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Evaluation of Phytochemical and Anti-diarrheal Activity of Methanol Stembark Extract of *Combretum hypopilinum* Diels (Combretaceae)

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of article.

Abstract

Introduction: The plant *Combretum hypopilinum* has many medicinal uses in Africa. Infusion of fresh or dry leaves are commonly taken as cholagogic, diuretic, and purgative and also to treat gastrointestinal disorders, including diarrhoea, dysentery, and stomach aches

Objective: To carry out phytochemical and antidiarrheal studies on the methanol stembark extract of *C. hypopilinum*.

Methods: The *C. hypopilinum* stembark (1kg) were extracted with methanol using cold maceration method for 9 days. The solvent was removed by rotary evaporator and the extract was partitioned with n-hexane, chloroform and ethyl acetate to give the n-hexane, chloroform, and ethyl acetate fractions respectively. The extract was subjected to preliminary phytochemical screening, acute toxicity study and antidiarrheal activity using animal models.

Result: The following metabolites: carbohydrates, alkaloids, phenol, steroids, terpenes, saponins, flavonoids and tannins were revealed when the phytochemical screening of the plant extract was carried out. The oral median lethal dose (LD_{50}) of crude extract in mice and rats was estimated to be above 5000 mg/kg. The antidiarrheal activity carried out at dose 150, 300 and 600 mg/bw i.p. using castor oil induced diarrheal, gastric-transit time and anti-enteropooling tests in mice and rats. The extract significantly ($P<0.05$) inhibited diarrheal in mice and rats with highest protection/ inhibition of 93.1% and 87.9% at doses of 600 and 300 mg/kg (dose dependently) which compared with atropine sulphate (86.2%) respectively, in the castor-oil induced diarrheal test in mice. The extract also significantly ($P<0.05$) inhibited diarrheal in charcoal meal test with the highest % protection of 24.38% at dose 600 mg/kg and compared with loperamide (28.80%). The anti-enteropooling test in rats showed 68% inhibition of diarrheal as compared to atropine sulphate with 78% inhibition.

Conclusion: The results indicated that the methanol stembark extract of *Combretum hypopilinum* possesses significant dose dependant antidiarrheal activity ($p<0.05$) in all the antidiarrheal tests. Thus, the traditional use of the plant for the treatment of diarrheal is highly justified in this study.

Keywords: Antidiarrheal,*Combretum hypopilinum*, Phytochemical constituents and Stembark.

INTRODUCTION

Natural product is a chemical compound or substance produced by living organism. In the broad sense, it includes any substance produced by life (Samuelson, 1999). Natural product can also be prepared by chemical synthesis and have played a central role in the development of the field of organic chemistry by providing challenging synthetic targets (Natural product foundation, 2013). Within the field of organic chemistry, the definition of natural product is usually restricted to mean purified organic compound isolated from natural sources (Hanson, 2003). Within the medicinal chemistry, the definition is often further restricted to secondary metabolites (William and Lemke, 2002). Secondary metabolites are not essential for survival, but nevertheless provide organisms that produce them with an evolutionary advantage (Maplestone, *et al.*, 1992). Natural

products have pharmacological activities that can be useful in treating various kinds of diseases, and may act as active substances for both traditional medicine and modern medicines (Raafat, 2013). Recently, there has been a great interest in herbal remedies for the treatment of number of ailments including diarrhoea (Viswanatha, *et al*., 2007).

