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ACTIVE ACH-INHIBITORY FRACTIONS FROM *WALTHERIA INDICA* L. (STERCULIACEAE) METHANOL LEAF EXTRACT

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ABSTRACT

Waltheria indica is used in many medicinal preparations in ethnomedical practice in Nigeria. Its use in treating diarrhea has been scientifically validated and reported. The present work examined the probable intestinal relaxant effects of organic solvent fractions obtained from the methanol extract of the leaves. The methanol extract (90 g) was suspended in water and partitioned repeatedly with chloroform (200 mL x 5) in a separating funnel. The aqueous and the chloroform fractions were concentrated under pressure and separately evaluated for relaxant effects on 80µg Ach-induced ileum contractility at concentrations of 20, 40 and 80 mg. The aqueous fraction was further subjected to vacuum liquid chromatography (vlc) using dichloromethane with increasing concentrations of ethyl acetate and later methanol. Seven fractions obtained were tested for effects on Ach-induced ileum contractions at concentrations of 10, 20 and 40mg and compared with atropine 20 µg. Both the aqueous and dichloromethane fractions significantly ($p<0.0.5$) inhibited the contractile effect of Ach on the isolated ileum. However, the inhibitory effect of the aqueous fraction was more pronounced particularly at 80mg as the C_{max} of Ach was reduced to 11.23 ± 1.00 % in contrast to 58.37 ± 3.16% produced by the chloroform fraction. The vlc fractions (fraction 2 and to some extent fraction 4) obtained from the aqueous fraction produced remarkable relaxant effects on the ileum particularly fraction 2 which at 20mg, completely abolished the contraction produced by the Ach, similar to the effect of Atropine (20 µg). The extract o*f W. indica* especially the aqueous fraction contains bioactive constituents that can be separated and purified for improved relaxant effects on the intestine. The results further provide evidence for the ethno-medical use of the plant in treating diarrhea.

KEYWORDS: ***Waltheria indica*; Aqueous fraction; Ileum contraction.**

INTRODUCTION

Traditionally, plants are used for the treatment of diseases in different parts of the world [1] and their use contributes significantly to primary health care delivery [2]. Diarrhea is a discomforting ailment characterized by hyper-secretion of abdominal fluid and/or increase in intestinal peristalsis leading to frequent passage of loose and watery stool [3]. There are quite a number of synthetic drugs that are used to attenuate the frequency or completely treat the condition. However, quest for more available, potent, safe and cheaper natural materials make the need to advance research into medicinal plants. Many plants are used in the treatment of diarrhea in ethnomedicine and *Waltheria indic*a is one of such plants used in South Western part of Nigeria. It is used for the treatment of sexually transmitted infections, urinary tract infections and a variety of infant illnesses. Its root's extract is reported to be used in treating ailments such as diarrhea, wounds and stomach ache [4]. Whole plants may be used to treat cough, haemorrhage, fever and malaria amongst others [5]. It has also been reported to be of tremendous use in South America and Hawai against pain, inflammation, diarrhea, dysentery,

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conjunctivitis, wounds, abscess, epilepsy, convulsions, anemia, erectile dysfunctions, bladder ailments and asthma [6]. The petroleum ether and methanol extracts of the leaves have been investigated and documented for anti-inflammatory activity [7].

In continuation of our reports on the antidiarrheal effect of the leaf extract of *W. indica* in laboratory animals [8], the present work was carried out to examine the relaxant effects of organic solvent and chromatographic fractions of the extract on isolated intestinal strips.

MATERIALS AND METHODS

Collection and extraction of the plant material

As stated in our earlier report [7], the leaves of *Waltheria indica* were collected in June 2012 by a plant collector, Alabi Najimdeen, at Iwo in Osun State. This was followed by authentication by Dr. Olufemi Soshanya of Forest Research Institute of Nigeria (F.R.I.N) Ibadan where herbarium specimen was deposited and FHI109766 issued as the herbarium number. The leaves were dried for three days at room temperature and further dried in an oven maintained at 50 ^{o}C for 4 h followed by pulverization using a laboratory milling machine (Chris Norris, England, UK).

