Nigerian Journal of Pharmaceutical and Applied Science Research, 9(3), 71-82; September 2020 (ISSN 1485-8059. Available at www.nijophasr.net)

Investigation of the *In vivo* Antidiarrhoeal Activity of *Hippocratea africana* Root Extracts by Model Infection and Protection Test in Mice against Bacterial Isolates from Infectious Diarrhoeal and Gastroenteritis Patients in Uyo, Nigeria.

[1*]Ekong U. S., [2]Udoh, D. I., [1]Alozie, M. F., [1]Udofa, E. J. and [1]Akpaide N. I.

[1]Pharmaceutical Microbiology/Biotechnology Unit, Department of Pharmaceutics and Pharmaceutical Technology, Faculty of Pharmacy, University of Uyo, Nigeria.

[2]Department of Microbiology, Faculty of Science, University of Uyo, Uyo, Nigeria.

ABSTRACT

Diarrhoea is the frequent passage of unformed, loose or watery stools within a day with or without the presence of blood and mucus. It is the major cause of morbidity and mortality in many rural communities and urban centres in Africa, particularly in children under the age of five. This calls for the development of cost effective alternative and affordable treatment strategies against the costly orthodox therapy for diarrhea by the use of herbal drugs from medicinal plants, one of which is *Hippocratea africana*. Pulverized *H. africana* roots were extracted by cold-marceration and stored at 4 ^{o}C until used.. Bacterial- diarrahoeic cultures were isolated from composite diarrhoeal stool samples by pour-plating in selective/differential media, and identified by standard microbiological procedures. *In vitro* anti-diarrhoeal activity of *H. africana* extracts was determined by the modified agar-well diffusion technique. Minimum inhibitory concentration (MIC), minimum biocidal concentration (MBC) and the mode of activity were determined by the reference standard agar-dilution test (ADT). *In vivo* anti-diarrhoeal activity and protection efficacy were assayed by mouse protection test (MPT) model. Forty eight(48) bacterio-diarrhoeic cultures were isolated and identified, with the numbers and percentages occurrence as follows; *Escherichia coli*, 11 (22.91%); *Salmonella typhi*, 6 (12.5%); *Shigella dysentariae*, 7 (14.6%); *Vibrio cholera* 7 (14.6%); *Pseudomonas aeruginosa*, 2 (4.2%); *Campylobacter jejuni*, 8(16.7%); *Staphylococcus aureus*, 3 (6.3%) and *Clostridium difficle*, 4 (8.3%). *H. africana* root extracts exhibited *in vitro* broad spectrum, concentration-dependent, bactericidal anti-diarrhoeal activity and potency against the bacterio-diarrhoeic isolates with significantly ($p<0.05$) lower MIC (3.13-12.5 mg/ml) and correspondingly lower MBC ~~values~~ (12.5 – 25.0 mg/ml) values respectively, compared with the control. In the *in vivo* MPT, *H. africana* root extracts offered appreciable (83.3 – 100.0 %) protection to the bacterio-diarrhoeic challenged experimental animals, compared with the none or averagely (0.00-50.0 %) protection in the control.

Keywords: Investigation, *In vivo*, Antidiarrhoeal-activity, Mouse Protection Test, *H. africana*, Root- extracts, Bacterio-diarrhoeic Isolates, Gastroenteritis.

INTRODUCTION

Diarrhoea is the frequent passage of unformed, loose or watery stools, (usually three or more times in 24 hours) (Njume and Goduka, 2012; Komal and Rana, 2013). It is also variously described as a physiological increase in the number of stools three or more per day; and increase in fluidity of the stools, and/or the presence of blood- mucus with increase neutrophil-polymorphs in stools; as well as the passage of or more loose/liquid stool per day or more frequently than is normal for an individual respectively (Njume and Goduka, 2012; Novaneethan and Gianella, 2012; Velazquez *et al;* 2012; Komal and Rana, 2013). Diarrhoea itself is not a disease, but a symptom of the most common clinical manifestation of several gastrointestinal diseases and infections (gastroenteritis), which may be caused by both infectious and non-infectious agents (Palombo, 2006; Njume and Goduka, 2012). Irrespective of aetiology, diarrhoea most of the time will occur when there is an imbalance between absorption and secretion; when the absorptive capacity of the intestine is exceeded and net secretion is greater than absorption (Nigro *et al*., 2000), as well as even minimal changes in normal intestinal fluid and electrolyte balance (Thapar and Sanderson, 2004; Njume and Goduka, 2012). The onset of the disease may be abrupt (acute) and self-limiting in immune-competent individuals; but chronic diarrhea may be persistent even with therapy, especially in people with an underlying debilitating clinical conditions such as HIV/AIDs and diabetes mellitus or individuals with an ageing immunity (Nigro *et .al;* 2000; Njume and Goduka, 2012). Diarrhoea is a major cause of morbidity and mortality in rural African and other developing countries communities, particularly in children under the age of five (Njume and Goduka, 2012). It is a common disease accounting for over 75% of childhood illnesses in Africa with a severity that seemed to largely depend on aetiology and age (Boadi and Kutinen, 2005).

