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Der Pharmacia Lettre, 2016, 8 (18):207-213
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Anti-diarrhoeal properties of the leaf extracts of *Combretum racemosum* p. Beauv in rodents

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ABSTRACT

To investigate on the anti-diarrhoeal properties of the aqueous and methanol leaf extracts of *Combretum racemosum* in rats. Preliminary phytochemical analysis and acute toxicity study were conducted using standard methods. The effect of the aqueous and methanol extracts of *C. racemosum* on gastrointestinal transit was determined in rats using the Charcoal meal model. The effect of both extracts on castor oil-induced diarrhea was determined. The anti-diarrhoeal activity of the extracts was investigated in the setting of prostaglandin E₂-induced enteropooling in rats. The effect of the extracts on the spontaneous rhythmic contractions of the isolated rabbit jejunum and on acetylcholine and histamine-induced contractions of the guinea-pig ileum were also investigated. Phytochemical analysis revealed the presence of alkaloids, glycosides, carbohydrates, reducing sugars, proteins, saponins, steroids, flavonoids, and terpenoids. Acute toxicity test showed LD₅₀ of >5000mg/kg body weight in mice. A significant reduction in gastrointestinal transit by both extracts was observed when compared with 2.5% dimethylsulphoxide (DMSO) and atropine respectively ($p < 0.01$). The extracts inhibited the spontaneous rhythmic contractions of the rabbit jejunum in a dose-dependent and reversible manner and also inhibited acetylcholine and histamine-induced ileal contractions. Significant inhibition of prostaglandin E₂-induced enteropooling was observed upon treatment with both extracts in a manner comparable to that of the standard antidiarrhoeal drug (loperamide). Both extracts showed significant anti-diarrhoeal activity against castor oil-induced diarrhea. The extracts of *Combretum racemosum* leaves have significant anti-diarrhoeal activity and justifies its traditional use in folk medicine for the treatment of diarrhoea.

Key words: *Combretum racemosum*, gastrointestinal transit, extracts, anti-diarrhoeal, castor oil, Prostaglandin E₂

INTRODUCTION

Diarrhoea is a major health burden particularly in developing countries where sanitation is inadequate. Diarrhoea has led to a significant increase in infant mortality and morbidity particularly in low income settlements [1,2]. Diarrhoea is characterized by the passage of three or more loose motions of stool per day. The incidence of

diarrhoeal diseases has remained astronomical in spite of the efforts organisations to curb diarrhoea. Plants serve as hypothetical sources for new drugs and chemical entities. These plants can be extremely useful as leads for synthetic modification and optimization of biological activity. Drug discovery from plants has become expedient because many allopathic drugs like diphenoxylate, loperamide and antibiotics that are available for the treatment of diarrhoea are not free of side effects. Several studies have evaluated the effectiveness of plant medicines in treating diarrhoea [3-8]. *Combretum racemosum* has been used in folk medicine for many years in eastern Nigeria as an anti-diarrhoeal and anti-ulcer herb. *C. racemosum* is used for the treatment of dysentery, cholera, ulcers, menorrhagia, helminthic infestations, and depression. The anti-ulcer activity of *C. racemosum* has been verified [9].

Combretum racemosum has a powerful folkloric reputation as an anti-diarrhoeal shrub. The identification and evaluation of drugs from natural sources as alternatives is crucial. Consequently, the aim of this study is to verify the anti-diarrhoeal effect of the leaf extracts of *C racemosum*.

MATERIALS AND METHODS

Plant collection and Taxonomy

The leaves of *C. racemosum* were collected from their natural habitat in Ifite-ani village, Agulu in Anambra state of Nigeria in the month of September, 2014. The plant was identified by a taxonomist at the Department of Plant Science and Biotechnology, University of Nigeria, Nsukka. A sample of the plant was deposited at the Department of Plant Science and Biotechnology, University of Nigeria, Nsukka for future reference (UNH/47b). The leaves were dried under the shade to a constant weight and reduced to fine powder with a mechanical grinder.

