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Evaluation of the Antidiarrhoeal Activity of the Methanol Pulp Extract and Fractions of *Annona muricata* Linn (Annonaceae)

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ABSTRACT

Diarrhoea is among the foremost preventable causes of death in developing nations, affecting primarily children and newborns. The study was designed to evaluate the antidiarrhoeal activity of the methanol extract and fractions of the pulp of *Annona muricata*. The methanol extract and fractions of the pulp of *A. muricata* were evaluated for antidiarrhoeal effect using a gastrointestinal motility test, castor oil-induced diarrhoea, castor oil-induced enteropooling and isolated rabbit jejunum. Acute toxicity test and preliminary phytochemical screening were also performed on the methanol extract. Phytochemical screening of the crude extract showed the presence of alkaloids, flavonoids, glycosides, saponins, tannins, terpenoids and steroids. There was no death or signs of acute toxicity even at the maximum dose of 5000 mg/kg. The hexane fraction (25 mg/kg) and methanol extract (25, 50 and 100 mg/kg) significantly ($p < 0.05$) inhibited gastrointestinal motility. There was a significant ($p < 0.05$) reduction in the number of watery diarrhoea produced in groups treated with the methanol extract (100 mg/kg) as well as the fractions (100 mg/kg). The volume of intestinal content was significantly ($p < 0.01$) reduced in a dose-dependent manner by the methanol extract and fractions. The methanol fraction (100 mg/kg) produced the highest inhibition (69.71%) in castor oil-induced enteropooling. The methanol and hexane fractions attenuated the rhythmic contractions of isolated rabbit jejunum. The findings demonstrated that the methanol extract of *A. muricata* and its solvent fractions have antidiarrhoeal activity, validating the folklore use of pulp for diarrhoea therapy.

Keywords: *Annona muricata*, Antidiarrhoea, Acetylcholine, Gastrointestinal motility, Rabbit jejunum

Introduction

Diarrhoea is the second largest cause of death in children under the age of five, after pneumonia, accounting for 9% of all child deaths globally.[1] The majority of diarrhoea-related morbidity and mortality occur in low- and middle-income nations, primarily in rural areas as well as urban suburbs and slums.[2,3] These regions are made up of Southern Asia and sub-Saharan Africa which includes Nigeria, where the prevalence is 18.8%.[1,3,4] Diarrhoea is defined as a change in regular bowel movements, as well as a rise in the water content, volume, and weight of the stool, or a frequency of three or more times per day stool evacuation.[5] Excessive loss of fluid resulting from diarrhoea is associated with an imbalance between the absorptive and secretory mechanisms of water and electrolytes in the intestinal tract, accompanied by hypermotility.[6] Currently available drugs such as antimicrobials, antisecretory, and antimotility are associated with some adverse effects like constipation, resistance, respiratory depression, lethargy, excitement, and coma.[7-9]
Many of the medications are also either not available or not affordable. As a result, finding a new antidiarrhoeal drug that is less expensive, safe, and efficacious than the current treatments is critical.[10]

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According to the World Health Organization, over 80% of the population in low-income nations relies on plant-based medications[11] and for these reasons, research into many of the folklore therapy by locals are encouraged to substantiate efficacy and provide guidance on their safety.
Annona muricata L., commonly known as soursop, graviola, guanabana and sirsak, is a member of the Annonaceae family.[12,13] It can be found in tropical and subtropical regions of the world, including India, Malaysia, and Nigeria.[14] The fruit is used as natural medicine for arthritic pain, neuralgia, arthritis, diarrhoea, dysentery, fever, malaria, rheumatism, skin rushes and worms.[15] The leaves are utilized in the treatment of cystitis, diabetes, headaches and insomnia.[15] In tropical Africa, the plant is used as an astringent, insecticide and piscicide agent. It is also used to treat coughs, pain and skin diseases.[16] The leaves of *A. muricata* are used as an ethnomedicine throughout South America and tropical Africa, notably Nigeria, to treat tumors and cancer.[17] In addition, the anti-inflammatory, hypoglycemic, sedative, smooth muscle relaxant, hypotensive and antispasmodic effects are also attributed to the leaves, barks and roots of *A. muricata*.[14,13] The anticancer[18], antioxidant[19], antiprotozoa[20], hepatoprotective and bilirubin-lowering[21], insecticidal[22], gastroprotective[23,24], antibacterial[25] and wound healing[26] activities have been reported. Although, the pulp of *A. muricata* is used by locals in many parts of Sub-Sahara Africa as antidiarrhoeal therapy, no prior research has been done on the antidiarrhoeal effects of the pulp extract of *A. muricata* and its solvent fractions. Thus, the study aimed at investigating the antidiarrhoeal effect of the methanol pulp extract and fractions of *A. muricata*.

