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Research Article

The Mechanisms of Anti-Diarrhoeal Activities of Some Derived Partitions of *Anacardium Occidentale* Leaf Extract

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Abstract

Aim: This study was designed to unearth the probable mechanism of action (*in vitro*- on isolated rabbit jejunum) of the anti-diarrhoeal properties of methanol, chloroform, and pet ether derived partitions from *Anacardium occidentale* leaf extract.

Methods: The rabbit jejunum of about 2-3 cm long was dissected out, placed on a Petri dish containing freshly prepared and oxygenated Tyrode solution at room temperature. The tissue was mounted and allowed to record its basal rhythmic contraction for 2 minutes before the effect of each partitioned extract was evaluated at different concentrations. The effects of standard autonomic modification drugs, acetylcholine, histamine, and the muscarinic inhibitor atropine sulphate at different concentrations were investigated in the presence/absence of varying concentrations of each partitioned extract. The data obtained were analysed using Graph pad Statistical package.

Results: The *in vitro* results showed that the different partitions of *A. occidentale* leaves provoked relaxing effect on acetylcholine- and histamine- induced contractions of isolated rabbit jejunum, which portrayed a concentration dependent effect across the various percentages investigated. It was observed that whereas, the partitions at all concentrations studied, partially inhibited acetylcholine-induced smooth muscle contractions, the histamine receptors where completely blocked, indicating more of an anti-histamine than anticholinergic mechanism of controlling diarrhoea for *A. occidentale* extract. The chloroform partition possessed potent active principles with overt anti-diarrhoeal activities principally via intestinal smooth muscle relaxation better than other partitions.

Conclusion: In conclusion, the different partitioned extracts of *A. occidentale* leaves has shown good anti-diarrhoeal effects, principally via the histaminic pathway. Hence, lending some credence to the scientific claim that *A. occidentale* leaves can be used to control diarrhoea.

Keywords: *Anacardium Occidentale;* Extract Partitions; Anti-Diarrhoea; Antihistamine; Anti-Cholinergic

Introduction

Many factors contribute to diarrhoea outbreak such as poor sanitation, consumption of infected food, intake of contaminated water and unclean environment. Parasites, bacteria, viruses, gastroenteritis, specific medicines, ingested poison, food intolerance, colitis, vitamin A deficiency, consuming unripe fruits, food allergies, reaction to rancid nuts or oils, lack of fiber in the diet, excessive bowel movements, infected fomites, polluted environment, seasonal rainfall, contaminated surfaces, enzyme deficiency, infections from insects and flies etc. can also cause diarrhoea. Depending upon the etiology, the stool frequency can be dramatic. Diarrhoea remains the second most common cause of death among children below the age of five following closely behind pneumonia [1].

Diarrhoea could occur in three (3) forms - secretory form (caused mostly by bacterial enterotoxins), exudative form (as a result of increased permeability of the intestinal mucosa either due to inflammation or infections) or osmotic form (commonly associated with maldigestion or mal-absorption of food). Typically, most diarrhoea syndromes are a combination of these forms. Therefore, a good anti-diarrhoeal agent should be able to principally decrease intestinal secretions, cause dysmotility or reverse the underlying problem that produced the observed changes in secretions or motility [2].

According to the United Nations International Children's Emergency Fund (UNICEF), an estimated over 5000 children die daily due to diseases with diarrhoeal complications [3]. Of this number, roughly 78% of the deaths occur in African and South- East Asian regions [1,4]. Animals are not left out of the condition, since there are associations with high morbidity rates seen especially in their new-borns. Despite the high rate of losses of lives especially among neonates, the condition has a lot of economic implication on food animals and livestock production with a resultant wasting among animals, as well as decreased animal proteins and very serious losses to livestock farmers.

The World Health Organization (WHO) classified diarrhoea as among the world's top rated communicable diseases with very high mortality rates especially among children below the age of five and infants [5]. It was estimated that in Africa alone, with the use of herbal medications, cases of diarrhoea among children below the age of five has decreased from 177.0 per 1000 live birth to 81.3 per 1000 live birth [6], which is an indication that alternative herbal remedy is gaining more focus especially in Africa.

Herbal plants have become one of the bases of modern medicine [7,8]. *A. occidentale* plant is one of these herbal remedies scientifically reported to control diarrhoea of both infectious and non-infectious origins [9-12]. Is has been shown that some derived partitions from the *A. occidentale* plant crude extract have an enhanced anti-diarrhoeal activity, there is need to unearth the probable mechanism of action of these extract partitions, hence, the aim of this study.