Extract preparation

Fresh leaves of *C. racemosum* (800 g) were washed with clean water and macerated in 200 mls of distilled water. The macerated leaves were strained through muslin and filtered through a Whatman no. 1 filter paper. The filtrate obtained was freeze-dried. A yield of 8.5% (w/w) of the crude aqueous extract (CAE) was obtained. Another 1000 g of powdered leaves of *C. racemosum* was macerated in 2 litres of methanol for 48hrs. The extract was filtered using a Whatman No.1 filter paper and the filtrate evaporated to dryness using a rotary evaporator (Model type 349/2 Corning Ltd). A yield of 11.7% (w/w) of the crude methanol extract (CME) was obtained. The CME was dissolved in 2.5 % aqueous solution of dimethyl sulphoxide (DMSO) to get the desired concentration for the experiment.

Phytochemical analysis

The crude extract of *C racemosum* was screened for secondary metabolites using standard methods [10].

Animals

Sixty (60) albino wistar rats of either sex weighing 140-180 g, thirteen (13) albino mice weighing 28-43 g, and a rabbit weighing 1.3 kg were obtained from the Animal House of the Faculty of Veterinary Medicine of the University of Nigeria, Nsukka. They were housed in clean gauzed cages under standard condition of temperature (25±3°C) and a 12:12 hour light/dark cycle. The period of acclimatization was two weeks during which the animals were given standard pellets (Guinea-feed Nigeria Plc) and water *ad libitum*. Protocols describing the use of rats and in accordance with the American Physiological Society's guiding Principles for Research involving Animals and Human beings were adhered to [11].

Acute toxicity test

The Lorke procedure for median lethal dose (LD₅₀) determination was used [12].

Gastrointestinal motility test

Thirty (30) albino Wistar rats of either sex were divided into six (6) groups of five (5) rats per group. The animals were starved for 24 hours prior to the experiment but allowed free access to water. Group B and C received 150 and 300 mg/kg body weight of CAE, group D and E received 150 and 300 mg/kg b.w of CME, while A and F received 2.5 % DMSO (5 ml/kg) and 10mg/kg b.w of atropine and served as negative and positive control respectively. All administration was through the oral route. Five minutes after drug administration, 0.5ml of a 10% charcoal triturated in 3% tragacanth was administered to each animal orally. The animals were euthanized thirty (30) minutes later and their abdomen opened. The percentage distance of the intestine (from the pylorus to the caecum) traveled by the charcoal diet marker in the treatment groups was determined as described by Akah *et al* [13].

Guinea-pig ileum preparation

A guinea pig was starved of food for 24 hours but allowed access to water. The guinea pig was euthanized and the ileum taken out. The ileum was cut into smaller pieces of about 2.0 cm and transferred into a shallow dish containing Tyrode's solution and aerated. The organ bath was properly washed and filled to the 30 ml mark with Tyrode's solution of the following composition (mM): NaCl 12, KCl 2.7, CaCl₂ 1.3, NaHCO₃ 12, MgCl₂ 0.5, Na₂HPO₄ 0.14, and glucose 5.5, from the reservoir. The preparation was set up as described by Anonymous [14]. The preparation was allowed to equilibrate for 60 minutes during which the bathing fluid was changed every 10 minutes to prevent the accumulation of toxic metabolites. The organ bath was maintained at 37°±1°C. The contractions evoked by the agonists-acetylcholine and histamine were recorded. The effects of the CAE and CME of *C. racemosum* on the isolated guinea pig ileum were determined. The effects of CAE and CME of *C. racemosum* on acetylcholine and histamine induced ileal contractions were also investigated. Each agonist/antagonist was used on a separate tissue and three separate determinations were made for each agonist/antagonist. The responses were recorded using a writing lever connected to a kymograph and stimulator (Bioscience 400 Kent Sheerness, UK).

Studies on the rabbit jejunum

A rabbit weighing 1.0kg was starved of food but not water overnight and sacrificed. The jejunum was obtained and transferred to a dish containing Tyrode's solution. A piece of jejunum, 2.5cm long, was mounted in a 20ml organ bath containing Tyrode's solution of the following composition (mM): NaCl 136.8, KCl 2.7, CaCl₂ 1.3, NaHCO₃ 12, MgCl₂ 0.5, Na₂HPO₄ 0.14, and glucose 5.5 using standard procedure [14]. One end of the tissue was tied to a transducer connected to a two-way channel recorder. The preparation was maintained at 37°±1°C and aerated. A tension of 1.0 g was applied. A 60 minute equilibration period was allowed during which the physiological solution was changed every 15 minutes. At the end of the equilibration period the effects of increasing concentrations of the crude extracts (CAE and CME) on the spontaneous contractions of the jejunum were evaluated. Responses were recorded using the isotonic transducer, 7006 (Ugo Basile, Italy) connected to the PowerLab recording device (LabChart Pro 6.0- ADI instrument). Determinations were done in triplicate.