The powdered plant material (1.5 kg) was extracted using Soxhlet apparatus with 7.5 L of methanol for 4-5 h in a batch of 180-200 g at room temperature. After concentration, the soft extract was kept in a refrigerator maintained at 4 °C until required.

Organic solvent partitioning of the methanol extract

About 90 g of the crude methanol extract was suspended in water and the solution partitioned exhaustively with chloroform (200ml x 5) in a separating funnel until a clear lower layer (chloroform) was obtained. The aqueous and the chloroform fractions obtained were separately concentrated to dryness under pressure using rotary evaporator maintained at 45 ^{o}C.

Source and maintenance of animals

Male albino Wistar rats (180-230 g) were purchased from Physiology Department in Ambrose Ali University Ekpoma, Nigeria. The animals were maintained in the Animal House of the Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Benin with adequate access to food (Topfeeds, Benin City) and water *ad libitum*. They were handled according to international ethics on the use of animals for experiments based on National Institute of Health protocols [9].

Effects of the organic solvent fractions on isolated rat ileum

The Albino Wistar rats were sacrificed using chloroform anesthesia. The abdominal cavity of the animals was immediately opened and the ileum 1-1.5 cm in length was removed and trimmed from surrounding tissue. The contents of the intestine were washed with Physiological Salt Solution (PSS); Tyrode solution composed of sodium chloride 40 g, sodium hydrogen bicarbonate 5 g, d-glucose 5 g, sodium dihydrogen phosphate 0.25 g, potassium chloride 1 g, magnesium chloride 0.5 g, and calcium chloride 1.32 g in 5 L of distilled water. Segments of the ileum were tied with silk threads at both ends (ileum tied in opposite directions) and suspended in a thermo-regulated 25mL organ bath containing Tyrode solution maintained at 37 ^{o}C. The ileum was attached to a tissue holder at the base of the organ bath and the other end to the isometric Ugo Basile channel recorder through an isometric transducer. The tissues were constantly bubbled with 95 % O_2 and 5% CO_2. A suitable weight or resting tension of 760 mg as applied to the individual tissue. The suspended ileum was allowed to equilibrate for 30-45 minutes before adding acetylcholine or the particular plant extract or the standard drugs. After the initial equilibration period, Acetylcholine at concentrations of 20-80 µg was added to the organ bath and the control concentration-response curve for acetylcholine was determined. Each time, acetylcholine was left in contact with the tissues for 30 seconds before adding the next concentration. Then the tissue was washed two times with Tyrode solution at the interval of 90 seconds. The effect of the aqueous fraction on the isolated ileum was evaluated by simultaneous administration of the various concentrations of Ach and 20, 40 and 80 mg of the fraction. Similar procedure was repeated for the chloroform fraction at the same concentrations.

Vacuum liquid chromatography (VLC) of the aqueous fraction

Part of the aqueous fraction obtained (38.50 g) was absorbed on a silica gel (30-70 µm) and loaded in a Sintered Glass (No. 3) attached to a Buckner flask connected to a vacuum pump. The mixture was eluted with 300 mL of dichloromethane with increasing concentration of ethyl acetate (25, 50, 75, 100 %), ethyl acetate with increasing concentrations of methanol (25, 50, 75% in ethyl acetate) and later methanol with 25 % in water. The ten (10) fractions obtained were concentrated and subjected to thin

layer chromatography in chloroform-methanol (3:2) on commercial silica gel plates (GF_{254}). The plates were sprayed with concentrated H_2SO_4 followed by heating in the oven at 110 ^oC for 5 minutes. Based on the thin layer chromatography profiles, the fractions were bulked together to obtain seven (7) fractions as 1 (A, B), 2 (C, D), 3 (E), 4 (F), 5 (G, H), 6 (I), 7 (J) [10].