Correspondence Author: E-mail: **ubsa4@yahoo.com**

Worldwide, an estimated nine million children, majority younger than 5 years old die annually due to diarrhea (WHO, 2009: De-Wet *et al.,;* 2010*).* Most of these deaths are reported to occur in rural African communities where healthcare facilities are inadequate, and the majority of the people lack access to clean and safe water, a major vehicle for transmission of infectious diarrhoea (Forsberg *et.al* 2009; Nwambete and Joseph, 2010). It is estimated that diarrhoea kills more young children around the world than malaria, HIV/AIDs and tuberculosis combined (Forsberg *et al.,* 2009; Nwambete and Joseph, 2010; Njume and Goduka, 2012). The causes of diarrhoea are wide and varied, the majority of which are linked to poor sanitary conditions, lack of access to clean and safe, drinking water as well as low socio-economic status (Mandomando *et al;* 2007; Aremu *et al.,* 2011; Njume and Goduka, 2012). Infectious diarrhoea, the most common form of diarrhoea worldwide, has been reported to be caused by viruses, bacteria or protozoa (Casbun- Jones, 2004; Palombo, 2006' Samie *et al;* 2009; Njume and Goduka, 2012; Velazquez *et.al.,* 2012). Amongst the viruses include: rotavirus, include: enteric adenovirus, norovirus, enteroviruses, caliciviruses and astroviruses, which infection by rotavirus is responsible for the most severe forms of diarrhoea especially in children accounting for 25 – 40% of cases (Cooke, 2010; Aremu *et al,*2011; Njume and Goduka, 2012). The bacterial agents include: enterotoxigenic *Escherichia coli (*ETEC), accounting for >25%; *Campylobacter jejuni accounting* for 18% (Aremu *et al.,* 2011; Njume and Goduka , 2012); diarrhoegenic *E. coli* (DEC) which include: enteroinvasive *E. coli* (EIEC), enteroggregative *E. coil* (EAEC), enterohaemorrhagic *E. coli* (EHEC), enteropathogenic *E.coli* (EPEC) and ETEC (Vilchez*et al.,* 2009), *Vibrio cholerae,* non-typhoidal *Salmonella, Shigella* species and *Salmonella typhi* (Palombo, 2006; Cooke, 2010; Njume and Goduka, 2012). Protozoal agents which have been incriminated as serious causative agents of diarrhoea in Africa and worldwide include *Cryptosporidium parvum, Giadia lamblia,* and *Entamoeba histolytica* (Haque *et al.,* 2009; Nkrumah and Nguah, 2011; Njume and Goduka, 2012). Non-infectious diarrhoea are reported to be caused by toxins, poisons, drugs, food allergens, prolonged antibiotic therapy resulting in the disruption of gut microflora, pseudomembranous colitis resulting from overgrowth and infection by *Clostridium difficle* (Njume and Goduka, 2012). However, the occurrence of diarrhoea may at times also be an indication of clinical conditions that are located out of the gastrointestinal tract, (GITd) (Mandomando *et al.,* 2007; Njume and Goduka, 2012). The indications for the presence of diarrhoea include: stool weight of greater than 200g/day for more than 30 days; more than 3 stools per day for more than 30 days; more than 3 stools per day for more than 7 days; or more than 3 stools per day, loosen than usual, for more than 3 days; or more than 3 stools per day with a change in frequency and consistency (Komall and Rana, 2013). The major symptoms of diarrhoea and other gastroenteritis are abdominal bloating, cramps, loose watery unformed stool with or without blood, urgency of stools, undigested stools, which irrespective of duration causes dehydration with overt signs of thirst, fatigue, dry-skin and tongue in the adult (Velazaquez *et. al.,* 2012; Komall and Rana, 2013). The excess loss of water and electrolytes in the stool often leads to dehydration, hyponatraemia, and hypokolaemia. According to WHO and other researchers, infectious diarrhoea is one of the most common cause of morbidity and mortality in many developing countries, including Nigeria, affecting mainly the infants and the children (Farthing, 2002; Boadi and Kutinen, 2005; Komall and Rana, 2013). Infectious diarrhea which may be acute (1-2 days) or chronic (≥ 4 weeks), can be classified as non-inflammatory due to enterotoxins; or inflammatory due to microbial invasion of the colon, triggering inflammatory response (Komall and Rana, 2013). There are two major health care systems used in the treatment of diarrhoea in the developing world: Orthodox (medications) and indigenous (plants /herbs) systems (Daniz-Santos *et al.,* 2006; Guiterez *et al.,* 2007; Singh and Sharma, 2011; Njume and Goduka, 2012). While, the orthodox system is well structured and highly developed, the indigenous systems are poorly organized, virtually unregulated and difficult to rationalize scientifically, even though it is readily available and cheap; but the two systems exist side by side (Tchacendo *et al.,* 2011; Njume and Goduka, 2012). The orthodox system, presents various medications for diarrhoea such as loperamide, codeine, diphenoxylate, lidamidine, clonidine, bismuth-subsalicylate, racecadotril, etc. But these drugs have many side-effects such as abdominal discomfort, dry mouth, nausea, constipation and headache (Nigro *et al*., 2000; Njume and Goduka, 2012; Komall and Rana, 2013). In most cases, oral rehydration therapy (ORT) is used as the first-line treatment of both infectious and non-infectious diarrhea (Bradhan, 2007; Asakitipi, 2010; Njume and Goduka, 2012; Komall and Rana, 2013). Other supplementation treatment include folate, vitamin A, zinc-sulphate, magnesium and copper, several absorbents like kaolin, pectin, activated charcoal and *Lactobacillus sporogenes* as well as antibrotics and 'over the counter (OTC) medicaments (Kormal and Rana, 2013). However, as reported, the OTC drugs possessed many side-effects such as abdominal discomfort, dry-mouth, nausea, constipation and headache. Consequently, this informed the renewed interest and much attention being focused on the many herbal remedies or medicinal plants available with anti-diarrheal activity with lesser side-effects than the conventional drugs. These medicinal plants or herbal remedies serve as an alternative measures to maximize the medicinal potentials of indigenous plants