Combretum hypopilinum of combretaceae family consist of 18 genera, the largest of which are combretum with about 370 species and terminalia with about 200 species (Gedson, *et al.*, 2002 and Lawrence, 1951). The plant *Combretum hypopilinum* was found to contain choline, vitexin, oxalic, mallic and gallic acid (Kaita and Muazu, 2008). The Protective effect of *Combretum hypopilinum* (Diels) root bark extract against CCl4-induced hepatotoxicity in wistar rats (Agbonon, *et al.,* 2018), have been studied

Diarrhea is defined as the passage of liquid or watery stools at least three times in 24-hours period or more frequent passage than its normal for the individual (WHO, 2013) or presence of blood and mucus as a result of an imbalance between the absorptive and secretory mechanisms in the intestinal tract (Mathan, *et al*., 1998). Base on clinical syndrome and duration there are basically four types of diarrhea: Acute watery diarrhea, acute bloody diarrhea (dysentery), persistence diarrhea and chronic diarrhea. Acute diarrhea is characterized by abrupt onset of frequency, water loose stool usually without visible blood which lasts less than fourteen days in duration (most episodes subside within seven days of onset). Although it is often mild, acute diarrhea can lead to severe dehydration as a result of large fluid and electrolytes loss. Ninety percent of acute diarrhea result from infection causes usually virus. Medication such as antibiotics and drugs that contain magnesium product are also common offender.

Recently dietary changes can also cause acute diarrhea. This includes coffee, tea, dietetic foods, gum that contains poorly absorbable sugars. Traveling to developing area of the world can result in exposure to bacterial pathogens common in certain areas, and also contaminated foods (Fletcher *et al.*, 2007).

Acute bloody diarrhea or dysentery involves diarrhea with visible blood and mucus in the feces, symptom includes anorexia, rapid weight loss and damage to the intestinal mucosa by invasive bacteria. The most important causes is *Shigella dysenteriae*, however it is *Shigella flexneri* that is the chief cause of endemic shigellosis in developing countries. Other causes of dysentery include *Campylobacter jejuni* and *Salmonella* (non typhoid specie).

Chronic diarrhea refers to diarrhea which is recurrent or long lasting due mainly to noninfectious causes. It may be caused by gastrointestinal disease, may be secondary to systemic disease, and may be psychogenic in nature (Biu, 2006). Medicinal remedies prepared from the indigenous plants are mostly the only readily accessible and affordable therapies for the control of diarrhea in many rural communities in developing countries (Green, *et al.,* 2011). There are some plants such as *Moringa olefeira*, *Ceramonda riliquar* commonly called locust beans (Lahssini, *et al.,* 2015*), Pudium guajava* commonly called guava (Kamath, *et al.,* 2014), *Catharanthus ruseus* (Njume and Godica, 2012) and *Scourine gaverosa* (Magaji, *et al.,*2007) have been used for the treatment of diarrhea. Some of these plants have been scientifically validated with active components isolated such as tannin, alkaloids saponins, flavonoids, steroids and terpenes (Teke, *et al.,* 2010 and Ojewole, *et al.,* 2008). Unfortunately, the drugs used for the treatment of diarrhea are not completely free from adverse effects ranging from mild to severe including, dryness of the mouth, drowsiness, dizziness, severe pain in stomach, abdominal bloating and vomiting, constipation, anxiety, confusion, depression, causes headache and muscle spasm (Brunner.,2010), and microorganisms sometimes tend to develop resistance towards the antibiotics used as anti-diarrhoeal drug (Soberon, *et al.,* 2007).Its therefore become necessary to design and investigate the ethno-medicinal claim of the plant for the treatment of diarrhea and to contribute to drug development by isolating and characterizing the compound(s) therein.

METHODOLOGY

Solvents used were of analytical grade which were methanol, hexane, chloroform, ethyl acetate and acetone. The reagents used were freshly prepared and include those for phytochemical screening such as, Molish reagent, Meyers reagent and Borntragers reagent, Syringes and needles, Mortar and pestle, Sample bottles, Beakers, separating funnel and conical flask of Pharmaceutical and Medicinal Chemistry laboratory, Ahmadu Bello University, Zaria.

Collection and identification of plant material

The stembark of *Combretum hypopilinum* plant sample was collected in Galadimawa, Giwa local government area, Kaduna state, botanical and authentification was made at the herbarium unit of the department of biological sciences, Ahmadu Bello

University, Zaria by Mallam Namadi Sanusi with the voucher specimen number of V/N 12063 deposited for future reference. Stem bark of the plant were air-dried under shade and later pulverized using mortar and pestle.