Effects of the VLC fractions on the Ach-induced contraction

The concentration of Ach (80 µg/mL) that produced the highest contraction was used. The effects of each of the bulked fractions (1-7) were separately evaluated on the isolated ileum at concentrations of 0.1, 0.2 and 0.4 mg/mL which implied 10, 20 and 40 mg. The activities of the fractions were compared with that of atropine 20 µg (obtained from 0.1mL of 200 µg/mL).

Statistical analysis

All the data obtained were expressed as mean ± SEM (standard error of mean) and n represents the number of animals used. Where applicable, the data were compared using one way analysis of variance (ANOVA) on Graph pad InstatR version 2.05a software (UK). $P < 0.05$ was taken as level of significance.

RESULTS

The administration of increasing concentrations of Ach was accompanied with corresponding increase in the contractility of the isolated rat ileum. A maximum contraction C_{max} of 100 % was obtained at Ach concentration of 80 µg/mL. Simultaneous administration of the different concentrations of the Ach with 20, 40 or 80 mg of aqueous fraction of *W. indica* was observed to remarkably and significantly ($p< 0.05$) inhibit the stimulatory effect of the Ach in the rat ileum in a concentration-dependent manner. For example, in the absence of the aqueous fraction, the Ach alone at a concentration of 8 µg/mL induced an ileal contraction of 63.36 ± 4. 07 %. This was reduced to 14.71 ± 0.50, 12.10 ± 0.76 and 5.29 ± 0.50 % respectively in the presence of the 20, 40 or 80 mg of aqueous fraction. Similarly, the C_{max} of the Ach was reduced to 56.86 ± 3.24, 21.43 ± 0.83 and 11.23 ± 1.00 % by the concentrations of the aqueous fraction respectively (Fig.1).

Similar to the result presented above, there was a corresponding increase in the contraction elicited by increasing concentrations of Ach with the C_{max} obtained at concentration of 80 µg/mL. The chloroform fraction equally produced remarkable inhibitory activities against the contractions induced by the various contractions of the Ach on the ileum. A percentage contraction of 90.05 ± 8.62 produced by Ach alone was reduced to 58.99 ± 4.52, 54.05 ± 3.19 and 48.37 ± 2.69 % in the presence of 20, 40, and 80 mg/mL of the fraction respectively. Also, the C_{max} produced at the concentration of 80 µg/mL was reduced to 75.38 ± 4.13, 66.37 ± 4.67 and 58.37 ± 3.16 % in the presence of 20, 40 and 80 mg of the fraction respectively (Fig.2).

The VLC fractions exhibited varying concentration dependent inhibitory effects on the Ach-induced ileal contraction. While fractions 1,3,5,6 and 7 (at concentrations of 10-40mg/mL) were observed to produce slight inhibitory effect on the contraction induced by the Ach, fractions 2 and 4 elicited significant inhibitory activities on the Ach. The presence of 10 mg of fraction 4 reduced the Ach C_{max} to 55.07 ± 1.45 and further reductions in the C_{max} were observed to be 24.64 ± 1.45 and 19.57± 2.18 % in the presence of 20 and 40 mg respectively. However, the Ach C_{max} was reduced t0 15.94 ± 0.00 % in the presence 10mg of fraction 2 while administration of 20 mg of the fraction completely abolished the contraction induced by Ach. Atropine (20 µg) was equally observed to completely block the effect of Ach (Fig. 3).

DISCUSSION

Plant extracts are known to contain different categories or groups of secondary metabolites which are responsible for the various effects observed in humans or animals when administered into them. The constituents may act synergistically with one another or some may possess higher activities than others. In an attempt to know which constituent(s) may be responsible for any activity a plant extract elicits, it is necessary to employ separation methods that will enable the constituents to be separated into different immiscible solvents. This explains the partitioning of the methanol extract of *W. indica* into chloroform and aqueous phases [11].

The constituents of the extract also vary in their polarities which can explain why they possess different dissolution properties in different solvents. Organic solvent partitioning of extracts is usually carried out to ascertain which fraction would possess better activity above the extract particularly with higher effects at similar or even lower concentrations. Such higher activity is an indication of the presence of the active constituents in the fraction which can be said to be in more concentrated form.