in diarrhoeal and other gastroenteritis chemotherapy. Thus, herbal remedies prepared from indigenous plants are almost always the only readily accessible and affordable alternatives therapies for the control of diarrhea in many rural communities in Africa and the developing world (Tona *et al.,* 1999; Lin *et al*., 2002; Agbor *et al*., 2004; Ashur *et al.,* 2004; Atta *et al*., 2004; Mabeku *et al* 2006*;* Palombo, 2006; Gutierezz *et al*., 2007; Hassan *et al.,* 2007; Appido *et. al.*, 2008; Birdi *et al.,* 2010; Singh and Sharma 2011; Njume and Goduka, 2012; Komall and Rana, 2013). In these communities, extracts decoctions, infusions and concoctions or ashes of various plants parts (roots, rhizomes, tubers, aerial-parts, stem-bark and leaves) are used as remedies for diarrhoea, gastroenteritis and other illnesses. The literature is very rich with information on the antidiarrhoeal activities of most of these indigenous African folkloric plants and some have been scientifically validated in animal models with isolated active components (Agbor*et al*., 2004; Dahiru *et al*., 2006; Palombo, 2006; Magaji *et al*., 2007; Ching *et al*., 2008; Appido *et. al.*, 2008; Ojewole *et. al.*, 2008; Abere *et al.*, 2010; Teke *et. al.*, 2010; De Wet *et. al.,* 2010; Maroyi, 2011; Bakere *et al*., 2011; Njume and Goduka, 2012; Komall and Rana, 2013). According to these authors, the anti-diarrhoeal activity of many of these plants have been found to be due to the presence of these phytochemicals; alkaloid, tannins, saponins, flavonoids, steroids, and /or terpenoids. One of such medicinal plants is *Hippocratea africana*, used in many African countries by traditional medicine practitioners for the treatment of various ailments and microbial infectious diseases (Okokon *et al.,* 2006; Okokon *et al.,* 2011; Ndem et *al.,* 2013; Komall and Rana, 2013). *H. africana* (Wild) Loess Hippocrateaceae inhabits the green forests and is a perennial climber with glaburous hairs and is widely distributed in tropical Africa, reproducing from seeds (Diazel, 1956; Eshiette *et al.* 2006; Ndem et *al.*, 2013). In Nigeria, it is called "Gody" in Hausa; "Ponju"-Owiwi" in Yoruba; and "Ipungwa" in Tiv; while the Ibibio and Annang tribes of the Niger-Delta region of South-South Nigeria called it "Eba Enang-Enang". Similarly, the Akan-Asante in Ghana called it "Nnoto", and the fula-pulaa in Senegal called it "Njabo" (Burkill, 1985' Eshiett *et al.,* 2006; Okokon *et al.,* 2006; Ndem *et al.,* 2013). The plant has been reported to contian many phytochemicals such as alkaloids, tannins, saponins, cardiac-glycosides, flavonoids, etc (Eshiett *et al.,* 2006). The plant is used in traditional medicine by locals to treat various diseases infections and ailments like fever, malaria body-pairs diabetes, ulcer as well as diarrhoea, etc. However, willing the scope of this study, only few vadilitions of the aforementioned folkloric claims particularly, the anti-diarrhoeal activity which mainly focused on the root extracts have been reported (Okokon *et al.,* 2011; Komall and Rana, 2013). Conversely, only a limited general information had been reported on the *in vitro* anti-diarrhoeal activity of *H. africana* root extracts (Okokon *et al.,* 2011; Komall and Rana, 2013; .Ekong and Ubulom, 2016); without any *in vivo* antidiarrhoeal activity. Thus, there is a dearth of information on the *in vivo* anti-diarrhoeal activity of *H. africana* root extracts, to lend and confirmed further pharmacological credence to its ethno-botanical use as herbal remedy for treatment and control of infectious diarrhoea. Hence, this base-line study was carried out to evaluate the *in vivo* anti-diarrhoeal activity by a model infection and protection test assay in mice, to confirm the efficacy or otherwise of the *in vivo* antidiarroeal activity of the root extracts of .*H. africana* against the establishment of infections by the clinically isolated diarrhoeic pathogens.

MATERIALS AND METHODS

Plant Material and Extraction

Fresh plant (*H. africana*) used in the study were collected from a fringe rain-forest in Ikono Local Government Area, Akwa Ibom State, South –South Nigeria. The plant was authenticated by a taxonomist at the Department of Botany and Ecological Studies, Faculty of Science, University of Uyo, Nigeria, and the Voucher Specimen deposited at the Department of Pharmacognosy and Natural Medicine, Herbarium, Faculty of Pharmacy, University of Uyo, Nigeria. The fresh roots were separated from the plant, air-dried and pulverized using pestle and mortar, weighed and stored in sealed polyethene bags, until extractions. The extraction of the pulverized-root material was done by cold- maceration following the methods of Harborne, (1983) with modifications.(Ekong and Ubulum, 2016). Exactly 100 g of the sample was macerated in 1litre distilled water and in 500 ml of 95% ethanol in 2 litre capacity flaks, tightly sealed and kept for three days with intermittent shaking. Thereafter, the macerated samples were filtered using cotton-wool and Whatman No.1 filter paper. The respective solvent- extracted crude filtrates were concentrated to dryness in water-bath at 40 ^{o}C and stored at 4 ^{o}C for the anti-diarrhoeal and other assays.

Stool Samples Collection, Isolation and Identification of Bacterial Diarrhoeal Cultures

Stools were collected from diarrhoeal patients and those with stomach-ache and gastroenteritis at the University of Uyo Health Centre, into samples-stopper Bijou bottles containing either 10 ml nutrient-broth (NB) or 10 ml normal-saline (NS). These were immediately taken to the Pharmaceutical Microbiology Laboratory, Faculty of Pharmacy, University of Uyo, Nigeria, for cultivation and isolation. Cultivation and isolation were aseptically carried-out by inoculating 1.0 ml aliquots of ten-fold serially diluted samples in both general-purpose and selective/differential media such as nutrient-agar (NA), mannitol-salt agar (MSA), MacConkey, agar (MCA), Eosin-methylene blue agar (EMB); Salmonella-Shigella agar (SSA), Triple sugar

iron agar (TSI), and Thiosulphate citrate-sucrose bile salt agar (TCBS), blood-free charcoal selective medium (CSM), Presten *Campylobacter* blood-free medium (PCM); cycloserine-cefoxithin, egg-yolk fructose agar (CCFA) (Ekong *et.al.,* 2015 a, b; Ekong and Ubulom, 2016) by the standard pour-plate technique and incubated at 37 ^{o}C for 48 h (Collins and Lyne, 1979). Isolated cultures were aseptically purified by twice repeated sub-culturing by streaking on the respective isolation media and maintained as slant cultures at 4 ^{o}C. Characterization and identification of the isolated and purified cultures were based on standard microbiological, biochemical and physiological procedures, (Konemann *et al.,* 1994, Ekong *et.al*., 2015 a,b; Ekong and Ubulum, 2016).

