.wt solvent fraction) to determine the most active solvent fractions.

Table 1
Anti-enteropooling activity of solvent extracts of *A. senegalensis* root bark in castor oil- induced diarrheal rats.

Group	Volume of intestinal fluid (mL)	Weight of intestinal fluid (mg)
DMSO	3.13 ± 0.24^{d}	2.53 ± 0.14^{b}
Tween	2.03 ± 0.41^{b}	2.50 ± 0.23^{b}
3.0 mg/kg b.wt. Loperamide	2.00 ± 0.23^{f}	2.73 ± 0.19^{e}
100 mg/kg b.wt. DR	3.23 ± 0.03^{e}	3.10 ± 0.26^{c}
100 mg/kg b. wt. HR	2.53 ± 0.15^{c}	3.37 ± 0.27^{d}
100 mg/kg b.wt. AR	1.16 ± 0.10^{a}	1.90 ± 0.16^{a}

*Values are mean of five replicates ± S.E.M. Values with different superscript down the column are significantly different ($p < 0.05$)

Column chromatography of the most bioactive solvent fraction

The most bioactive solvent fraction was subjected to column chromatography. Ethylacetate: butanol: water: acetic acid (50:40:5:5) was used for eluting the solvent fraction. Eluents were collected in 100 mL. The sub-fractions were spotted on a thin layer chromatography (TLC) plates. Sub-fractions with similar retention factor (Rf) on the TLC plate were pooled together. All sub-fractions were evaluated for anti-enteropooling activity at 25 mg/kg b.wt sub-fractions

Identification of the compounds present in the bioactive sub-fraction by Gas chromatography - mass spectrometry (GC-MS) analysis

Identification of the compounds present in the sub-fraction was performed using GC-MS equipment (Thermo Scientific Co), Thermo GC – Trace Ultra version 5.0 Thermo MS DSQII model. Experimental conditions of the GC-MS system were as follows; DB S -MS capillary standard a non-polar column with a dimension of 30 Mts, 10: 0.25 mm and film thickness of 0.25 µm.

Determination of electrolyte concentration in the intestinal fluid of castor oil-induced diarrheal rats administered the bioactive sub-fraction

The rats were fasted for twelve hours. Diarrhea was induced to all rats by administering 1 mL of castor oil orally. One hour later, 25 mg/kg b. wt. of the bioactive sub-fraction reconstituted in water was administered to the treatment group. One hour after, the rats were sacrificed by ether anesthesia. The small intestine of each rat was carefully removed and milked into a sample bottle. The concentration of sodium ion and potassium ion in the intestinal fluid was determined using the method described by Wooten and Freeman [19]. The small intestine was placed in 0.25 M sucrose for biochemical analysis.

Biochemical analysis

The small intestine was homogenized in 0.25M sucrose (1:4) and was used to determine malondialdehyde (MDA) concentration, antioxidant enzyme (catalase, SOD, GPx), Na^{+} - K^{+} ATPase and cyclooxygenase II (COX-II) activity as described by Kunchandy and Rao [20], Aebi [21], Sun and Zigman [22], Flohe and Guenzler [23], Suhail and Rizvi [24] and Guenzle *et al* [25]. respectively.

Statistical analysis

Statistical analysis was computed using SPSS software version 24.0. Data were statistically analyzed with one-way analysis of variance (ANOVA) and Duncan multiple range test. For all the tests, results with $p < 0.05$ were taken to imply statistical significance.

Results

Anti-enteropooling test

The result of the anti-enteropooling activity of solvent extracts of *A. senegalensis* root bark is shown in Table 1. Aqueous root bark extract (AR) significantly decreased ($p < 0.05$) the volume and weight of intestinal fluid when compared to the negative control and the loperamide group.

Table 2
Anti-enteropooling activity of solvent fractions from aqueous root bark extract (AR) of *A. senegalensis* in castor oil-induced diarrheal rats.