Study area

This study was carried out in the animal research laboratory in the Department of Physiology and Pharmacology, College of Veterinary Medicine, Michael Okpara University of Agriculture, Umudike (MOUAU).

Experimental animal

Three (3) matured rabbit bucks weighing between 1.8 kg and 1.82 kg body weight were used for the study, one buck for each partitioned extract of *A. occidentale*.

Materials and Methods Plant Collection

Fresh leaf samples of *Anacardium occidentale* pre-screened for efficacy in experimentally induced diarrhoea [12], were collected within the premises of Veterinary College, MOUAU, in the month of June 2017. They were identified and authenticated by a taxonomist from the department of Botany, College of Natural Sciences, MOUAU. Voucher specimens were deposited at the herbarium of the College of Veterinary Medicine (CVM), Michael Okpara University of Agriculture, Umudike (MOUAU), and voucher number assigned was MOUAU/CVM/VPP/2017/015.

Plant extraction and partitioning

The leaves were collected, separated from debris, washed in running tap water, and subsequently air-dried to obtain crisp leaf samples, before being pulverized into fine powder using a stainless steel laboratory blender [13]. The plant material was extracted using cold maceration method (1:5 w/v in MeOH) for 48- 72 hours and with intermittent vigorous agitation every 2 hours. The weighed crude extract obtained was partitioned using the solvent-solvent protocol described by Kupchan and Tsou [14] as modified by Houghton and Raman [15] in a 9:1 concentration of extracting solvent (methanol). Thereafter, the resultant solution was then partitioned successively in equal volume, immiscible organic solvents of increasing polarity, first petroleum ether (Pet Ether) which was well shaken, then chloroform ($CHCl_3$). Before adding the next solvent, the partition formed by the former was decanted. Thereafter the next solvent was added, shaken well and allowed to settle. At the end, all the three (3) fractions obtained (methanol, chloroform and pet ether fractions) were evaporated to dryness using a rotary evaporator, transferred into pre-weighed, labelled, clean beakers. The percentage yields of the different partitions were calculated.

$$\% \text{ Yield} = \frac{W_1}{W_2} \times \frac{100}{1}$$

Where;

W_1 = Weight of material extracted

W_2 = Weight of plant material

Experimental procedure

Effects of crude extract partitions on isolated rabbit jejunum

The study of the activities of the test partitions on an isolated rabbit jejunum were determined using the method described by Amos., *et al.* [16].

A matured rabbit buck fasted overnight (18 h) was properly restrained and exsanguinated; the fur around the rabbit abdomen was carefully shaved and a mid-line incision through the skin and abdominal muscle along the linea alba made using a pair of coarse scissors. A segment of rabbit small intestine (specifically, the jejunum) of about 2-3 cm long was dissected out and placed on a Petri dish containing freshly prepared and oxygenated Tyrode solution at room temperature. Using a pair of fine curved forceps, a length of thread was passed through both ends of the dissected guts and was mounted on to an aerated (95% O_2/5% CO_2), 30 ml organ bath connected to a pressure transducer (H.L Scientific Industries, Amabala Cantt, India) maintained at 37 ± 1^0C. The intestinal content was removed by 3x intermittent flushing with Tyrode solution over 2 minutes. Mounted intestinal tissues recorded basal rhythmic contraction for about 2 minutes before the effect of the test partition were evaluated (at different concentrations).

Thereafter, a 60-min equilibration period was allowed during which, the physiological solutions were changed at 15-min intervals. At the end of the equilibration period, the effects of standard autonomic modification drugs, acetylcholine (0.33 mg/ml - 0.99 mg/ml), histamine (0.33 mg/ml - 0.99 mg/ml) and the muscarinic inhibitor atropine sulphate (0.33 mg/ml - 0.66 mg/ml) were investigated in the presence/absence of varying concentrations of either the partitions (0.1, 0.2, and 0.4 mg/ml). The contact time for each assay was 1 min, thereafter 3 brisk washes and a 15-min rest period was observed before the next addition. All assays were repeated in triplicates to ensure consistency of responses. Three different rabbit bucks were used for each partition.