Standardization of Isolated Diarrhoeal Cultures Innocula Density

Inocula of the isolated and purified bacterial cultures were standardized to 0.5 MacFarland nephelometer turbidity, with cell density approximately 1.5 x 10^{8}cfu/ml, following the methods of Tilton and Howard (1987), Baron and Finegold (1990), with modifications (Ekong *et al.,* 2004). Inocula of Gram-positive bacteria were diluted to factor 3; while those of the Gram-negative bacteria were diluted to factor 5 (Ekong *et al.,* 2004).

Anti-diarrhoeal Activity Spectra of *H. africana* Root Extracts against Isolated Diarrhoeal Cultures

Anti-diarrhoeal activity of *H. africana* root extracts *in vitro* on the isolated diarrhoeal cultures was evaluated by spread plating 0.1ml aliquots of the standardized broth cultures on diagnostic sensitivity test agar (DSTA), plates following the agar-well diffusion technique (Collins and Lyne, 1979) with modifications (Ekong *et al.,* 2004). The assay plates were held at 4 ^{o}C for 1 h, before incubation at 37 ^{o}C for 24 h (Ekong *et.al.,*2004*).* Wells of equal diameter similar to the assay plates filled with ciprofloxacin,(CFX) on DSTA seeded plates of the respective cultures, under the similar assay conditions, as the test plates served as positive control. Inoculated DSTA plates without the extracts, but with distilled water served as the negative control. In the respective assay conditions, the sizes of inhibition zone diameter (IZD) obtained that is less than, equal to, or greater than the well-diameter (4mm), correspondingly indicated inactivity (resistance, R), indifference (intermediate, I), and activity (sensitivity, S) respectively. Potency of the root extracts was evaluated by activity-index, calculated as the ratio of extract activity to that of the positive control (Ekong *et .al.,* 2015 a, b).

Determination of Mode of Anti-diarroeal Activity of *H. africana* Root Extracts on Isolated diarrhoeal cultures

The mode of anti-diarrhoeal activity of the *H. africana* roots extracts against the isolated diarrhoeal, cultures was assessed by determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), which respectively measures the -static and -cidal activities. MIC was determined by separately streaking the standardized cultures on DSTA plates containing two-fold serially diluted concentrations of the extracts obtained by macro-broth dilutions, following the reference standards agar-diluting technique (ADT) and the assay plates incubated at 37 ^{o}C for 24 h (Collins and Lyne, 1979;Tilton and Howard, 1987; Baron and Finegold, 1990). Inoculated DSTA plates without the extracts concentrations served as negative control. The MICs of the extracts were taken as the least concentration that inhibited the growth of the bacterial culture assayed by the streak growth on the DSTA test plates compared to those in the control plates. MBC of the root extracts were determined by further overnight incubation of the non-growth MIC plates at 37 ^{o}C. Thereafter, the MBC assay plates were observed for the presence or absence of growth. The MBCs were determined as the least concentrations of the root extracts that killed the cultures evidenced by no-growth on the streak-lines, from which the mode of activity was determined (Ekong *et al.,* 2004).

Experimental Animals

The experimental animals used were albino mice of body weights within the range of 20.0 – 35.0g. The animals were obtained and breed at the Animal House, Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Uyo, Nigeria. The animals were kept in clean and well-ventilated cages and allowed access to food and water *ad libitum.*

***In vivo* Antidiarrhoeal Activity and Protection Efficacy of *H. africana* Root Extracts**

The *in vivo* efficacy of the anti-diarrhoeal activity and protection potential of *H. africana* root extracts against the establishment of infections by the isolated diarrhoeal pathogens was conducted using albino-mice (20.0 – 35.0g) body weights, following the animals protection model of Ekong *et al.,* (2004); using the acute-toxicity profile of *H. africana* roots, previously determined as follows: aqueous root (843.43 mg/kg) and ethanol root (649.24 mg/kg) (Ekong and Effiong, 2016). In the assays, the mice were grouped into six sets of six mice per group. Each group was challenged intraperitoneally with 0.5 ml of fresh and young standardized isolated diarrhoeic cultures, previously sensitive to the root extracts to establish infections. At an interval of one and five hours respectively, the animals were intraperitoneally injected with *H. africana* root extracts at the dose equivalent to the MIC of a given bacterial isolate in the study, but less than the LD_{50} of the root extracts for the mice (Ekong *et al.,* 2004). The animals were allowed access to food and water *ad libitum*, throughout the five day study period. The number of

deaths and other clinical and physiological observations per group of the challenged mice were recorded. Similarly, for negative control, the assay conditions were similar as described above, but normal saline instead of the extracts were intraperitoneally administered after one and five hours. The animals were also allowed access to food and water *ad libitum* and daily observations including deaths were recorded, within the five day study period.

Statistical Analysis

The results obtained were differently expressed as multiple comparison of the means ± standard error of mean (SEM) and in simple percentages: The significant differences between the mean values were determined by Analysis of Variance (ANOVA) in a completely randomized design (CRD) at $p<0.05$, followed by Turkey Kramer Multiple comparison, post values of $p<0.05$ (Ekong and Effiong, 2016).

