In Vitro Susceptibility Study of *Salmonella Spp.* and *Vibrio Cholerae* to Crude Extracts of *Euphorbia Kamerunica* and *Cola Gigantea*

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***Abstract*: - Treatment of *Salmonella* and *Vibro* spp with synthetic antibiotics have progressively declining efficacy due to resistances posed by the target organisms. This study seeks to investigate the susceptibility of these organisms to two age-long anti-diarrhea herbs: *Euphorbia kamerunica and Cola gigantean. Vibrio cholerae* and type cultures of *Salmonella typhi, Salmonella arizona* and *Salmonella enterica* were collected from the Microbiology Laboratory, University of Nigeria, Nsukka. *Euphorbia kamerunica* succulents and *Cola gigantea* bark were collected from Neke-Oghe in Ezeagu L.G.A of Enugu State. The herbs were identified in the herbarium at the Botany Department of the University of Nigeria, Nsukka. Crude extracts of the herbs were made using n-hexane and hot water. Known concentrations of the extracts were used to test the sensitivity of the organisms. Gram-negative antibiotic discs were also used for comparison. Higher sensitivities of the organisms were observed with n-hexane extract of *E. kamerunica* (75%) and *C. gigantea* (54.2%) but lower sensitivity with their hot water equivalents: 0% and 25% for *E. kamerunica* and *C. gigantea* respectively. *E. kamerunica* was generally more inhibitive to the organisms than *C. gigantea.* The bactericidal effects were also more pronounced with the n-hexane extracts of the herbs. The organisms showed varied sensitivity to the antibiotics. Statistical analysis using t-test carried out to compare the herbal and antibiotic treatments indicated a significant difference with the hot water extracts of *E. kamerunica* and *C. gigantea;* $t_{cal} > t_{tab}$ but no significant difference with the n-hexane extract of *E. kamerunica;* $t_{tab} > t_{cal}$.**

I. INTRODUCTION

V. cholerae, causes diarrheal disease and is serologically classified into 'O' antigenic group. Strains belonging to O group 1 (O1) are responsible for cholera. Strains other than O1 are called non-O1; they can cause only sporadic infections and do not have the potential to cause epidemics. Strains of serovar O1 consist of two biotypes, classical and El Tor. *V. cholerae* includes both pathogenic and nonpathogenic strains that vary in their virulence gene content.

Cholera is acquired through ingestion of contaminated food or water. A London physician and epidemiologist John Snow, was the first to show that illness was associated with sewage (Thompson and Swings, 2006). The incidence rate is more than five million cases annually, most of which occur in Asia and Africa, with 8% of cases requiring hospitalization. Despite primarily affecting developing countries, cholera remains a serious public health problem for some developed countries (Pazzani *et al*., 2006*).* Risk factors inclue: poor sanitation, limited health care, and unsafe drinking water (Heidelberg *et al*., 2000).

The pathogenic process begins with its ability to cross the acid barrier of the stomach, colonize the epithelium of the small intestine, and producing enterotoxins (and possibly other toxins) which disrupt ion transport by intestinal epithelial cells. The subsequent loss of water and electrolytes leads to the severe diarrhea characteristic of cholera. After an incubation period ranging from hours to a few days, profuse watery diarrhea (rice-water stools) begins (Strohl *et al*., 2001). The incubation period of cholera can range from several hours to 5 days and is dependent in part on inoculum size (Kaper *et al*., 1995). Cholera is a medical emergency that can have a favourable prognosis with properly organized management (Ndour *et al*., 2006). The disease runs its course in 2 to 7 days; the outcome depends upon the extent of water and electrolyte loss and the adequacy of water and electrolyte repletion therapy (Finkelstein, 1996).

Cholera is managed most importantly by fluid and electrolyte replacement through the administration of Oral Rehydration Salt until the disease runs its cause. Antimicrobial therapy such as tetracycline, fluoroquinolones and azithromycin are also helpful.

Salmonella species cause typhoid fever, paratyphoid fever, and the foodborne illness- salmonellosis (Ryan and Ray, 2004). Early symptoms include nausea, vomiting, abdominal cramps, diarrhea (sometimes bloody), fever, and headache. The infection can cause other health problems, like meningitis and pneumonia (Ryan and Ray, 2004).