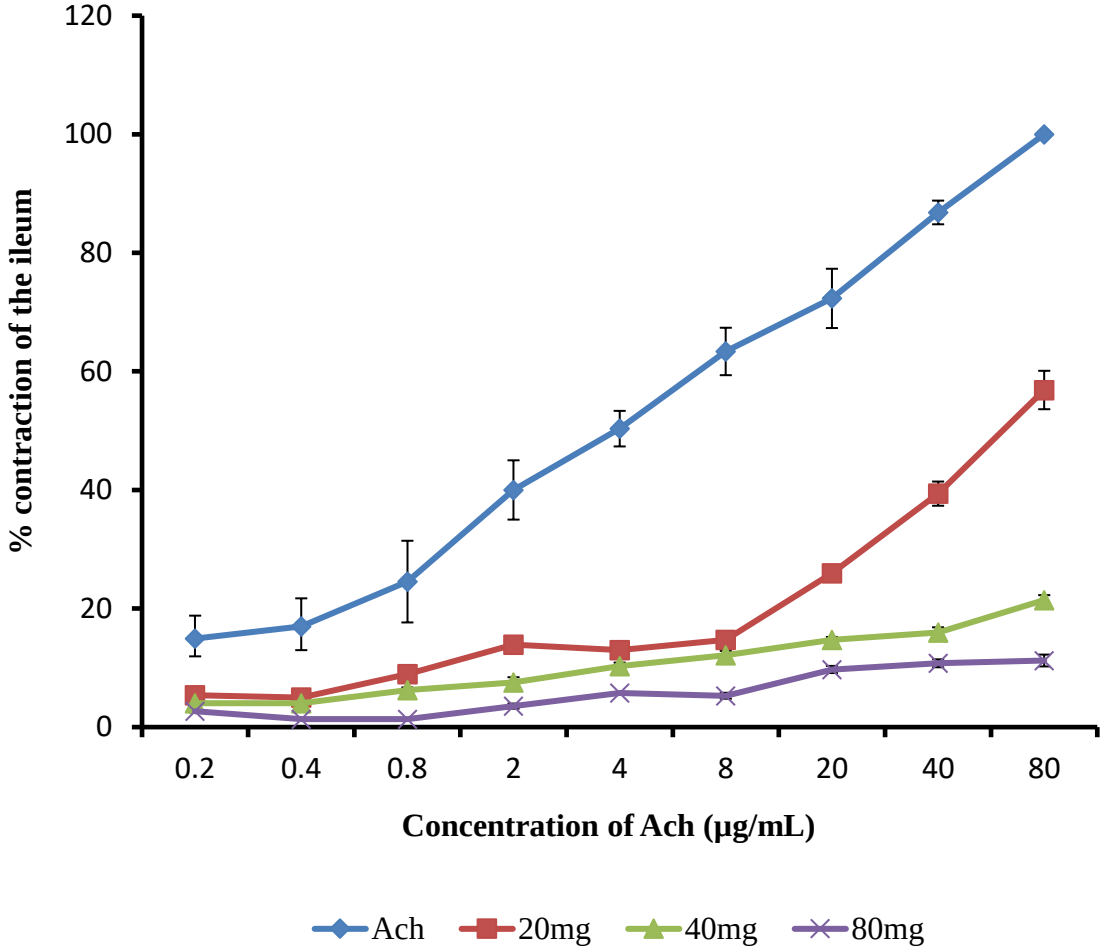

Figure 1: Relaxant effect of the aqueous fraction of *W. indica* on Ach-induced rat ileum contraction. The fraction significantly inhibited the contractile effects of Ach in a concentration-dependent manner.