Group	Weight of int. fluid (mg)	Volume of intestinal fluid (mL)
Control	1.25 ± 0.34^{b}	1.95 ± 0.06^{b}
3 mg/kg b.wt. Loperamide	1.77 ± 0.07^{b}	1.93 ± 0.18^{b}
50 mg/ kg b.wt. HFAR	1.00 ± 0.09^{b}	1.84 ± 0.15^{b}
50 mg/kg b.wt. EFAR	2.07 ± 0.08^{c}	3.05 ± 0.23^{c}
50 mg/kg b.wt DFAR	0.84 ± 0.07^{a}	1.03 ± 0.03^{a}
50 mg/kg b.wt AFAR	1.32 ± 0.11^{b}	1.38 ± 0.12^{b}

*Values are mean of five replicates ± S.E.M. Values with different superscript down the column are significantly different ($p < 0.05$)

Table 3
Retention factor (R_f) of sub-fractions from dichloromethane fraction of aqueous root bark extract (DFAR) of *A. senegalensis.*

Sub-fraction	R_f
DFAR1	0.61
DFAR2	0.62
DFAR3	0.63
DFAR4	0.65
DFAR5	0.75
DFAR6	0.70
DFAR7	0.71
DFAR8	0.71
DFAR9	0.72
DFAR10	0.40

Table 4
Anti-enteropooling activity of sub-fractions from dichloromethane fraction of aqueous root bark extract (DFAR) of *A. senegalensis* in castor oil-induced diarrhoeal rats

Fractions	Weight of intestinal fluid (mg)	Volume of intestinal Fluid (mL)	Descriptive feature
Control	7.74 ± 0.38^{c}	5.00 ± 0.12^{c}	Liquid
25 mg/kg b.wt DFAR1	2.47 ± 0.12^{a}	2.60 ± 0.13^{a}	Solid
25 mg/kg b.wt DFAR2	2.47 ± 0.17^{a}	2.37 ± 0.18^{a}	Liquid
25 mg/kg b.wt DFAR3	3.30 ± 0.14^{b}	3.07 ± 0.26^{b}	Liquid

*Values are mean of five replicates ± S.E.M. Values with different superscript down the column are significantly different ($p < 0.05$)

Bioactivity guided fractionation of most active crude extract

The anti-enteropooling activity of solvent fractions from the aqueous root bark extract of *A. senegalensis* in castor oil-induced diarrheal rats is presented in Table 2. The dichloromethane partitioned fraction of the aqueous root bark extract of *A. senegalensis* (DFAR) significantly decreased ($p < 0.05$) the weight and volume of the intestinal fluid when compared to the negative control and loperamide.

Table 3 shows the R_f of each of the 10 sub-fractions obtained from column chromatography of DFAR. Sub-fractions 1 to 4 had R_f values ranging between 0.61 - 0.62; 5 to 9 had R_f values ranging between 0.70 to 0.72 while 10 had R_f value of 0.40. Based on the similarities in R_f, they sub-fractions were pooled into three sub-fractions.

Table 4 shows the anti-enteropooling activity of sub-fractions from dichloromethane fraction of aqueous root bark (DFAR) of *A. senegalensis* in castor oil-induced diarrheal rats. Sub-fractions 1, 2 and 3 (DFAR1, DFAR2 and DFAR3) significantly decreased ($p < 0.005$) both the weight and volume of intestinal fluid. The highest anti-enteropooling activity was exhibited by sub-fractions 1 and 2, but the intestinal fluid of sub-fraction 1 (DFAR1) was solid while sub-fraction 2 was liquid. Sub-fraction 1 was thus chosen to be the most bioactive sub-fraction.

Identification of the compounds in the bioactive sub-fraction from A. senegalensis *root bark*

Figure 1 shows the GC-MS chromatogram of sub-fraction 1 of the dichloromethane fraction of aqueous root bark extract (DFAR1) of *A. senegalensis* Table 5. shows the identified compounds in DFAR1. Four peaks were obtained from GC-MS analysis of DFAR1 and the compounds were identified from the retention time (RT) obtained as shown in Table 5. Androstan-3-one, 17 hydroxy-2-methyl (2β, 5β, 17β), (a steroid) is the major compound in the sub-fraction with 51.98 peak area. Other compounds present in the sub-fraction includes 3-tetradecen-5-yne and pyrazolo (3, 4, b) pyridine-3-one.