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Materials and Methods

Drug, chemical and reagents

Atropine sulphate injection (Jeil Pharm.Co Ltd, India), Distilled water, Molisch's reagent, Fehling solution, Benedict's reagent, Mayer's reagent, Dragendorff's reagent, Wagner's reagent, Picric acid solution, Castor oil (Bell's, England), Glucose, Methanol (BDH Chemicals Ltd, England), Ethylacetate and n-hexane (Wanes LA, Netherland), activated charcoal (Olchla Industrial Area, India)

Experimental animals

Adult Sprague Dawley rats (180-200 g), adult Swiss mice (18-24 g) of both sexes and New Zealand rabbits (1.5 - 3.0 kg) bred in uniform condition at the animal facility of the Pharmacology and Toxicology Laboratory Department, University of Nigeria Nsukka, were used. They were housed under standard conditions of temperature (25 ± 2^o C), standard feeds (Ladokun Feeds, Ibadan, Nigeria) and water ad libitum. The experimental protocols were approved by the ethics committee of the Faculty of Pharmaceutical Sciences, University of Nigeria (FPSRE/UNN/22/0001) and were in conformity with the ethical guidelines of the National Code of Conduct for Animal Research Ethics (NCARE).

Plant collection and identification

The *A. muricata* fruits were collected at Eha-Alumona Nsukka in Enugu State between the months of July and August, 2018. The fruit was identified and authenticated by Mr. A.O. Ozioko, at the International Centre for Ethnomedicines and Drug Development (InterCEDD) Nsukka. The specimen voucher with No InterCEDD/16099 was deposited in the Centre's herbarium

Preparation of pulp extract

The fresh *A. muricata* fruits were collected, the back peeled off, the seeds removed and chopped into small cubes and sun–dried to a constant weight. Resulting from this process is dried pulp which was pulverized into fine powder using a blender, weighed and stored in an air- tight container for further use. The powder (350 g) was cold macerated with 2.5 L of methanol. The mixture was allowed to stand with occasional agitation for 48 h after which it was filtered. The filtrates were concentrated to dryness in vacuo at 40°C using a rotary evaporator to give a residue referred to as the methanol extract of *A. muricata* (MEAM).

Furthermore, a part of *A. muricata* extract was subjected to successive fractionation using solvents of different polarities (n-hexane, ethylacetate and methanol). Each solvent (2.5 L) was added intermittently to each phase and was allowed to stand for 48 h with consistent agitation. The fractions were then concentrated by rotary evaporation and dried in an oven at 40°C. The n-hexane (HF), ethylacetate (EAF) and methanol (MF) fractions were stored in airtight containers in a refrigerator until used.

Phytochemical screening of extract

Qualitative phytochemical analysis of the methanol extract was performed according to standard procedures.[27,28]

Acute toxicity test (LD_{50})

Lorke's approach was used to determine the acute toxicity of the methanol extract in mice.[29] In the first phase, mice were placed in three groups (n=3) and were administered 10, 100, and 1000 mg/kg methanol extract. The mortality rate and behavioural changes in the treated mice were monitored for 24 h. Because no deaths were reported in the first phase, a new batch of 3 mice (one for each group), received 1600, 2900, and 5000 mg/kg of the extract. The treated animals were observed for mortality or signs of acute intoxication for 24 h. The geometric mean of the highest non-lethal dose and the least toxic dose was used to derive the LD_{50}.