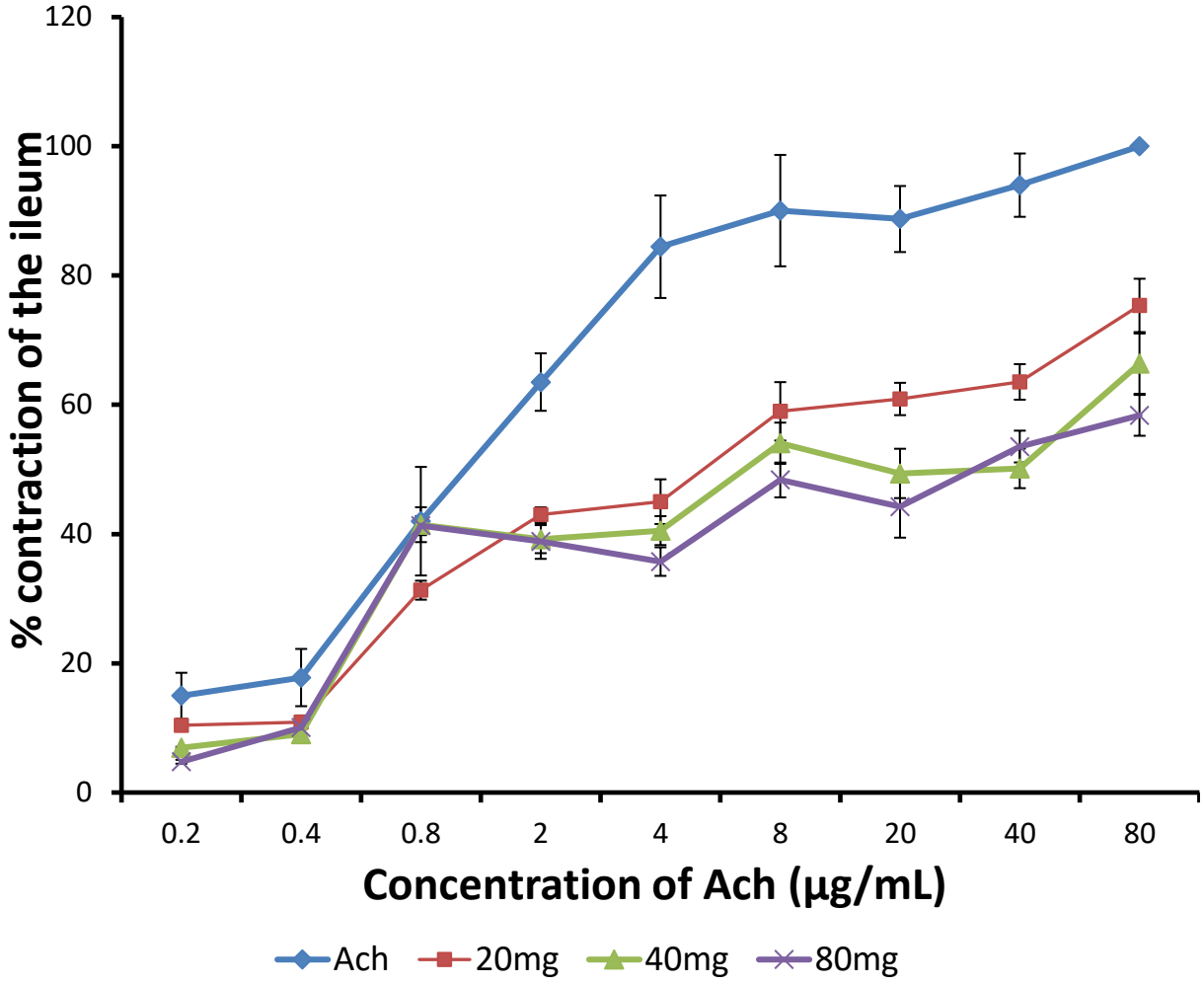

Figure 2: The relaxant effect of the chloroform fraction of *W. indica* on Ach-induced rat ileum contraction. The fraction remarkably inhibited the stimulatory effect of the Ach on the rat ileum.