RESULTS

Table 1: Isolation, characterization, identification, distribution and percentage occurrence of bacterial diarrhoeic isolates fromDiarrhoeal Patients

Media/Colony Description	Colony characteristics	Biochemical tests								sugar fermentation test					Probable Organism	Number/ percentage occurrence n= 48(100%)
	Colony morphology	Gram reaction	Motility	Indole	Catalase	Coagulase	Citrate	Urease	Oxidase	Man.	Suc	Man	Lac.	Glu		
MCA – red, no mucoid EMB –green metallic sheen CLED- yellow colonies	short rods +	-	+	+	-	-	-	-	-	A	AG	A	AG	A	*Escherichia coli* (EC)	11(22.9)
MCA – Light purple colonies SSA – black colonies TSI –black colonies	straights rods	-	+	-	-	-	-	+	-	A	A	-	-	A	*Salmonella typhi* (ST)	6(12.5)
MCA- light purple colines SSA- black colonies TSI –black colonies	rods	-	-	-	-	-	+	-	-	AG	A	A	-	A	*Shigella dysentariae* (SD)	6 (2.3)
MCA- colourless colonies TCBS – yellow colonies	curved rods	-	+	-	-	-	+	-	+	AG	-	A	-	-	*Vibrio cholera* (VC)	7(14.6)
NA – blue–green spreading colonies EMB – non-pigment light purple colonies	short rods	-	+	+	+	-	+	+	-	-	A	A	-	A	*Pseudomonas aeruginosa* (PA)	2(4.2)
MSA- yellow colonies BA- yellow colonies with β-haemolysis	cocci in cluster	+	-	-	+	+	-	-	-	A	A	AG	A	AG	*Staphylococcus aureus* (SA)	4(8.3)
CSM – Gray/flat colonies PCM- Gray/flat colonies	Curved Rods	-	+	+	+	-	+/-	-	+	-	-	-	-	-	*Campylobacter jejuni* (CJ)	5 (16.7)
CCFA/ CDC- NBA - Gray/slightly raised, flat and spreading with rhizoid margins	Straight Rods	+	-	-	-	-	-	-	-	A	A	A	A	A	*Clostridium difficle* (CD)	4(8.3)

NA = Nutrient agar; BA = blood agar; MSA = Manitol of salt agar; MCA = macConkey agar; EMB = Eosin methylene blue agar; SSA = Salmonella – Shigella agar; CLED = cystrine electrolyte deficient agar; TSI = Triple sugar iron agar; TCBS = Thiosulphate Citrate-Sucrose Bile Salt agar; CSM = Blood-free charactral based selective medium; Presten*Campylobacter* blood-free medium;CCFA = Cycloserine-Cefoxithin, egg-yolk, fructose agar*; DCD-NBA= CDC,* Non-selective Blood Agar*;* + = positive reaction or growth; - = Negative/ No reaction or growth; A = Acid production; AG = Acid/gas production

Table 2: *In vivo* antidiarrhoeal activity and potency of *H. africana* roots extracts against clinically isolated pathogenic diarrhoeal bacteria

Bacterial isolates	Antidiarrhoeal activity/inhibition zone diameter (mm) Roots		Control	Relative potency of Root Extracts	
	AQ	ETH	CFX	AQ	ETH
EC	36.1 ± 0.23	30.1 ± 0.36	43.2 ± 0.21	85.6	70.1
ST	32.2 ± 0.40	27.2 ± 0.46	40.4 ± 0.21	79.7	67.3
SD	38.5 ± 0.61	30.1 ± 0.12	45.3 ± 0.41	85.0	66.4
VC	36.4 ± 0.40	29.3 ± 0.31	40.2 ± 0.21	80.7	65.0
PA	27.7 ± 0.58	23.2± 0.35	40.3 ± 0.14	88.7	57.6
SA	32.2 ± 0.32	30.4± 0.40	40.3 ± 0.42	76.1	71.9
CJ	34.5± 0.74	28.6± 0.14	40.3 ± 0.14	85.6	71.0
CD	33.8± 0.23	24.3±0.31	40.2 ± 0.21	84.1	60.4

A total of 48 bacterial cultures were isolated from the composite stool samples of diarrhoaeic and other gastrocenteritis patients (Table 1). The number and percentages occurrence of diarrhoeal cultures isolated were as follows: *Escherichia coli* (EC), 11 (22.9%); *Salmonella typhi* (ST), 6 (12.5%); *Shigella dysentariae* (SD) and *Vibrio cholerae* (VC), 7 (14.6%) respectively; *Pseudomonas aeruginosa*, (PA), 2 (4.2%), *Campylobacter jejuni* (CJ),8(16.7%); *Clostridium difficle* (CD), 4(8.3%) and *Staphylococcus aureus* (SA), 3(6.25%). The result indicates that Gram negative bacteria were the predominant cultures

isolated, and lessly the Gram-positive cultures lead by EC as the most frequently isolated cultures while SA and PA were the lessly isolated cultures in the study. The *in vitro* anti-diarrhoeal activity and potency of the *H. africana* root extracts against the isolated diarrhoeal cultures indicated a broad-spectrum anti-diarrhoeal activity, significantly ($p < 0.05$) higher in the aqueous than the ethanol root extract, compared with the control (Table 2). The result shows that all the isolated cultures from the diarrheal stool samples were sensitive and susceptible to the activity of the root extracts, without any recorded intermediate, and resistance (inactivity), with corresponding high potencies within the range (57.6 – 85.6). The MIC and MBC of *H. africana* root extracts against the diarrhoeaic isolates in the study, shows predominantly lower MIC values (3.13 – 12.5 mg/ml) and correspondingly lower MBC values (12.5 – 25.0 mg/ml) for all the isolated diarrhoaeic cultures (Table 3). The result indicates that the aqueous extract recorded significant lower MIC values compared with the ethanol extract against the isolates. Equally, the aqueous extract recorded a significantly ($p< 0.05$) lower and uniform MBC value (12.5 mg/ml) against all the isolates, compared with higher values by the ethanol extract. Furthermore, the result indicated that *H. africana root* extracts exhibited a concentration-dependent bactericidal anti-diarrhoeal activity against all the isolated cultures. The results of the *in vivo* anti-diarrhoeal activity of *H. africana* extracts assayed by mouse protection test indicated some notably physiological signs of infections in the mice for all the isolates (Table 4). The prominent physiological signs noticed were; pyrexia, reduced body and gait movement, pruritis, convulsion, dullness, closed-eye-lids, anorexia, chills, crawling, frequent watery and fluidy stooling, as well as weight loss and finally dead. Within the study period, the prominent convalescing signs noticed were; improved movement semi-solid stools and reduction in number of stooling; appetite and feedings, leading to begins normally and active. Furthermore, the *in vivo* mouse protection test to assay the efficacy or otherwise, indicated that *H. africana* aqueous root extract effectively prevented death in all the groups on day 1; with only one death recorded per each group of isolates between Day 2 – Day 5 and offered 83.3-100 % protection against the establishment of infections by the isolated cultures to the challenged experimental animals (Table 5). The sequence of protection efficacy of *H. africana* root aqueous extract was in the descending order: SD, VC, CJ, SA and CD (100.0%); ST, EC, and PA (83.3%).The result indicates the excellent activity of *H. africana root* aqueous extract against all the isolated diarrhoeic cultures in the study.