Gastrointestinal motility test

A gastrointestinal motility test was conducted according to the method previously described by Tenorio *et al*.[30] The adult Wistar rats were starved of food for 24 h prior to the experiment but were allowed free access to water. They were randomly assigned to the following groups: Group 1 received distilled water (5 mL/kg,), Group 2 received Atropine (5 mg/kg), Groups 3-5 received the extract (25, 50, and 100 mg/kg), Groups 6-8 received hexane fraction (25, 50, and 100 mg/kg), Groups 9-11 received ethylacetate fraction (25, 50, and 100 mg/kg) and Groups 12-14 received methanol fraction at the doses of 25, 50 and 100 mg/kg, respectively. Previous pilot study conducted on the methanol extract produced significant antidiarrhoeal activity with lower dosages than higher dosages, thus, informed the choice of dosages used in this study. All rats received 0.5 mL of charcoal meal 1 h after treatment. Rats were sacrificed 1 h later, and the small intestine from the pyloric sphincter to the ileocecal junction was excised. The distance traversed by charcoal was measured and expressed as a percentage of the overall length of the small intestine using the equation below:

$$Intestinal\ transit\ (\%) = \frac{D}{L} \times 100 \text{ - - ---------- (1)}$$

Where D = distance covered by charcoal meal and L= total length of the small intestine

Castor oil-induced diarrhoea

The castor oil-induced diarrhoea study was carried out according to methods previously described by Mekonnen *et al*.[8] Rats of both sexes were allotted into five groups and fasted for 18 h prior to the test. Distilled water (5 mL/kg,) was administered to Group 1 as control and Group 2 received Atropine (5 mg/kg). Groups 3-5 received the extract (25, 50, and 100 mg/kg). All doses were administered orally. One hour later, all the rats received 1 mL of castor oil orally. Animals were individually placed in cages lined with adsorbent papers and observed for 4 h for the presence of diarrhoea defined as watery (wet), unformed stool. The activity of each group was expressed as percent inhibition (%) of diarrhoea. The percent inhibition of defecation was calculated as follows:

$$Inhibition\ of\ defecation\ (\%) = \frac{A - B}{A} \times 100 \text{ - - - - - (2)}$$

Where A = number of wet droppings in control and B = number of wet droppings of treated.

A similar study was also performed with the extract fractions at three dose levels (25, 50, and 100 mg/kg).

Castor oil-induced enteropooling

The castor oil-induced enteropooling was carried out according to a method previously described by Sisay *et al*.[31] Rats of either sex were randomized into five groups and fasted for 18 h prior to the test. Group 1 received distilled water (5 mL/kg) as a negative control, Group 2 received atropine (5 mg/kg), while Groups 3-5 received 25, 50 and 100 mg/kg of methanol extract. After 1 h of the different treatments, all the animals were given 1 mL of castor oil orally. The animals were sacrificed after 1 h and the small intestine from the pylorus to the caecum was isolated. The intestinal contents were weighed and volume measured using a graduated cylinder. Percentage reduction in the volume of intestinal was calculated as:

$$Volume\ reduction\ (\%) = \frac{Vc - Vt}{Vc} \times \frac{100}{1} \text{ - - - - - - - (3)}$$

Where, V_c = Mean volume of the intestinal content of the control group and V_t = Mean volume of the intestinal content of the test group.

$$Weight\ reduction\ (\%) = \frac{Wc - Wt}{Wc} \times \frac{100}{1} \text{ - - - - - - - (4)}$$

Where W_c = Mean weight of the intestinal content of the control. While W_t = Mean weight of the intestinal content of the test group.

A similar study was also performed with the extract fractions at three dose levels (25, 50, and 100 mg/kg).