Statistical Analysis

Data obtained were analysed using Graph pad (prism) statistic package and expressed in terms of means ± standard errors as well as percentage fold-changes, where appropriate. Dose dependent effect on anti-diarrhoea index *in vivo* (ADI), peristaltic index (PI), etc., were subjected to One-way analysis of variance (ANOVA) coupled with appropriate post-hoc statistics, while multiple comparisons (comparing the effect of the partition-treated groups with the control-untreated group, and between the 3 partitions) was conducted using multiple linear regression analysis (MLRA), while statistical confidence was set at 95% ($P < 0.05$).

Ethical Considerations

All procedures were carried out in strict compliance to the institutional ethical instructions for the work, as well as adequate consultations to the Experimental Ethic Committee (EEC) guidelines to laboratory animal care and use [17].

Results

The plant derived partitions yielded 8 grams (40%), 7 grams (35%), and 4 grams (20%) of methanol, chloroform and petroleum ether fractions, respectively.

Effect of different partitions of the extract (*A. occidentale*) on the isolated rabbit jejunum

Result of the effect of *A. occidentale* extract partitions on the isolated rabbit jejunum as presented in Table 1, showed that the relaxing effect (percentage activity) of each partition was increased as their concentrations increased in a concentration dependent manner. The highest response effect of the chloroform fraction (88.81%) and methanol fraction (83.65%), and Pet ether fractions (81.41%) occurred at 0.8 mg/ml concentration. The comparative study of the three fractions suggests that the relaxant effect occur in the decreasing order: Chloroform > Methanol > Pet ether.

FBC dose of partitions (mg/ml)	Basal Rhythmic Contraction (mm)	Response to isolated rabbit jejunum (mm)	Percentage relaxing activity (%)
Chloroform 0.1	9.00 ± 0.40	1.97 ± 0.02	77.91 ± 1.07
0.2	9.00 ± 0.40	1. 62 ± 0.17	81.94 ± 1.82*
0.4	8.75 ± 0.25	0.97 ± 0.08	88.81 ± 1.04***
Methanol 0.1	7.50 ± 0.28	2.08 ± 0.07	72.27 ± 3.68
0.2	5.75 ± 0.25	1.33 ± 0.19	77.08 ± 2.91
0.4	7.25 ± 0.25	1.13 ± 0.14	83.65 ± 1.94*
Pet ether 0.1	5.25 ± 0.25	1.58 ± 0.21	69.75 ± 4.66
0.2	4.75 ± 0.25	1.13 ± 0.14	76.50 ± 2.36
0.4	5.25 ± 0.25	0.95 ± 0.15	81.41 ± 3.51

Table 1: Effect of *A. occidentale* extract partitions on the isolated rabbit jejunum.

*p < 0.05, **p < 0.01 ***p < 0.001, when compared with control group.

Effect of acetylcholine on the isolated rabbit jejenum

Upon administration of standard muscarinic (Acetylcholine), after the smooth muscle was allowed to record it basal rhythmic contraction for 2 minutes, a forceful contraction of the rabbit Jejunum was elicited which increased in both amplitude and duration in a concentration dependent manner (Table 2).

Ach induced its maximum response (contractile effect) on the isolated rabbit jejunum at the highest concentration (11.50 mm, 0.99 mg/ml) and the increase was significant ($p < 0.05$). The concentration of 0.66mg/ml and 0.99mg/ml elicited a maximum response of 9.25mm, and 9.75mm with percentage activity of 64.51, and 71.66, respectively. The percentage activity was higher and significant ($p < 0.05$) at the highest concentration of Ach used (0.99 mg/ml, 83.61%).

FBC dose of Acetylcholine (mg/ml)	Basal rhythmic contraction (mm)	Response to isolated rabbit Jejunum (mm)	Percentage contracting activity (%)
0.33	3.25 ± 0.25	9.25 ± 0.47	64.50 ± 3.47
0.66	2.75 ± 0.25	9.75 ± 0.25*	71.70 ± 2.89*
0.99	1.87 ± 0.12	11.50 ± 0.29**	83.60 ± 1.34**
R^2		0.737	0.713

Table 2: Effect of acetylcholine on the isolated rabbit jejunum. *p < 0.05, **p < 0.01 ***p < 0.001, when compared with control group.

Effect of atropine sulphate on the isolated rabbit jejunum

Atropine (standard anti-muscarinic) at 0.33 and 0.66 mg/ml inhibited the normal smooth muscle contraction by 65.90% (from 9.25mm to 3.12mm) and 75.69% (from 8.25mm to 2.00mm), respectively. This tissue response to atropine sulphate occurred in a concentration dependent manner (Table 3).