Table 3: MIC, MBC and mode of activity of *H. africana* roots extract against isolated diarrhoeaic bacteria pathogens

Bacterial isolates	MIC (mg/ml)		MBC(mg/ml)		MIC/MBC Index	
	AQ	ETH	AQ	ETH	AQ	ETH
EC	6.25	6.25	12.5	12.5	-	-
ST	6.25	6.25	12.5	25.0	-	-
SD	6.25	6.25	12.5	12.5	-	-
VC	6.25	6.25	12.5	12.5	-	-
PA	6.25	12.5	12.5	25.0	-	-
SA	3.13	6.25	12.5	12.5	-	-
CT	6.25	6.25	12.5	25.0	-	-
CD	6.25	6.25	12.5	25.0	-	-

- = cidal activity

Table 4: Signs and symptoms of model infection, *in vivo* antidiarrhoeal activity and mouse protection assay of *H. africana* aqueous root extract on diarrhoeal bacterial isolates.

Text isolates	Physiological observation of *in vivo* protection within 5 days									
	Assay (Days)					Control (Days)				
	1	2	3	4	5	1	2	3	4	5
EC	Pyrexia reduced movement	Fatigue	Convulsion	Dead	Normal and active	Chills convulsion	Weal/ crawling	Dead	Dead	Dead
ST	Chills pyrexia	Tremor	Reduced appetite	Dead	Normal increase feeding	Childs pyresia	Anorexia	Anorexia	Weight loss	Dull
SD	Anorexia pyrexia	Dull	Improve movement	Active & normal	Active	Pyrexia	Crawling	No movement	Dead	Dead
VC	Pyrexia no movement	Anorexia shuffle	Active eyelid edema	Improves feeding	Active normal	Pyrexia	Convulsio n	Crawling	Weight loss	Dead
PA	Pyrexia & Chills	Convulsion	Weight lost	Dead	Dead	Anorexia pyrexia	Crawling	Weak crawling	Dead	Dead
CJ	Pyrexia & Chills	Convulsion	Weight lost	Dead	Active	Anorexia pyrexia	Crawling	Weak crawling	Dead	Dead
SA	Pyrexia	Reduced movement	Puritus	Improve feeding	Active	Pyretic itchy body	Weight loss	Weak crawling	Dull	Dead
CD	Pyrexia	Reduced movement	Puritus	Improve feeding	Active	Pyretic itchy body	Weight loss	Weak crawling	Dull	Dead

Table 5: Model infection, *in vivo* anti-diarrhoeal activity and protection efficacy of *H. africana* aqueous root extract against establishment of infections by diarrhoeal bacterial isolates

Test isolates	Number of animals used	No of death within five days												No. of survivors within five days		Percentage death per group %		Percentage protection per group	
		Test						Control											
		1	2	3	4	5	Total	1	2	3	4	5	Total	Test	Control	Test	Control	Test	Control
EC	6	-	-	1	-	-	1	-	1	1	3	-	5	5	1	16.67	83.3	83.3	16.7
ST	6	-	-	1	-	-	1	-	1	1	1	-	3	5	3	16.7	50.0	83.3	50.0
SD	5	-	-	-	-	-	0	-	-	2	1	-	3	6	3	0.00	50.00	100.0	50.00
VC	6	-	-	-	-	-	0	-	1	1	1	-	3	6	3	0.00	50.0	100.0	50.0
PA	6	-	-	1	-	-	1	-	1	2	3	1	6	5	0	16.7	100.0	83.3	0.00
CJ	6	-	-	-	-	-	0	-	1	2	2	-	5	6	1	0.00	83.3	100.0	16.7
SA	6	-	-	-	-	-	0	-	1	2	1	-	4	6	2	0.00	66.7	100.0	33.3
CD	6	-	-	-	-	-	0	-	1	1	1	-	3	6	3	0.00	50.0	100.0	50.0