Castor oil induced diarrhea

Male rats (140-185g) were employed for this evaluation. They were all screened initially by administering 1.0ml of castor oil orally and only those with demonstrable diarrhoea were used for the study. Six groups of five (5) animals each were employed. All groups received castor oil at a dose of 1ml/animal orally [15]. Thirty minutes after castor oil administration, animals in group A received 5 ml/kg of 2.5% DMSO, group B received 150 mg/kg CAE, group C received 300 mg/kg CAE, while the fourth, fifth, and sixth groups received 150 mg/kg CME, 300mg/kg CME, and diphenoxylate (10 mg/kg) respectively. Drug administration was by the oral route. After this administration, the animals were placed separately in metabolic cages with filter paper, which was changed every hour. The severity of diarrhoea was assessed each hour for 6 hours. The total number of faeces and diarrhoeal faeces defaecated and the total weight of faeces were recorded within a period of 24 h and compared with the control group. The total number of diarrhoeal faeces of the control group was considered 100%. The results were expressed as a percentage of inhibition of diarrhoea [16].

Prostaglandin-E₂ induced enteropooling

The effect of the extracts on Prostaglandin- E₂ induced enteropooling was investigated using the method described by Rao *et al.* with slight modification [17]. Five groups of male albino rats of five (5) animals each (190-220) were fasted for 18 hours before the experiment but had access to water. Group A was treated with 5ml/kg 2.5% DMSO orally followed by intraperitoneal injection of 1ml of 5% ethanol in normal saline and served as normal control. Group B was treated with Prostaglandin E₂ (100 µg/kg) p.o., Group C and D received 150 and 300 mg/kg of aqueous extracts of *C. racemosum* through the oral route while Group E and F received graded doses of CME orally respectively. Animals in group G received Loperamide (2mg/kg p.o.). Thirty minutes later, all the rats except those in group A were treated with Prostaglandin E₂ (100µg/kg in 5% ethanol in normal saline, i.p.). These rats were sacrificed 30 minutes after the administration of Prostaglandin E₂. The intestine (from pylorus to the caecum) was dissected and the contents milked into a graduated cylinder and measured. The percentage reduction of intestinal secretion (volume) was calculated.

Statistical analysis

Data were analyzed with the student's t-test. The results were expressed where appropriate as mean ± standard error of mean. Mean values of test groups were compared with those of control groups and regarded as significant at P<0.05.

RESULTS

Phytochemical screening

The phytochemical analysis of the plant leaves revealed the presence of alkaloids, saponins, flavonoids, terpenoids, glycosides, resins, and steroids.

Acute toxicity test

None of the animals (mice) died after receiving an oral dose of 5,000 mg/kg body weight.

Gastrointestinal motility test

Both CAE and CME produced significant inhibition of gastrointestinal transit when compared with the control group ($p < 0.05$; $p < 0.001$). The inhibition was dose dependent. This inhibition was also comparable with that of atropine - a standard anti-spasmodic agent (Table 1).

Table 1: The effect of the extracts of *C. racemosum* on gastrointestinal transit in rats

Drug	Dose (mg/kg)	% gastrointestinal movement.	% inhibition of gastrointestinal movement.
2.5% DMSO	10 ml/kg	93.6 $\pm$ 1.39	-
Atropine	10	40.08 $\pm$ 2.90**	56.4
CAE	150	44.60 $\pm$ 1.81**	52.4
CAE	300	26.70 $\pm$ 2.26***	71.5
CME	150	32.80 $\pm$ 1.54***	65.0
CME	300	18.40 $\pm$ 1.91***	80.3

*Data expressed in mean $\pm$ SEM, ** $p < 0.01$; *** $p < 0.001$ when compared with the negative control (2.5% DMSO) group. CAE: Crude aqueous extract; CME: Crude methanol extract*

Effect of the extracts on isolated rabbit jejunum

The extracts (CAE and CME) produced inhibition of the intrinsic rhythmic contractions of the isolated rabbit jejunum in a dose-dependent and reversible manner (Figure 1).