Studies on isolated rabbit jejunum

The abdomen of an adult rabbit was dissected after they were euthanized.[32,33] The jejunum was cut into 2-3 cm segments and dissected free of mesentery. The lumen was flooded with a Tyrode solution that contained the following components (mM/L) : NaCl 134; KCl 2.7; $CaCl_2$ 1.8; $NaHCO_3$ 11.9; $MgCl_2$ 1.0; NaH_2PO_4 0.4 and glucose 5.56. The tissue was mounted in a 20 mL organ bath containing Tyrode solution at 37 ± 1^{o}C and bubbled with air. The physiological salt solution was replaced every 15 min throughout a 1 h

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equilibration period with a resting tension of 0.5 g. Acetylcholine, atropine, crude extracts, and fractions were all tested for their effects. Responses were recorded on the student kymograph through an isometric pressure transducer. Each concentration of agonist was given 1 min of contact time before being washed off three times. Between drug additions, the tissue was given a 3 min rest interval. The extract and fractions and atropine were tested for their ability to relax spontaneous rhythmic contractions of the isolated rabbit jejunum.

Statistical analysis

Results were expressed as the mean ± standard error of the mean (SEM) and analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's Post Hoc test using GraphPad prism version 7.0. $p < 0.05$ and $p < 0.01$ were considered statistically significant.

Results and Discussion

Preliminary phytochemical tests showed that the methanol extract of *A. muricata* contains alkaloids, tannins, flavonoids, saponins, terpenoids, glycosides, and steroids (Table 1).

Previously, some of these secondary metabolites have been demonstrated to possess antidiarrhoeal effect.[34] Tannins have been reported to provide antispasmodic and muscle relaxant actions, flavonoids decreased prostaglandin E_2-induced intestinal secretion, saponins suppress histamine release, and terpenoids inhibit prostaglandin release, according to studies.[23] By suppressing intestinal secretion and motility, all of these effects help to prevent diarrhoea.[35] As a result, these secondary metabolites may be implicated in the antidiarrhoeal action of the plant's extracts. Furthermore, no signs of toxicity were uncovered in the acute toxicity study, indicating that the extract could be considered reasonably tolerable and safe even at 5000 mg/kg.

In the gastrointestinal motility test model, the methanol extract significantly ($p < 0.05$) inhibited (77.2, 71.3 and 64.7% inhibition) gastrointestinal motility in a dose-dependent manner (100, 50 and 25 mg/kg respectively) as shown by a reduction in gastrointestinal movement of a charcoal meal (Figure 1). The n-hexane (25 mg/kg) and methanol (50 mg/kg) fractions caused higher percentage inhibitions (71.1 and 63.7%) than ethylacetate fraction (45.6%) (Figure 1). Reduced gastrointestinal motility lengthens the time gastrointestinal contents spent in the intestine, which may help with water and electrolyte absorption.[36] As a result, the antidiarrhoeal action can be linked, at least to some extent, to its antimotility action.

In the castor oil-induced diarrhoea model, the extract produced a dose-dependent decrease in the number of watery diarrhoea. At a dose of 100 mg/kg, the extract significantly ($p < 0.05$) decreased the number of watery diarrhoea induced by castor oil (2.00 ± 1.00) compared to the control group (4.40 ± 0.80) Table 2. The hexane fraction caused a reduction in the number of watery diarrhoea in a non-dose dependent manner. Significant ($p < 0.05$) inhibition of 54.5 and 50.0% in the diarrhoeal episodes was observed at 25 and 100 mg/kg respectively. The methanol fraction (25, 50 and 100 mg/kg) caused significant ($p < 0.05$) inhibition of 54.5, 81.8 and 77.3% in the diarrhoeal episodes (Table 2). In the intestine, lipases hydrolyze castor oil or its triglyceride to glycerol and ricinoleic acid, which operates predominantly in the small intestine to promote fluid and electrolyte secretion and expedite intestinal transit.[37] Furthermore, the release of prostaglandin and the irritant impact of ricinoleic acid enhance it. The release of these prostaglandins causes a rise in intestinal motility and secretions into the intestinal lumen.[38] The antidiarrhoeal action of the methanol extract and fractions of *A. muricata* against experimentally induced diarrhoea by castor oil could be linked to its anti-electrolyte permeability effect. The usefulness of *A. muricata* pulp as a potential antidiarrhoeal agent is evidenced by the considerable reduction in the number of faecal droppings.