Preparation, Extraction and Partitioning of the Plant Material

The powdered stembark (1 kg) was extracted with methanol using cold maceration method for 9 days with occasional shaking. The solvent was removed using rotary evaporator and percentage yield was calculated. The extract (240g) was suspended into1000 ml of distilled water to obtain the water soluble and water insoluble parts. The water insoluble part was partitioned with n-hexane, chloroform, ethyl acetate and acetone to give the n-hexane, chloroform, ethyl acetate and acetone fraction respectively

Preliminary phytochemical screening

The method of Sofowora (1984) and Silva *et a*l. (1998) were applied for the detection of plant secondary metabolites of the methanol stembark extract of the plant.

Experimental Animals

Apparently, 50 locally breed Swiss albino mice of either sex (19-27g body weight) and 25 rats of either sex (50-70g body weight) were acquired from Animal House facility of the Department of Pharmacology and Therapeutics Ahmadu Bello University, Zaria, Nigeria. The animals were fed with laboratory diets and water *ad libitum* and maintained under standard conditions in cage at room temperature.

Drugs and dosages

The chemicals and drugs administered were loperamide, atropine sulphate, castor oil, charcoal meal, normal saline and *C.hypopilinum* methanol stem bark extract (150, 300 and 600 mg/kg extract)for the mice and rats, all were administered intraperitorially (i p).

Protocol was approved by institutional animal ethical committee according to Ahmadu Bello University academic guidelines for the use and care of experimental animals. All experiment was conducted during the day time.

In-vivo Antidiarrheal Studies

Castor Oil Induced Diarrhea

The experiment was performed according to the method described by Awouter, *et al.,* 1978). Mice were fasted for 18 hours which were randomly divided into five group 0f five mice each. Animals in group i-iii received stembark extract of *C. hypopilinum* (150, 300 and 600 mg/kg respectively) and group iv received atropine sulphate (0.5 mg/kg) while the group v received normal saline (10 ml/kg). One hour after administration all mice received 0.5 ml castor oil and then were placed individually in cages whose floor was lined with pre-weighed filter paper. Observation was made for four hours based and the numbers of faeces (wet and dry) were counted per one-one hour. Percentage protection against diarrhea was calculated with respect to the number of wet feces using the formula below.

WFC = wet of feces in negative control group

WFE = wet of feces in test group

Intestinal Motility Test

Intestinal motility test was done according to the method of Mascolo*et al.* (1994). The twenty five mice were fasted for 12 hours and divided into five groups of five mice each. Animals in group i received normal saline 10 ml/kg p.o, group ii-iv received graded dose of stembark extract of *C. hypopilinum* (600, 300 and 150 mg respectively) while group v received loperamide (3 mg/kg). Sixty minutes after administration each animal was given 0.33 ml charcoal meal (3% activated charcoal suspended in 0.3 g acacia). All mice were sacrificed after 60 minutes of charcoal meal administration by cervical dislocation. The small intestines were then removed, extended and placed lengthwise on a moist paper horizontally on a clean surface. The distance in centimeters travelled by the charcoal meal (serving as a marker) from the pylorus was measured, and the whole length of the intestine from pylorus down to the caecum was equally measured.

The periodic index (PI)/Intestinal transit was calculated as a percentage of distance travelled by charcoal meal relative to the length of the intestine.

DPC = distance from pylorus to caecum

A=movement of charcoal meal in negative control group
B = movement of charcoal meal in test control group

Enteropooling Test

The method described by Robert *et al*. (1976) was adopted. Twenty-five rats were fasted overnight and then randomly divided into five groups of five rats each. Group i-iii (150, 300 and 600 mg/kg extract), group iv pre-treated with atropine sulphate as positive control while group v received 10ml/mg normal saline, Sixty minutes later 0.5 ml castor oil was administered to all animals and 60 minutes after all the rats were sacrificed by light ether anesthesia and their small intestines removed, the intestine content were collected by milking into a graduated syringe, and the volume measured and recorded. Volume obtained from negative control group (group v) was used to compare with the rest of the groups. Values less than the negative control groups were considered as protection from diarrhea.