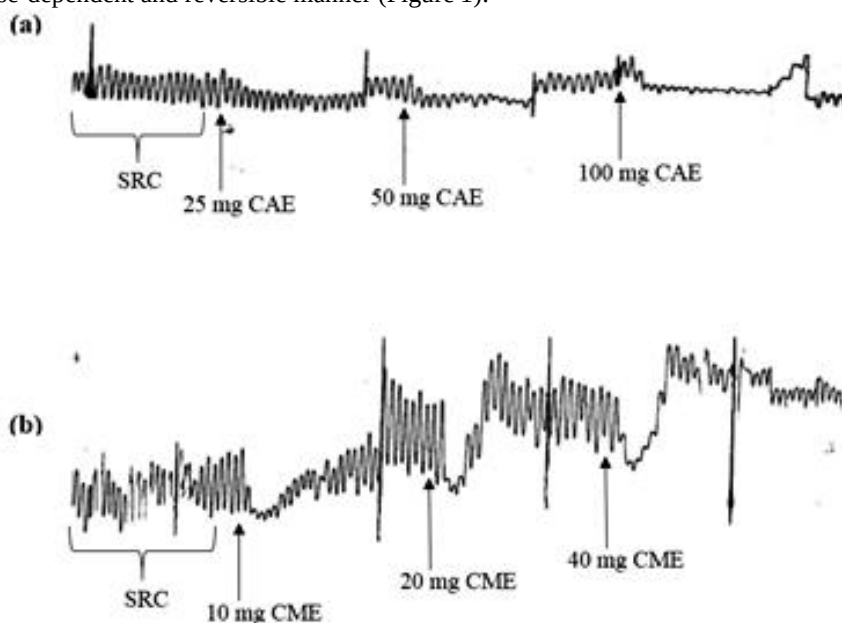


Figure 1: Effect of the aqueous (a) and methanol (b) extracts of *C. racemosum* on the spontaneous rhythmic contractions of the rabbit jejunum. [SRC - Spontaneous rhythmic contractions; CAE – Crude Aqueous Extract; CME – Crude Methanol Extract]

Effect of the extracts on isolated guinea-pig ileum

Both extracts did not evoke contractile responses from the isolated guinea-pig ileum. However, both extracts antagonized acetylcholine and histamine induced contractions in a dose-dependent and reversible manner (Tables 2 and 3). The CME and aqueous extracts had IC_{50} values of 131 μ g/ml and 31.60 μ g/ml respectively against acetylcholine-induced contractions. Also, the IC_{50} values against histamine-induced ileal contractions were 84.58 mg/ml and 329.97 μ g/ml for the aqueous extract and CME respectively.

Table 2: Effect of the aqueous and methanol extracts on acetylcholine (ach) induced contractions of the guinea-pig ileum

Agonist (20µg) + Dose of extract	% inhibition of maximal response	
	CAE	CME
10µg	2.88 ± 0.60	20.36 ± 0.48
20µg	9.37 ± 0.44	43.52 ± 0.37
40µg	24.13 ± 0.65	60.00 ± 0.85
80µg	40.92 ± 1.18	74.29 ± 0.40
160µg	55.47 ± 0.63	81.30 ± 1.35
320 µg	64.12 ± 0.71	90.16 ± 0.54
640 µg	84.17 ± 0.65	-
	IC ₅₀ = 131 µg/ml, r ² = 0.9877	IC ₅₀ = 31.60 µg/ml, r ² = 0.9586
(CAE: Crude aqueous extract; CME: Crude methanol extract)		

Table 3: Effect of the aqueous and methanol extracts on histamine induced ileal contractions

Histamine (2µg) +Doses (mg) of extract	% inhibition of Maximal response.	
	CAE	CME
2	5.40 ± 0.20	5.20 ± 0.15
4	9.80 ± 0.42	18.70 ± 0.42
8	12.70 ± 0.50	30.48 ± 0.80
16	20.00 ± 0.38	38.00 ± 0.70
32	29.62 ± 0.84	44.50 ± 0.52
64	40.10 ± 1.09	53.40 ± 0.81
128	69.80 ± 0.35	79.64 ± 0.38
	IC ₅₀ = 84.58 mg/ml, r ² = 0.8650	IC ₅₀ = 329.97 µg/ml, r ² = 0.9557
(CAE: Crude aqueous extract; CME: Crude methanol extract)		