- = No death recorded

DISCUSSION

Infectious diarrhea is a common, composite gastroenteritic infectious disease, accounting for up to 75% childhood illness in Africa with a severity that depends to some extent on aetiology and age (Casburn-Jones and Farthing, 2004;Boadi and Kutinen, 2005; Cooke, 2010; Njume and Goduka, 2012). Infectious diarrhoea, which may be exudative or secretory, has a wide and varied cause, the majority of which are related to unhygienic and poor sanitary conditions, as well as low socio-economic status (Mandomando et *al*., 2007; Aremo *et al.,* 2011; Njume and Goduka, 2012). Exudative and secretory infectious diarrhoea being the most common forms of diarrhoea worldwide have been reported to be predominantly of microbial origin and caused by viruses, bacteria and protozoans (Casburn –Jones and Farthing, 2004; Cooke, 2011; Njume and Goduka, 2012; Noveneethan and Giannella, 2012; Velazquez *et .al.,* 2012). Amongst these microbial groups, infections with bacteria predominates, mostly by enterotoxigenic bacteria (Notably, Enterotoxigenic *E. coli*, (ETEC); *Vibrio cholerae*, *Clostridium botulinum*, *Clostridum perfringens*, *Campylobacter jejuni*, *Klebsiella pnuemoniae*, *Yersinia enterocolitica* and *Aeromonas hydrophilia*); and enteroinvasive bacteria (mostly Enteroinvasive *E. coli*, EIEC; non-typhoidal *Salmonella* species*; S. typhi, S., enterotides, Shigella* species *Campylobacter jejuni*; *Yersinia enterocolitica*, and *Vibrio parahaemolyticus*); where ETEC and *Campylobacter* account for over 25 % and 18 % of worldwide diarrhoeal cases respectively (Aremu *et al*., 2011, Njume and Goduka, 2012). Accordingly, in this study, the bacterial species isolated from the diarrhoeal and gastroenteritis stool samples are the aucthocthonous gut microflora: *Escherichia coli, Salmonella typhi; Shigella dysentariae; Vibrio cholerae; Pseudomonas aeruginosa, Campylobacter jejuni, Clostridium difficle* as well as the allochthonous *Staphylococcus aureus.* However, under certain physiological conditions imbalances, these bacterial gut microflora isolates are liable and become the underlying etiologic agents of the many mild/opportunistic and fatal human infections, notably amongst which is exudative/secretory infectious diarrhoea (Rosenberg and Cohen,1983; Konemann *et al*.,1994; Payne *et al*., 2006; Samie *et al*., 2009; Reis and Horn, 2010). The study corroborated the earlier report that exudative and secretory infectious diarrhoea caused by bacteria, viruses and protozoans are the most common form of diarrhoea worldwide (Casburn-Jones and Farthing, 2004; Cooke, 2011; Njume and Goduka, 2012; Velazquez *et al,* 2012). Thus, in this study, the isolated diarrhoeic cultures could be enterotoxigenic or enteroinvasive which could have elaborated potent enterotoxins, thereby causing the associated diarrhea given the notified clinical and physiological signs and symptoms of diseased state. This assertion is in line with and confirmed the earlier report that several enterotoxigenic and enteroinvasive bacteria such as enterotoxigenic *Escherichia coli, Salmonella typhi, Salmonella typphimurium, Clostridium difficle, Aeromonas hydrophilia, Yersinia enterocolitica, Campylobacter jejuni, Vibrio cholerae* and *Klebsiella pneumoniae* cause diarrhoea by the production of potent enterotoxins. The enterotoxins from these bacteria have their effects on the enterocyte functions by stimulating the secretion of transepithelial electrolytes, increasing the osmotic flux of water and ions in the intestinal lumen, specifically heat-labile (LT) and heat-stable (ST) enterotoxins from *E. coli, V. cholerae* and *C. jejuni*; increase net fluid secretion by affecting the enzyme adenylate cyclase or guanilate cyclase by activation of the cAMP (cylic 3^1, 5^1-adenosine monophosphate) in the mucosal epithelium, which induces an increase of intestinal secretion, resulting in diarrhoea (Casburn- Jones and Farthing, 2004; Novaneethan and Giannellia, 2012; Velazquez *et al*., 2012; Njume and Goduka, 2012). Furthermore, in the study, *Escherichia coli* was the most frequently isolated culture, accounting for 22.9% occurrence, followed by *Campylobacter jejuni*, (16.7%); *Shiegella dysentarie* and *Vibrio cholera* (14.6%) respectively and *Salmonella typhi* (12.5%). These percentages of frequency of isolation and occurrence, indicates the propensity of the isolates to cause diarrhoeal