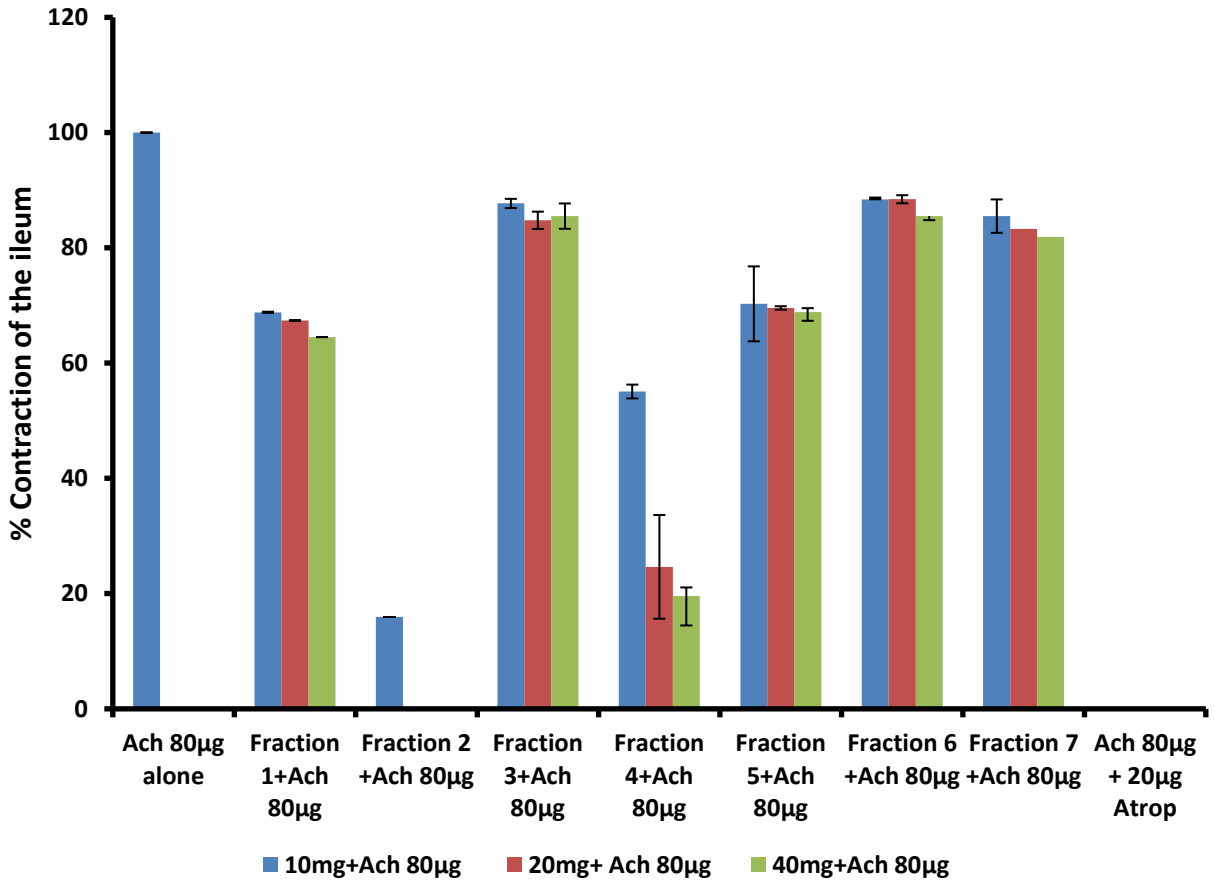

Figure 3: The inhibitory effects of the VLC fractions on the Ach-induced rat ileal contractions. At 20 and 40 mg, Fraction 2 showed the highest inhibitory effect against the contraction induced by Ach while atropine also effected inhibition at 20µg.

This work has shown that the inhibitory effects of the organic solvent fractions of the methanol extract of *W. indica* resulted in remarkable improvement over the crude extract earlier reported [8]. The aqueous fraction was discovered to inhibit the contractions of the rat ileum produced by Ach more than the chloroform fraction at various concentrations. For instance, while the C_{max} produced by 80 µg/mL of Ach alone was reduced to 11.23 ± 1.00 % in the presence of 80 mg of aqueous fraction, similar concentration of the chloroform fraction was observed to reduce the C_{max} to 58.37 ± 3.16 %.