Effect of the extracts on castor oil induced diarrhea

The CAE and CME were found to be effective against castor oil induced diarrhoea on experimental rats at two dose of 150 and 300 mg/kg body weight (Table 4). Single oral doses of *Combretum racemosum* extracts of 150 and 300 mg/kg body weight produced significant decrease in the severity of diarrhoea in terms of reduction in the rate of defecation and consistency of faeces in albino rats. The percentage inhibition for the number of wet faeces as well as wet mass indicates the presence of antidiarrhoeal activity in extract when compared with that of the control group. Experimental results indicate that the activity was more pronounced at the dose of 300 mg/kg body weight. The percentage of inhibition of number of wet faeces as well as wet mass by the methanol extract (300 mg/kg) were found to be 71.63% and 67.19% respectively while that of the aqueous extract (300 mg/kg) were found to be 63.55% and 62.69% respectively. These were significant (p<0.001) and very much comparable to that of standard drug diphenoxylate (80.37% and 69.66% respectively; Table 5).

Table 4: Effect of the extracts on prostaglandin-E₂ induced enteropooling

Group	Dose (mg/kg)	Volume of intestinal content (ml)	% reduction of intestinal secretion (volume)
3% Tween 80 + 5% ethanol in saline	10ml/kg + 1ml	4.94 ± 0.15	
3% Tween	10ml/kg	7.38 ± 0.16	
CAE	300	3.55 ± 0.17	51.9*
CME	300	1.81 ± 9.354E-02	75.5*
Loperamide	2	2.16 ± 0.12	70.7*

* P < 0.001; CAE: Crude aqueous extract; CME: Crude methanol extract

Table 5: Effect of the extracts on castor oil induced diarrhea

Group	Dose (mg/kg)	Total number of Faeces	Total number of diarrhoeal faeces	% inhibition of diarrhoeal faeces	Total weight of Faeces (g)	% inhibition of Wet Mass.
Castor oil + 3% Tween 80 (negative control)	1ml + 10ml/kg	27.80 ± 1.75	21.40 ± 1.32	-	8.90 ± 0.54	-
CME	150	20.20 ± 1.16**	9.00 ± 0.71***	57.94***	4.80 ± 0.57**	46.07**
	300	18.20 ± 1.85***	6.20 ± 1.39***	71.03***	2.92 ± 0.38***	67.19***
CAE	150	23.80 ± 1.28	11.80 ± 0.80**	44.85**	4.16 ± 0.71***	53.25***
	300	19.20 ± 1.16***	7.80 ± 0.66***	63.55***	3.32 ± 0.46***	62.69***
Diphenoxylate	10	9.00 ± 1.00***	4.20 ± 0.58***	80.37***	2.70 ± 0.51***	69.66***

Data expressed in mean ± SEM, *p<0.05; **p<0.01; ***p<0.001 when compared with the negative control. CAE: Crude aqueous extract; CME: Crude methanol extract

Effect of the extracts on prostaglandin E₂ induced enteropooling

The aqueous and methanol extracts at dose level of 300 mg/kg significantly inhibited prostaglandin E₂-induced enteropooling in terms of volume of intestinal content and in a manner comparable to that of the standard antidiarrhoeal drug – loperamide (2 mg/kg b.w).

DISCUSSION

Anti-diarrhoeal drugs attenuate gastrointestinal movement and/or gastrointestinal secretions. Ricinoleic acid is the effective agent of castor oil formed in the upper small intestine. The substance is poorly absorbed and consequently induces drastic changes in electrolyte movement, mucous epithelial absorbency, and intestinal activity. This ultimately evokes a hypersecretory response and diarrhoea. Interaction of ricinoleic acid with sodium and potassium in the intestinal tract lead to the formation of ricinoleate salts and these salts are probably responsible for diminution in the absorptive function of the intestinal mucosa. Ricinoleate salts inhibit sodium-potassium ATPase, stimulate adenyl cyclase, release prostaglandins or some other metabolite of arachidonic acid, and act as calcium ionophore [18]. As a calcium ionophore, ricinoleate brings about the influx of extracellular calcium and this activates calmodulin-dependent secretory mechanisms [19]. Ricinoleic acid evokes the release of prostaglandins (PG) due to its local inflammatory and irritant actions. The release of PG, particularly PGE₂, results in decrease in absorption and an increase in the net secretion of water and electrolytes into the small intestine [3]. Agents that inhibit PG biosynthesis or release delay castor oil-induced diarrhoea.