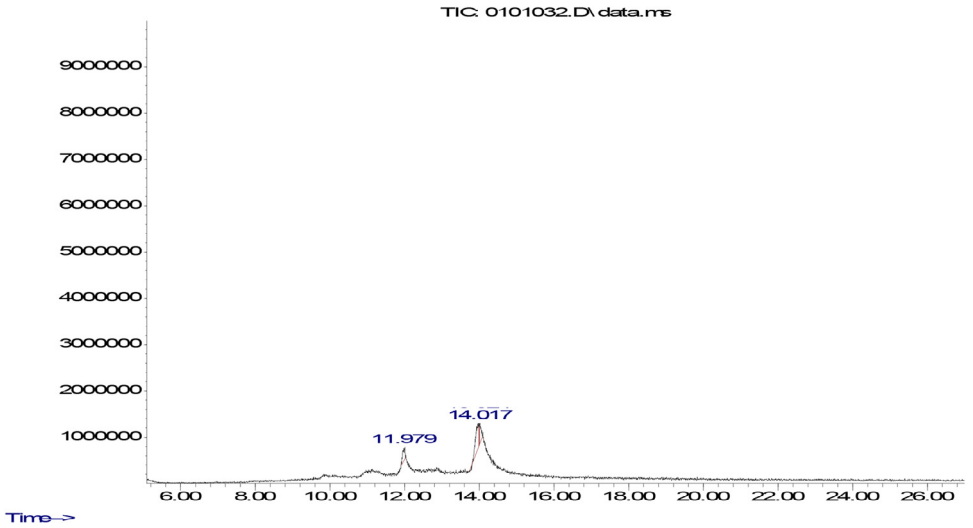

Figure 1. GC-MS chromatogram of sub-fraction 1 of dichloromethane fraction from aqueous root bark extract (DFAR1) of *Annona senegalensis*

Table 5
Chemical compounds present in sub-fraction 1 of dichloromethane fraction from aqueous root bark extract (DFAR1) of *Annona senegalensis*

S/N	Compound name	RT(min)	% peak area	Class of compound
1	3-Tetradecen-5-yne	11.98	19.36	Essential oil
2	Androstan-3-one, 17 hydroxy-2-methy, (2β,5β, 17β)	13.973	51.98	Steroid
3	Butanimide, N(3 methylphenyl) 2,2,3,3,4,4,4,heptafluoro-	13.996	9.52	
4	Pyrazolo(3,4,b)pyridine-3-one	14.017	19.14	Nitrgen containing heterocyclic.

Table 6
Electrolyte concentration of intestinal fluids of castor oil-induced diarrheal rats treated with DFAR1.

	Control	DFAR1
Na^+ (mmol/L)	55.73 ± 0.15^b	52.26 ± 0.09^a
K^+ (mmol/L)	1.64 ± 0.05^a	1.69 ± 0.09^a
Cl^- (mmol/L)	22.30 ± 0.11^a	28.31 ± 0.15^b

Mechanism of anti-enteropooling action of DFAR1

Electrolyte concentration in the intestinal fluid of castor oil-induced diarrheal rats treated with bioactive sub-fractions from A. senegalensis *root bark*

Table 6 shows the electrolyte concentration in the intestinal fluids of castor oil induced diarrheal rats after administration of the anti-enteropooling sub-fraction obtained from *Annona senegalensis* root bark. The concentration of K^+ in the intestinal fluid was not significantly different ($p > 0.05$) from the control. Administration of DFAR1 significantly decreased ($p < 0.05$) concentration of Na^+ in the intestinal fluid but significantly increased the concentration of Cl^- when compared with the diarrheal control.

Values are mean of five replicates ± S.E.M. Values with different superscript along the row are significantly different ($p < 0.05$).