The inhibitory effect of the aqueous fraction was also observed to be more than that produced by the extract in which C_{max} was reduced to 58.37 ± 3.16 % at the same concentration (as noted in our previous reports [7]). Furthermore, the EC_{50} (Effective concentration of Ach required to produce 50 % contraction of the rat ileum) in the presence of Ach alone was calculated to be 1.25 µg/mL, administration of 20, 40, and 80 mg of the chloroform fraction increased the values to 5.25, 6.5 and 33.75 µg/mL respectively. However, at a concentration of 20 mg of the aqueous fraction, the EC_{50} increased to 65.71 µg/mL while higher concentrations (40 and 80 mg) would have required more quantities of Ach. All these point to the fact the aqueous fraction had higher inhibitory effects than the chloroform fraction. This was achieved by separation of chloroform soluble constituents of the crude extract from the more polar ones which are soluble in water. Similar reports have been made on the potency of aqueous fraction of *Brachystegia eurycoma* Harms stem bark over the chloroform fraction [12].

Vacuum liquid chromatography is usually employed to further purify an active fraction by eliminating the seemingly inactive or less active ones. It offers bulk separation of constituents particularly when appropriate choice of solvents is made. In the present work, application of this procedure resulted in the elimination of the inactive and weak fractions thereby producing the most active fraction 2 which reduced the C_{max} of Ach (80 µg/mL) to 15.94 ± 0.00% at a concentration of 10 mg while the contractions were completely abolished by administration of 20 and 40 mg. Fraction 4 also showed ileum relaxant effects but not as effective as fraction 2 at all concentrations. This implied that the major ileum relaxant constituents of *W. indica* can be found in the aqueous fraction and in particular the fractions 2 and 4 obtained from the vacuum liquid chromatography. This again underscores the relevance and significance of partitioning and chromatographic separation in bioactivity guided evaluation of medicinal plants.

Diarrhea is a product of various pathological mechanisms, one of which is the increase in peristalsis leading to cramp feelings as a result of persistent contraction of the ileum which is usually accomplished by stimulation of the muscarinic receptors in the intestine. Ach used in this work is a known and major excitatory neurotransmitter of the enteric nervous system whose effect is mediated by stimulation of M2 and M3 (to a less extent) muscarinic receptors [13,14]. The stimulatory effect of Ach results in increase in both frequency and amplitude of contraction of the intestinal muscles [15]. The effect of Ach is usually antagonized or completely abolished by the administration of atropine depending on the concentration used. The inhibitory effect of atropine on Ach was also established in this work as the ileum contraction induced by the latter was completely nullified with the administration of the former. The fact that the aqueous fraction and the vacuum liquid chromatographic fractions 2 and 4 effectively attenuated or abolished the contraction elicited by Ach implied that the fractions contain high concentrations of constituents that seem to possess anti-muscarinic property like atropine. Although atropine is known to be an alkaloid, the nature of the constituents in these active fractions need to be determined and more work is being carried out to ascertain their nature and identities.

CONCLUSION

Methanol extract of *W. indica* has been established to possess remarkable antidiarrheal property. Although, the chloroform fraction of the extract showed some relaxant effect on isolated ileum, the aqueous fraction was more effective and seems to contain the major antidiarrheal constituents of the plant extract. Application of the vacuum liquid chromatography for further separation of the constituents in the aqueous fraction was observed to narrow the active constituents to fractions 2 and 4 with the former (at very low concentrations) having highly remarkably inhibitory effects to Ach- induced ileum contraction. The results general further justify the ethnomedicinal use of *W. indica* leaves as an antispasmodic or antidiarrheal crude drug. Subsequent reports will definitely reveal the nature and identities of the active constituents in the most potent fractions.

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