FBC dose of Atropine sulphate (mg/ml)	Basal Rhythmic Contraction (mm)	Response to isolated rabbit jejunum (mm)	Percentage activity (%)
0.33	9.25 ± 0.47	3.12 ± 0.12	65.90 ± 2.38
0.66	8.25 ± 0.25	2.00 ± 0.00	75.69 ± 0.69

Table 3: Effect of atropine sulphate on the isolated rabbit jejenum.

Competitive inhibition on contractile effect of ach by the different partitions

The relaxant effect of the different partitions on Ach induced contraction of the rabbit jejunum as presented in table 4, showed that the different partitions did not significantly ($p > 0.05$) inhibit the contractile effect of Ach on the isolated smooth muscle of the rabbit jejunum as their various percentage inhibiting effect were < 50% when compared to the response (competitive inhibitory effect) of the anti-muscarinic agent, atropine sulphate (93%). However, chloroform partition performed better with inhibitory effect of 31.81% at its highest concentration of 0.8 mg/ml than other partitions.

Parameter	Treatment (mg/ml)	Mean ± S.E (mm)	Percentage inhibitory effect of treatment (%)
Response to isolated smooth Muscle	Acetylcholine (Ach)	11.00 ± 0.40	-
	Ach+ Crude extract		
	0.66 + 0.2	10.25 ± 0.25	6.81
	0.66 + 0.4	8.50 ± 0.28	22.72
	0.66 + 0.8	8.00 ± 0.00	27.27
	Ach + methanol fraction		
	0.66 + 0.2	10.75 ± 0.25	2.27
	0.66 + 0.4	10.00 ± 0.40	9.09
	0.66 + 0.8	9.37 ± 0.23	14.80
	Ach + Chloroform fraction		
	0.66 + 0.2	8.25 ± 0.25	25.00
	0.66 + 0.4	9.25 ± 0.25	15.90
	0.66 + 0.8	7.50 ± 0.28	31.81
	Ach + Pet ether fraction		
	0.66 + 0.4	9.75 ± 0.12	11.36
	0.66 + 0.8	9.25 ± 0.25	15.90
	Ach + Atropine sulphate		
	0.66 + 0.2	0.72 ± 0.07	93.45
	0.66 + 0.4	1.07 ± 0.14	90.27

Table 4: Competitive inhibition of contractile effect of Ach by the treatment.

Result presented in Mean ± S.E.

Effect of histamine on the isolated rabbit jejunum

The contractile effect of histamine on the rabbit jejunum as presented in Table 5, occurred in a concentration dependent manner with the highest activity (87.26%) observed at 0.99 mg/ml, while 0.33 mg/ml did not elicit up to 50% contractile activity on the isolated jejenum.

FBC Dose of *A. occidentale* (mg/ml)	Basal rhythmic contraction (mm)	Response to isolated rabbit jejunum (mm)	Percentage activity (%)
Histamine			
0.33	2.17 ± 0.11	4.22 ± 0.19	48.53 ± 1.24
0.66	0.77 ± 0.08	2.07 ± 0.07	63.23 ± 3.23**
0.99	0.35 ± 0.06	2.60 ± 0.19	87.26 ± 2.87***
R^2			0.926

Table 5: Effect of histamine on tension generated by the rabbit jejenum.

*p < 0.05, **p < 0.01 ***p < 0.001, when compared with control group.

Competitive inhibition on contractile effect of histamine by the different partitions

The result of the inhibition effects of the treatments on the contraction induced by Histamine on Rabbit jejunum as presented in Table 6, showed that the different partitions did significantly ($p < 0.05$) inhibited the contractile effect of histamine on the isolated rabbit jejunum as their individual percentage blocking effect were > 50% and these blocking effects were concentration dependent, when the two concentrations (0.4 and 0.8 mg/ml) were compared, except with the pet ether partition where the lower concentration recorded a higher blocking effect than the high concentration.

The inhibiting effects of the partitions (at the highest concentration 0.8 mg/ml) occur in the order: chloroform fraction > methanol fraction > petroleum ether fraction. However, the chloroform partition at the 0.8 mg/ml concentration completely abolished (91%) the response effect of histamine, with a better effect than other partitions.