infections. These findings are in line with the report that diarrhoea infection with bacteria such as ETEC and *Campytobacter jejuni* respectively account for 25% and 18% of diarrhoeal cases in the developing world (Arema *et al.,* 2011; Njume and Goduka, 2012; Velazquez *et al.,* 2012). Equally *Escherichia coli* being the most isolated and with the highest percentage occurrence could be termed diarrhoeagenic, as its occurrence may possibly correlates with an ability to establish diarrhoeal infection. Thus, according to several reports, diarrhoeagenic *E. coli* (DEC), which include, ETEC; enteroinvasive *E. coli (*EIEC); enteroaggressive *E. coli* (EAEC); enterohaemorrhagic *E. coli* (EHEC) and enteropathogenic *E. coli* (EPEC) are amongst the major bacterial causes of secretory /exudative infectious diarrhoea in the world (Casburn-Jones and Farthing, 2004; Cooke, 2010; Njume and Goduka, 2012; Velazquez *et al.*,2012). The excellent and predominantly high *in vitro* anti-diarrhoeal activity of the *H. africana* root extract obtained in the study, could be attributed to and confirmed the relative presence and solubility of the aforementioned phytochemicals (alkaloids, saponnis, tannins, carbohydrates, resins and other phenolic compounds such as flavonoid, terpenes, sesquiterpenes, diterpenes and terpenoids) in water and ethanol as solvents for extraction. Moreover, in this study, the higher antidiarrhoeal activity of the aqueous extract compared to the ethanol extract is in contrast to the generally low activity by aqueous extracts compared with other solvents extracts (Ekong and Ubulom, 2016). However, irrespective of the assumed phytochemical disparity and the corresponding activity differential between the *H. africana* root extracts, the degree of antimicrobial and other biological activities of medicinal plants is universally acknowledged as the functions of the phytochemicals present. Thus, in the study, the presence of the aforementioned phytochemicals could be responsible for the antidiarrhoeal activities of *H. africana root* extracts. Evidently, this assertion is in line with the widely reports that the presence of phytochemicals are responsible for the antidiarrhoeal activity of medicinal plants (Appidi *et al.,* 2008; Ojewole *et.al.,* 2010; Teke*et.al.,* 2010; Njume and Goduka, 2012; Velazquez *et al.,* 2012; Komall and Rana, 2013). The relatively moderate antidiarrhoeal activity of the *H. africana* root ethanol extracts, could be linked to its moderate extractable or solubility strength compared to water (Ekong and Nnatu, 2016; Ekong and Effiong, 2016; Ekong and Ubulum, 2016). This is an indication of the nature and types of the phytochemicals, which may likely be moderately soluble in ethanol, the more stronger and polar solvent compared to water, the least polar solvent. Hence, this finding, may possibly confirmed our earlier assertion that *H. africana* root extracts phytochemicals are likely water-soluble metabolites (Ekong and Nnatu, 2016). However, this finding in the study is in contrast to the widely documented facts of the excellent extractable and solubility properties of the organic solvents, notably ethanol as vehicle, as well as the corresponding antimicrobial activity of the ethanol extracts. Nevertheless, the excellent *in vitro* potencies of the extracts, particularly, the aqueous extracts, against the diarrhoeagic isolates is an indication that the aqueous extract may be used as the first-lines antidiarrhoeics, or as an alternatives herbal remedy to the orthodox antibiotics chemotherapy and medication in the local communities due to lack of access and affordability of the orthodox medication. Generally, as previously reported, the excellent extractable and solubility properties, antimicrobial activity and potency, as well as interaction studies of *H. africana* extracts especially the aqueous extracts give the direction and informed why the locals usually prepare the concoctions, decoctions and infusions of *H. africana* in water and lessly with other organic solvents, as herbal remedies for the treatment of many infections, diseases and ailments, including diarrahoea (Ekong and Nnatu, 2016; Ekong and Effiong, 2016; Ekong and Ubulum, 2016; Ekong *et al.,* 2016). In the study the remarkable predominantly lower MIC and MBC values obtained, confirmed the high potency of the *H. africana* root extracts particularly the aqueous extract against the clinical diarrhoeal and gut isolates. These findings further supported and confirmed the excellent antimicrobial activity and superior and moderate anti-diarrhoeal activity of the aqueous and ethanol extracts respectively. In this study, the MBC values established basically a concentration-dependent-biocidal mode of antidiarrhoeal activity for the *H. africana*root extracts, against the clinical diarrhoeic isolates. This may be an indication that the extracts should be infrequently administered for optimal potency and performance as an antibacterial agent in exudative/secretary-infectious diarrhea and in other infections, diseases and ailments. In the study, the model infection and protection test in mice measured the *in vivo* antimicrobial activity efficacy and potency of *H. africana* root aqueous extracts in preventing the successful establishment of diarrhoael infections and diseases by the challenged diarrhoeal pathogens. The model infection and protection test in mice, measured the *in vivo* potency and efficacy of antibiotics, other anti-infectives and chemotherapeutic substances including herbal remedies, in preventing the successful establishment of infections in the challenged animals (Ekong *et al.,* 2004). Accordingly, in the study, *H. africana* root aqueous extract demonstrated an excellent *in vivo* antidiarrhoeal potency and efficacy, which is not only limited to *in vitro* conditions, as it offered between (83.3-100.0 %) protection against the successful establishment of diarrhoeal infections by the stools-isolated diarrhoeaic cultures. These cultures have been reported to and could be enterotoxigenic or enteroinvasive, producing potent enterotoxins in the intestinal mucosa, resulting in exudative and secretory infectious diarrhoeal infections, hence diarrhoeagenic, particularly *Escherchia coli* which could be ETEC,

EIEC, EAEC, EPEC or EHEC, (Casburn-Jones, and Farthing, 2004; Cooke, 2010; Njume and Goduka, 2012; Velazquez *et al.,* 2012; Novaneethan and Giannellia, 2012). Hence, from the *in vivo* protection study, it could be asserted that *H. africana* root extracts, effectively exhibited biocidal activity as the possible mechanism of action against the isolated cultures, thereby eradicating the enterotoxigenic and diarrhoeagenic cultures from invasion and production or inactivation of enterotoxins in the intestinal lumen, which could have stimulated the secretion of transepithelial electrolytes, increasing osmotic flux of water and ions, increased net-fluid secretion by activiation of cAMP in the mucosal epithelium, which induces an increase of intestinal secretions and causes diarrhoea, as compared to the control which offered little or no protection to the challenged animals. The biocidal mode of anti-diarrhoeal activity of *H. africana* root extracts is evident by the physiological signs noticed in the convaslecing assay animals including; infrequent and reduced stooling; semi-solid to solid stools, improved and normal feeding, as well as increase in body-weight and normal movement; compared to the control animals which presented diarrahoeal and diseased conditions and deaths within the study-period. Hence, the relatively little or no protection, which may be self-limiting and attributed to chemo-tactic factors and other immunologic defense mechanisms in the mice (host). Consequently, from the *in vivo* study, *H. africana* root aqueous extract may be prepared in the form of concoction, decoction or infusion as herbal remedies and used clinically in treatment of diarrhoeal and other infections/diseases caused by the isolated organisms.

CONCLUSION

The results of the study indicated that *H. africana* root extracts exhibited excellent boadspectrum, concentration-dependent, bactericidal anti-diarrhoeal activity against the bacterio-diarrhoeic isolates, and offered appreciable degree of protection to the experimentally challenged animals. Thus, the study provides further credence and evidence for the previously reported *in vitro* biocidal diarrhoeal activity of *H. africana* root extracts against diarrhoeagenic cultures isolated from composite stools samples. It also, provides a confirmation of the efficacy of *H. africana* root extracts in protecting experimental and challenged animals against the successful establishment of diarrhoeal and other infections/diseases. Therefore, the study may serve as a base-line information, giving the direction for the decoction, infusion or concoction of *H. africana* root extracts to be used as herbal remedies and alternatives to the lessly access, costly and at times unaffordable orthodox medicines in the treatment of diarrhoeal infections and disease

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