All the doses of tested extract and fractions of *A. muricata* produced significant inhibition against castor oil induced enteropooling which might be due to the inhibition of prostaglandin synthesis.

At 25, 50 and 100 mg/kg, the extract produced 63.27, 69.70 and 72.11% inhibition of intestinal content volume respectively. The inhibition produced by 100 mg/kg (72.11%) was comparable to atropine (71.85%) (Table 3). The fractions of *A. muricata* produced a significant ($p < 0.05$, 0.01) dose-dependent decrease in the volume of intestinal content. The hexane fraction at doses 25, 50 and 100 mg/kg offered 48.53, 67.56 and 68.63% inhibition respectively while ethylacetate fraction caused 49.06, 53.35 and 52.01% inhibition at 25, 50 and 100 mg/kg respectively. The methanol fraction at 100 mg/kg gave the highest inhibition of 69.71% amongst the fractions. This was comparable to atropine which produced inhibition of 71.85% (Table 3). The extract's anti-enteropooling activity is more important since it helps to alleviate diarrhoea by preventing enteropooling. The methanol extract and fractions decreased intestinal fluid accumulation. This indicates that the plant extract and fractions may reduce water and electrolyte secretion into the intestinal lumen while increasing absorption. This may reduce intestinal overload and distension, resulting in a decrease in intestinal motility (allowing for more time for absorption) and faecal water content, and hence a reduction in the total number of defecation episodes and diarrhoeal drops in the treated groups.[39]

Acetylcholine, a cholinergic agonist, caused a dose-dependent rise in rhythmic contractions of the rabbit jejunum. Endogenous and exogenous acetylcholine, as well as other parasympathomimetic drugs, have this effect.[40] The findings are consistent with current literature, which claims that acetylcholine causes an increase in contractions in the rabbit jejunum because the drug molecules bind to muscarinic receptors in the jejunum's smooth muscles.[41] Noradrenaline also had its characteristic sympathomimetic impact on the jejunum, relaxing it and lowering contractions.[42] The binding of noradrenaline molecules to the many adrenergic receptors in the jejunum's smooth muscle produced this action.[42]

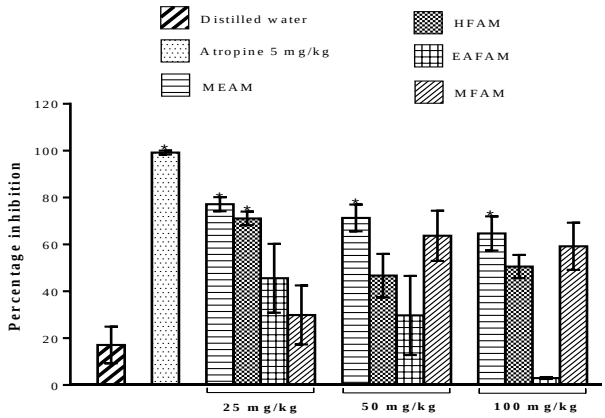

Figure 1: Effect of methanol extract and fractions of *A. muricata* on gastrointestinal motility.

Values are expressed as mean ± SEM; n = 5; *P < 0.05 significant relative to control; MEAM= methanol extract; HFAM = n-hexane fraction; EAFAM = ethylacetate fraction; MFAM = methanol fraction.

Table 1: Phytochemical screening of the methanol extract of pulp of *Annona muricate*

Phytochemical constituents	Relative abundance
Alkaloids	+
Flavonoids	+
Glycosides	+
Saponins	+
Tannins	+
Terpenoids	+
Carbohydrates	+
Proteins	-
Reducing sugar	+
Steroids	+

Key: + = Present; - = Absent

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Table 2: Effect of fractions of *A. muricata* on castor oil induced diarrhea