Treatment	Mean ± S.E	Percentage inhibitory effect of treatment (%)
Histamine	2.07 ± 0.07	-
Histamine + Methanol partition 0.66 + 0.4	0.30 ± 0.06	85.55
0.66 + 0.8	0.23 ± 0.13	88.88
Histamine + Chloroform partition 0.66 + 0.4	0.21 ± 0.11	89.85
0.66 + 0.8	0.17 ± 0.04	91.78
Histamine + Pet ether partition 0.66 + 0.4	0.25 ± 0.12	87.92
0.66 + 0.8	0.40 ± 0.04	80.67

Table 6: Comparative inhibition of treatments on histamine induced contraction of the rabbit jejunum (blocking effects).

Note: Values presented in Mean ± S.E.

Discussion

It is common knowledge that medicinal plants contain several secondary metabolites, which are actually developed as part of their defence mechanisms but implicated for most observed pharmacological activities and others [18]. The increased need for a better and potent alternative agent against diarrhoea has necessitated the constant screening of herbal remedies of origin [7].

The phytochemical analysis of the *A. occidentale* leaf crude extract revealed the presence of alkaloids, glycosides, flavonoids, tannins, saponins, anthraquinone, steroids, phenolics, resins, terpenoids, and cardiac glycosides(not published yet), and most of these secondary metabolites such as; tannins, alkaloids, saponins, flavonoids, steroids, phenolics and reducing sugars, have been shown to possess anti-diarrhoeal property [8,19] Apart from the presence of flavonoids, alkaloids, etc, in the different partitions, the presence of anthraquinone and phenols were detected in the chloroform partition, while absent in other partitions. This could among other scientific interpretations be the reason chloroform performed more satisfactorily as an antidiarrhoeal agent than other partitions. The acute toxicity of *A. occidentale* for both the crude and the different partitions have been reported by [20]. Symptomatically diarrhoea could occur as result of altered bowel function, in which case there

is an impaired intestinal absorption, excessive intestinal secretion of water and electrolyte, and a rapid bowel transit, hyper-motility [21]. Some anti-diarrheal agents such as anticholinergic, and narcotic (opioid) drugs work by decreasing peristaltic movements or increasing segmental contractions in the small intestine, which decreases the movement of faeces, controlling diarrhoea [22,23].The different partitions (0.2 - 0.8 mg/ml) elicited concentration dependent relaxing effect on the isolated rabbit jejunum, although the response was short-lived in the partitions compared to the relaxation effect produced by the standard anti-muscarinic agent, atropine sulphate. The highest effect of the chloroform fraction (88.81%), methanol fraction (83.65%), and pet ether fractions (81.41%) occurred at 0.8 mg/ml in a concentration dependent manner. The comparative study showed that, though the three partitions induced a relaxing effect on the isolated rabbit jejunum above 50%, even at the lowest concentration, the response (relaxant) was highest with the chloroform partition, this further provides credence to the *in vivo* study where the chloroform partition showed better anti- diarrhoeal activity.

Acetylcholine is a neurotransmitter commonly associated with parasympathetic effects. It binds to muscarinic receptors on smooth muscles causing the receptor-operated channel to open thus allowing sodium influx, which causes depolarization of the cell membrane. This depolarization opens voltage- dependent calcium channels and calcium ions enter the cell to induce the release of calcium from sarcoplasmic reticulum. The cytosolic calcium then binds to calmodulin to elicit a contraction [24]. Similarly, histamine binds to H1 receptor on gastrointestinal smooth muscle to initiate the same sequence of events [25]. An elevation of intracellular Ca^{2+} level by influx from extracellular compartment or release from intracellular store also results in contraction [26].

The results of this study suggest that the different partitions could have prevented the calcium influx through the voltage operated channels by inhibiting the calcium induced-calcium release mechanism preventing the release of calcium (which induces contraction of the smooth muscle resulting in diarrhoea) from the sarcoplasmic reticulum, closing of sodium and calcium ion channels, activation of second messengers like cAMP or preventing binding of calcium to calmodulin. This partial inhibition of acetylcholine and complete inhibition of histamine induced contraction of the rabbit jejunum by the different partitions may suggest that the plant extract interacts more with histaminic receptors than the muscarinic receptors in other to control diarrhoea.

Conclusion

In conclusion, the inhibiting effects of the different partitions (at the highest concentration 0.8 mg/ml) which completely abolished the response effect of histamine induced contractions occurred in the order: chloroform partition > methanol partition > petroleum ether partition. And the ability of the plant partitions in controlling diarrhoea (as an anti-diarrheal agent) was mostly via the histaminic pathway than the muscarinic pathway. This however lends credence to its folkloric use as an anti-diarrhoeal medicinal plant.

Sponsor

Self-sponsored.

Conflict of Interest

There is no conflict of interest.

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