Effect of administration of DFAR1 on malondialdehyde concentration and activities of antioxidant enzymes, Na^+- K^+ ATPase and cyclooxygenase II

Table 7 shows the effect of administration of DFAR1 on MDA, antioxidant enzymes, Na^+-K^+ ATPase and cyclooxygenases II. There was no significant difference ($p > 0.05$) in small intestine MDA concentration of rats administered DFAR1 and the control. The small intestine SOD and GPx activities of rats administered DFAR1 significantly decreased ($p < 0.05$) from 5.59 ± 0.68 to 4.01 ± 0.34 and 8.01 ± 0.31 to 6.90 ± 0.17 respectively. The activity of the small intestine catalase significantly increased ($p < 0.05$) when compared with the control. There was no significant difference ($p < 0.05$) in the small intestine activity of Na^+-K^+ ATPase activity of rats administered DFAR1 and the control group. The activity of COX-II significantly decreased ($p < 0.05$) when compared with the control.

Table 7
Small intestine malondialdehyde concentration, antioxidant enzymes, Na+- K+ ATPase and COX-II activities.

	Control	DFAR1
MDA (mg/dL)	12.47 ± 0.11[a]	11.83 ± 1.03[a]
Catalase (U/L)	2.28 ± 0.19[a]	3.00 ± 0.26[b]
GPx (U/L)	5.54 ± 0.68[b]	4.01 ± 0.34[a]
SOD (U/L)	8.01 ± 0.31[b]	6.90 ± 0.17[a]
Na^+- K^+ ATPase	6.10 ± 1.01[a]	5.22 ± 0.78[a]
COX II	11.23 ± 0.85[b']	6.78 ± 0. 23[a]

Values are mean of five replicates ± S.E.M. Values with different superscript along the row are significantly different ($p < 0.05$).

Discussion

The significant decrease in the weight and volume of intestinal fluid by aqueous root bark (AR) extract indicates the anti-enteropooling activity of this extract. This indicates that the extract enhanced proper water and electrolyte re-absorption. A substance that promotes water reabsorption in the small intestine has antidiarrheal potential [26]. The significant reduction in weight, volume, and liquidity of the intestinal fluid of rats administered sub-fraction 1 of dichloromethane fraction of aqueous root bark extract (DFAR1) indicates that anti-enteropooling compounds are present in the sub-fractions.

The main compounds in sub-fraction DFAR1; androstan-3-one, is an aldosterone derivative. Aldosterone (a steroid) stimulates electrogenic Na^+ absorption in the distal colon via the electrogenic sodium channel (ENaC) [26]. ENaC is the rate-limiting factor for electrogenic Na^+ absorption in the descending colon [27]. It is downregulated in inflammatory bowel disease (IBD) [28]. Also, activation of cystic fibrosis transmembrane conductance regulator (CFTR) that occurs in secretory diarrhea inhibits Na^+ absorption via ENaC [27]. Thus, the decrease in intestinal fluid Na^+ concentration by DFAR1 indicates that androstan-3-one stimulated ENaC to enhance Na^+ absorption. The reabsorption of Na^+ via the ENaC channel plays an important role in re-absorption of water [29,30]. Water flows in the direction of Na^+ flow by osmosis. Therefore, the stimulation of Na^+ absorption is responsible for the anti-enteropooling activity of the sub-fraction. It, therefore, implies that the major compound in DFAR1, androstan-3-one, 17 hydroxy-2-methy, (2β,5β, 17β), an aldosterone derivative, is responsible for the anti-enteropooling activity of the sub-fraction. Drug stimulation of ENaC channel is a good target for diarrhea treatment because of its distal location in the GI tract in the intestine in which highly efficient Na^+ absorption occurs [27]. Therefore, the aldosterone derivatives present in DFAR1 will be a good anti-diarrheal drug candidate via its anti-enteropooling activity.