Statistical analysis
The results were expressed as Mean±SEM and the mean values of control groups were compared with the mean values of the treated groups using one-way ANOVA followed by post hoc Dunnets t-test for multiple comparison. The results obtained were considered statistically significant at (P< 0.0

Results
Result of Preliminary Phytochemical Screening
The preliminary phytochemical screening of the methanol stem bark extract of *C. hypopilinum* revealed the presence of the carbohydrates, saponins, flavonoids, glycosides, tannins, phenol steroids terpenes and alkaloids as shown in Table 1 below.

Table 1: Result of Phytochemical Constituents of ME, HF and EA

Constituent	Tests	¤ Observations	ME	HF	EA	F
Carbohydrates	a. Molisch`s test	Reddish colour	+	-	-	-
Alkaloids	b Fehling`s test	Red precipitate	+	-	+	+
	a Mayer`s test	White-yellow ppt	+	-	+	+
Steroida &Terpenes	b. Dragendorf`s test	Orange-brown ppt	+	-	-	-
	a. Lieberman-Buchard	Brown ring at interface	+	+	-	-
	b. Salkowski test	Reddish colour	+	+	-	-
Flavonoids	a.Ferric chloride test	Green ppt.	+	-	+	+
	b.NaOH test	Yellow colour	+	-	+	+
Anthraquinones	a. Borntrager`s test	Pinkish colour	-	-	-	-
Saponins	b. Frothing test	Froth persist for 15 minutes	+	-	+	+

Present = +
Absent = -

Result of acute toxicity studies
The oral median lethal dose (LD_{50}) of the methanol stembark extract was found to be greater than 5000 mg/kg

Castor oil –Induced Diarrheal
This result of castor oil-induced diarrheal is shown in table 4

Table 2: Effect of methanol crude extract (MCE) on Castor oil - Induced Diarrhea in Mice

Treatment	Dose mg/kg	Mean no. of Wet Feaces	Mean no. of Dry Feaces	% inhibition
Normal saline	10 ml/kg	2.90 ± 0.19	0.20±0.09	0
MCE	150	1.90 ± 0.39	1.00±0.11	34.5
MCE	300	0.35 ± 0.19*	1.50±0.22	87.9
MCE	600	0.20 ± 0.14*	1.55±0.18	93.1
Atropine Sulphate	0.5	0.40± 0.15*	1.90±0.24	86.2

Values presented as Mean ± SEM. Data analyzed using one-way ANOVA followed by Dunett'sPostHoc test. *Significant at $p<0.05$ when compared to the NS group. n = 5.

Gastro-Intestinal Motility Test

This result of gastro intestinal motility test using charcoal meal is shown in Table 5

Table 3: Effect of methanol crude extract (MCE) on Gastric Transit Time in Mice

Treatment	Dose mg/kg	Length of Intestine	Movement of Charcoal (cm)	% Protection
Normal saline	10 ml/kg	42.00 ± 1.40	42.00±1.39	0.00
MCE	150	43.00 ± 2.04	42.42±0.2.58	0.00
MCE	300	44.7 ± 1.52*	38.24±1.37	8.90
MCE	600	46.1 ± 2.47*	31.76±3.67	24.38
Loperamide	0.5	42.2± 1.68*	29.90±4.55	28.80

Values presented as Mean ± SEM. Data analyzed using one way ANOVA followed by Dunett's Post Hoc test. *Significant at $p<0.05$ when compared to the NS group. n = 5.