Diarrhoea results from loss of equilibrium between the secretory and absorptive mechanisms in the intestinal tract accompanied by urgency resulting in an excess loss of fluid and electrolytes in the faeces with consequent electrolyte depletion. Hypermotility predominates in some other types of diarrhoea. In this study, the aqueous and methanol extracts of *C racemosum* exhibited significant anti-diarrhoeal activity against castor oil-induced diarrhea in rats. The results were similar to that of the standard drug diphenoxylate (10 mg/kg) with respect to the severity of diarrhoea. The anti-diarrhoeal action of both extracts could be due to increased absorption or decreased secretion as well as prevention of PG release by ricinoleic acid. It has been shown that E type prostaglandins cause diarrhoea through their effects on gastrointestinal motility, glucose absorption, water and electrolyte movement [20,21]. Both extracts significantly reduced prostaglandin E₂-induced enteropooling as evident from the marked reduction in the volume of the intestinal contents in a manner comparable to the reference drug, loperamide. Apart from the regulation of the gastrointestinal tract, loperamide has been reported to decrease colon flow rate and attenuate transit in the small intestine [22]. It is possible that the aqueous and methanol extracts of *C racemosum* produced their anti-diarrhoeal action by reduction in the synthesis or action of PGE₂. Moreover, the extracts significantly reduced gastrointestinal transit in rats in an analogous manner to atropine- a standard anticholinergic agent. Cholinergic stimulation causes diarrhea by increasing gastrointestinal (GI) motility. The probable mode of action for the observed significant inhibition of GI motility by the extract, is possibly the prevention of cholinergic transmission or by an anti-cholinergic effect on the epithelium of the gastric mucosa [23]. In-vitro pharmacological studies showed that the aqueous and methanol extracts of *C racemosum* inhibited guinea-pig ileal contractions produced by acetylcholine and histamine in a dose-dependent and reversible manner. The methanol extract was more potent against acetylcholine and histamine-induced ileal contractions. The extracts also, dose-dependently, inhibited the intrinsic rhythmic contractions of the isolated rabbit jejunum. This inhibition was remarkable for the methanol extracts and most importantly reversible - a desirable quality of a potent and novel antispasmodic agent [24]. These actions show that the extracts possess spasmolytic, anti-cholinergic, anti-histaminergic, and possibly anti-secretory mechanisms of action. Phytochemical screening revealed the presence of saponins, steroids, terpenoids, alkaloids, flavonoids, reducing sugars, proteins, and glycosides. Earlier studies showed that anti-dysenteric and anti-diarrhoeal properties of medicinal plants were due to alkaloids, saponins, flavonoids, steroids and/or triterpenes [3,25,26]. The anti-diarrhoeal activity of flavonoids has been attributed to their capability to inhibit intestinal motility and hydro-electrolyte secretion which are altered in the diarrhoeal state. Experiments have shown that flavonoids inhibit the intestinal secretory response induced by prostaglandin E₂. In addition, flavonoids have antioxidant properties which are thought to be responsible for the inhibitory effects exerted upon several enzymes particularly cyclooxygenase 2 [18]. The spasmolytic effects of flavonoids may be considered a nonspecific action since they were also observed to inhibit BaCl₂-induced contractions, electrically-induced contractions and those induced by a plethora of agonists, such as acetylcholine, serotonin, histamine and certain prostaglandins [27]. Alkaloids are known to inhibit the release of autocoids and prostaglandin, thereby inhibiting the secretion induced by castor oil. Sesquiterpenes, diterpenes, terpenes and terpenoid derivatives are have all been reported to inhibit the release of autocoids and

prostaglandins [28]. Therefore, these may be responsible for the mechanism of the anti-diarrhoeal activity of both extracts.

In conclusion, the results of this investigation revealed that both extracts of *Combretum racemosum* contain pharmacologically active substance(s) with anti-diarrhoeal properties. These properties confirm the use of *C. racemosum* as an anti-diarrhoeal drug by traditional healers in south-east of Nigeria. Further research is currently on-going in our laboratory using bioassay guided phytochemical and pharmacological studies in order to characterize the active principle(s).

Conflict of Interest Statement

The authors declare that there are no conflict of interest.

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