Malondialdehyde is an index of lipid peroxidation. Castor oil-induced diarrhea increases the formation of malondialdehyde in the gastrointestinal mucosa due to oxidative stress indicating an increase in lipid peroxidation [30]. The non-significant difference in malondialdehyde concentration indicates that DFAR1 did not inhibit lipid peroxidation by attenuating oxidative stress. The sub-fraction thus does not have antioxidant property.

Catalase and glutathione peroxidase (GPx) activities are depleted in castor oil-induced diarrhea and leads to oxidative stress [1,31]. Oxidative stress is involved in intestinal hypersecretion of electrolytes and water into the intestinal lumen. The significant decrease in the activity of GPx further confirms that the sub-fraction did not attenuate oxidative stress caused by castor oil-induced diarrhea. Thus, the anti-enteropooling activity of DFAR1 is not due to the attenuation of intestinal hypersecretion of electrolytes and water due to oxidative stress. The significant increase in catalase may be due to an increase in the generation of H_2O_2 peroxide which usually accompanies intestinal hypersecretion [30]. Catalase catalyzes the dismutation of hydrogen peroxide into water and oxygen [32]. The activity of an enzyme increases as its substrate concentration increases. The significant decrease in superoxide dismutase activity in rats administered DFAR1 may be attributed to the decreased generation of superoxide. Increased SOD activity correlates with an increase in fluid accumulation in castor oil-induced diarrhea [33]. Thus, a decrease in SOD activity may contribute to the anti-enteropooling activity of the sub-fraction.

Active extrusion of Na^+ ions across the basolateral membrane is mediated by the sodium pump (Na^+- K^+ ATPase) [34]. Na^+- K^+ ATPase maintains the electrochemical gradient required for Na^+ absorption and eventually water re-absorption. The activity of the pump is inhibited in all types of diarrhea. The non-significant increase in the activity of the pump upon administration of DFAR1 to castor oil-induced rats suggest that the sub-fraction does not contain substances capable of stimulating the pump and that the pump did not contribute to the anti-enteropooling activity of DFAR1.

The significant decrease in the activity of cyclooxygenases II in rats administered DFAR1 indicates that the sub-fraction is an inhibitor of prostaglandin synthesis. Cyclooxygenase is the key enzyme responsible for the synthesis of prostaglandins. Cyclooxygenase II is the inducible isoform and it is the target of anti-inflammatory drugs because it is responsible for the biosynthesis of prostaglandin in acute inflammation [35]. Prostaglandins elicit net secretion of fluid by inhibiting sodium absorption [36]. Prostaglandin is also known to cause inflammation [37]. Inflammation causes inflammatory bowel disease (IBD) whose symptoms are diarrhea. One pathophysiology of inflammatory associated diarrhea is epithelial barrier dysfunction which leads to reduced expression and/or function of ENaC and may cause transport disturbances [38]. Thus, under inflammatory conditions electrogenic sodium absorption is reduced secondary to decreased expression/function of ENaC

which in turn leads to inhibition of water absorption. [39] Inhibition of prostaglandin synthesis contributes to the anti-enteropooling activity of DFAR1 by restoring the inhibition of Na^+ absorption triggered by prostaglandin and prevents ENaC dysfunction caused by inflammation. Thus, the compound 3-tetradecen-5-yne (a triterpene) present in DFAR1 is responsible for inhibition of COX-II. Triterpenes and polyunsaturated fatty acids have been reported to have COX II inhibitory activity [40].

Conclusion

Anti-enteropooling compounds are present in the dichloromethane fraction of aqueous root bark extract (DFAR) from *A. senegalensis*. The sub-fraction is rich in androstan-16-ene-3-one, (an aldosterone derivative and a steroid) and 3-tetradecen-5-yne (an essential oil) that act as anti-enteropooling compounds. These compounds exert their anti-enteropooling activity by enhancing Na^+ absorption probably by stimulating ENaC channel, decreasing superoxide dismutase activity, and inhibiting COX-II activity. Androstan-16-ene-3-one and 3-tetradecen-5-yne are potential compounds in the development of antidiarrheal drugs.

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Declaration of Competing Interest

Authors declare that there is no conflict of interest.

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