Anti-enteropooling Test

This result of anti-enteropooling test in rats is shown in Table 6

Table 4: Effect of methanol crude extract (MCE) on enteropooling in Rats

Treatment	Dose mg/kg	Intestinal Content Volume(ml)	% Protection
Normal salin	10 ml/kg	0.64 ± 0.08	0.00
MCE	150	0.52 ± 0.05	18.75
MCE	300	0.34 ± 0.02	46.87
MCE	600	0.20 ± 0.03*	68.75
Atropine Sulphate	0.5	0.14 ± 0.02*	78.13

Values presented as Mean ± SEM. Data analyzed using one way ANOVA followed by Dunett's Post Hoc test. *Significant at $p<0.05$ when compared to the NS group. n = 5.

Discussion

The methanol stem bark extract of *C. hypopilinum* revealed the presence of carbohydrates, alkaloids, flavonoids, saponins, tannins, glycosides, steroids and triterpenes according to preliminary phytochemical investigation. The n-hexane fraction revealed the presence of steroids/triterpenes nucleus and the ethylacetate fraction revealed the presence of glycosides,flavonoids and saponins while the chloroform fraction shows glycosides, alkaloids, flavonoids and saponins.The oral median lethal dose (LD_{50}) of the crude methanol extract in mice and rats was estimated to be above 5000 mg/kg this showed that methanol stembark extract is moderately non-toxic when administered orally. The castor oil-induced diarrhea model is used for the evaluation of the antidiarrheal property of drugs. Ricinoleic acid, the active component of castor oil, is responsible for its diarrhea-inducing property (Roe, 1989). Diarrhea originates from an imbalance between the absorptive and secretory mechanisms in the intestinal tract

resulting in an excess loss of fluid in faeces (Jia, *et al*., 2008). Loperamide is known to increase the permeability of electrolytes at the level of the intestinal mucosa, alongside, the secretion of cholecystokinin in the duodenum, leading to hyper secretion which inhibits fluids' reabsorption (Ramesh, *et al.,* 2010). The tested extract may contain active substances that have increased the absorption of water and electrolytes from the gastrointestinal tract of mice. In contrast, the active principle found in castor oil, ricinoleic acid, irritates the intestinal mucosa and this result in the biosynthesis of inflammatory mediators such as Prostaglandins (PgE2) and Histamine and consequently an increase in intestinal motility and hyper secretion (Nwidu, *et al*., 2011; Kaur, *et al*., 2014). It is obvious that the extracts may have the capacity of inhibiting the effect of ricinoleic acid on the muscosa of the intestine. In castor oil induced diarrhea, the anti-diarrheic effect of the *C. hypopilinum* could result from inhibition of prostaglandin/histamine synthesis or by installing an anti-secretory mechanism. Loperamide used in this study as reference drug act by inhibiting the peristaltic activity, through indirect effect on circular and longitudinal muscle of the intestinal wall, also by stimulating the absorption of water and electrolytes, by increasing the intestinal transit time (anti-spasmodic) of the bowel content (Misra, *et al*., 2014). It is possible that the *C. hypopilinum* methanol stembark extract act in the same manner. Tannins could act by reducing intracellular calcium ion concentration or by activating the calcium pomp which would lead to muscle relaxation. The study showed that the methanol extracts of *C. hypopilinum* at a dose of 300 and 600 mg/kg exhibited a significant inhibition of castor oil-induced diarrhea in experimental mice, similarly significant inhibition in two doses (300 and 600 mg/kg) in the gastro-intestine motility test was exhibited (dose dependence). Tannins, alkaloids, saponins, sterols and terpenoids present in plants have been shown to be responsible for antidiarrheal activity (Zia-Ul-Haq, *et al*., 2012).

Conclusion

From the results, it showed that methanol stembark extract of *C. hypopilinum* possesses antidiarrheal activity which may be due to the presence of flavonoids, tannins, terpenes, saponin, or steroids. The pharmacologically activity could result from their ability to increase the absorption of water and electrolytes from the gastrointestinal tract and to inhibit prostaglandin/histamine synthesis, intestinal motility and hydro-electrolytic secretions. The result from this study showed that *C. hypopilinum* is a good candidate for the development of an antidiarrheicphytomedicine.

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