Treatment	Dose (mg/kg)	Number of watery diarrhoea	% Inhibition
Distilled water (control)	5 mL/kg	4.40 ± 0.80	-
Atropine	5	0.40 ± 0.20	90.9**
MEAM	25	3.20 ± 0.80	27.3
	50	2.60 ± 0.80	40.9
	100	2.00 ± 1.00	54.5*
HFAM	25	2.00 ± 0.40	54.5*
	50	1.60 ± 0.70	28.0
	100	2.20 ± 0.90	50.0*
EAFAM	25	1.00 ± 0.60	77.3*
	50	1.00 ± 0.60	77.3*
	100	0.60 ± 0.40	86.4**
MFAM	25	2.00 ± 0.70	54.5*
	50	0.80 ± 0.40	81.8**
	100	1.00 ± 0.40	77.3*

Values are expressed as mean ± SEM; n = 5; *p < 0.05; **p < 0.01 relative to distilled water; HFAM = n-hexane; EAFAM = ethylacetate fraction; MFAM = methanol fraction

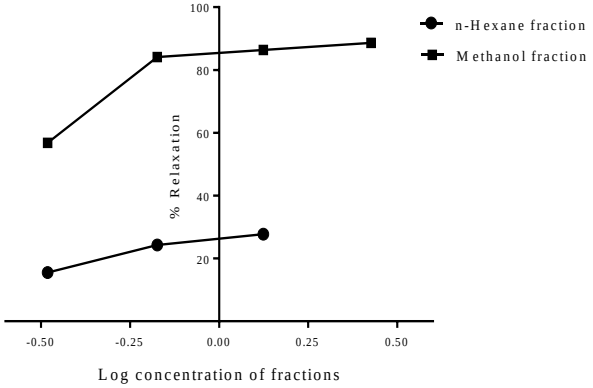

Figure 2: Effect of fractions of *A. muricata* on isolated rabbit jejunum

The n-hexane and methanol fractions of *A. muricata* showed a similar effect to that of noradrenaline by decreasing contractions in a dose-dependent manner (Figure 2). As a result, *A. muricata* fractions could have achieved this by binding to adrenergic receptors in the same way that noradrenaline does, and so could be considered a sympathomimetic drug. The ability of sympathomimetic drugs to inhibit intestinal motility is well documented.[42,43]

Table 3: Effect of fractions of *A. muricata* on castor oil induced enteropooling

Treatment	Dose (mg/kg)	Weight of small intestine (g)	Volume of intestinal content (ml)	% Inhibition
Distilled water (control)	5 mL/kg	2.66 ± 0.10	3.73 ± 0.10	-
Atropine	5	1.01 ± 0.11	1.05 ± 0.09	71.85**
MEAM	25	1.27 ± 0.18	1.37 ± 0.19	63.27*
	50	1.05 ± 0.08	1.13 ± 0.09	69.70**
	100	1.12 ± 0.25	1.04 ± 0.24	72.11**
HFAM	25	1.91 ± 0.09	1.92 ± 0.08	48.53*
	50	1.19 ± 0.22	1.21 ± 0.22	67.56**
	100	1.16 ± 0.12	1.17 ± 0.10	68.63**
EAFAM	25	1.84 ± 0.12	1.90 ± 0.10	49.06*
	50	1.55 ± 0.21	1.74 ± 0.23	53.35*
	100	1.86 ± 0.23	1.79 ± 0.17	52.01*
MFAM	25	1.83 ± 0.08	1.89 ± 0.09	49.33*
	50	1.45 ± 0.18	1.59 ± 0.18	57.37*
	100	1.10 ± 0.14	1.13 ± 0.11	69.71**

Values are expressed as mean ± SEM; n = 5; *p < 0.05; **p < 0.01 relative to distilled water; HFAM = n-hexane; EAFAM = ethylacetate fraction; MFAM = methanol fraction

Conclusion

The methanol pulp extract and fractions of *A. muricata* possessed antidiarrhoeal activity as revealed by reductions in the diarrhoeal droppings, intestinal fluid accumulation, and gastrointestinal motility. The results suggest that this may be the basis for the use of the pulp extract of *Annona muricata* in traditional settings to treat diarrhoea.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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