Currently, there are two recognized species of non-typhoid Salmonella: *S. enterica* and *S. bongori*, with six main subspecies: *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV), and *indica* (VI) (Janda and Abbott, 2006). Historically, serotype (V) was *bongori*, which is now considered its own species. *Salmonella* infections are

zoonotic; they can be transmitted by humans to animals and vice versa. Infection via food and water is also possible. *S. typhi* is adapted to humans and does not occur in animals. Enteritis *Salmonella* (e.g., *Salmonella enterica subsp. enterica* serovar Enteritidis) can cause diarrhoea, which usually does not require antibiotic treatment. However, people at risk such as infants, small children, the elderly, HIV patients and those with suppressed immunity can become seriously ill. Children with sickle cell anemia who are infected with salmonella may develop osteomyelitis (Giannella, 1996). Between 1990 and 2005 the number of officially recorded cases decreased from approximately 200,000 cases to approximately 50,000. It is estimated that every fifth person in Germany is a carrier of *Salmonella*. In the USA, there are approximately 40,000 cases of *Salmonella* infection reported each year (CDC, 2008). According to the World Health Organization, over 16 million people worldwide are infected with typhoid fever each year, with 500,000 to 600,000 of these cases proving to be fatal.

Different extracts of ***Euphorbia Kamerunica*** and ***Cola gigantean*** have been used in susceptibility studies and have been found to be effective against varieties of organisms including bacterial and fungal species. (Agbebi *et al*, 2012; Agyare et al, 2012; Ogunnusi and Oso, 2014; Adindu e al, 2016; Tarh and Iroegbu, 2017).

II. MATERIALS AND METHODS

Plant Sample Collection and Crude Extraction:

The plant samples were collected from Neke-Oghe in Enugu state. The bark of *C. gigantea* and the stem of *E. kamerunica*, were room-dried and ground into powder to aid extraction and phytochemical analysis. N-hexane and hot water were used to extract the constituents of ground, dry *E. kamerunica* succulent and *C. gigantea* bark. Approximately 25g of each plant was measured and put in respective conical flasks; 250ml of n-hexane was added to each flask and swirled to mix. The mixture was allowed to stand for 24 hrs, after which it was filtered through Whatman® no.1 filter papers and the filtrate was collected into conical flasks, poured into trays and allowed to air-dry. The same procedure was carried out for each plant using hot water. However, the mixture was allowed to stand until it cooled to about 40˚C before filtering. Dry samples were later scraped off and collected in universal bottles. These were preserved for further use in the refrigerator at 4˚C.

Antibiogram

A 500mg/ml concentration of the extract of each plant sample was made by adding 1g of the extract into 2ml of the extracting reagent or solvent (n-hexane or water). A two-fold serial dilution was then made to give 5 concentrations of the extract: 250, 125, 62.5, 31.25 and 15.625 mg/ml respectively in different test tubes. A 0.5 McFarland standard and normal saline was also prepared according to Manufacturer's specifications.

The organisms were then standardized by adding aliquots gradually from peptone water into normal saline in Bijou bottles, until they were just as 'cloudy' as the 0.5 McFarland standard. They were then allowed to stand on the bench, awaiting inoculation onto MHA plates.

Agar Diffusion Method

The Minimum Inhibitory Concentration (MIC), which is the smallest concentration able to stop the growth of the organism, was carried out in the study. Already prepared MHA plates were seeded with 0.1ml of the McFarland standardized organism (containing $1\times 10^8 \times 0.1$ CFU/ml). The excess was drained off after spreading evenly with a flamed glass spreader and the plates were allowed to stand for a few minutes for proper diffusion.

An 8mm cork borer was used to bore seven wells in the medium (MHA) and the six concentrations of the extract were then introduced into each well. The middle well, the seventh, contained the control, which was either hot water or n-hexane. The plates were then allowed to stand for 15 minutes before incubation to aid diffusion. Incubation was done at 37˚C for 18 hrs before reading their Inhibition Zone Diameters (IZD), taken in millimeters using a calibrated metre rule.

Agar Dilution Method

The Minimum Cidal Concentration (MCC), which is the smallest concentration able to kill the test organism, was carried out by further serially diluting the MICs two-fold to give 15.6, 7.8 and 3.9 mg/ml for each extract. About 1ml of these concentrations was added to 9ml of molten MHA and poured in Petri dishes and the mixtures were allowed to solidify.

About 0.1ml of each test organism was then spread-inoculated with a flamed glass spreader onto sterile MHA plates and allowed to stand for 15mins before incubation at 37˚C for 18 and 24 hrs.

Antibiotic Sensitivity Tests

Approximately 0.1ml of the each organism was seeded on already prepared MHA plates and then spread with a glass spreader and the excess drained off. Gram negative antibiotics of known concentrations (Maxicare® Medical Laboratories) were used against the organisms. The antibiotics and their concentrations are as follows:

Septrin (SXT)	30µg
Chloranphenicol (CH)	30µg
Sparfloxacin (SP)	10µg
Ciprofloxacin (CPX)	10µg
Amoxacillin (AM)	30µg
Augmentin (AU)	30µg
Gentamycin (CN)	10µg

Pefloxacin (PEF)	30µg
Tarivid (OFX)	10µg
Streptomycin (S)	30µg

Each disc was pressed gently down the media to ensure efficient contact. Plates were then incubated at 37˚C for 24 hrs.

III. RESULTS

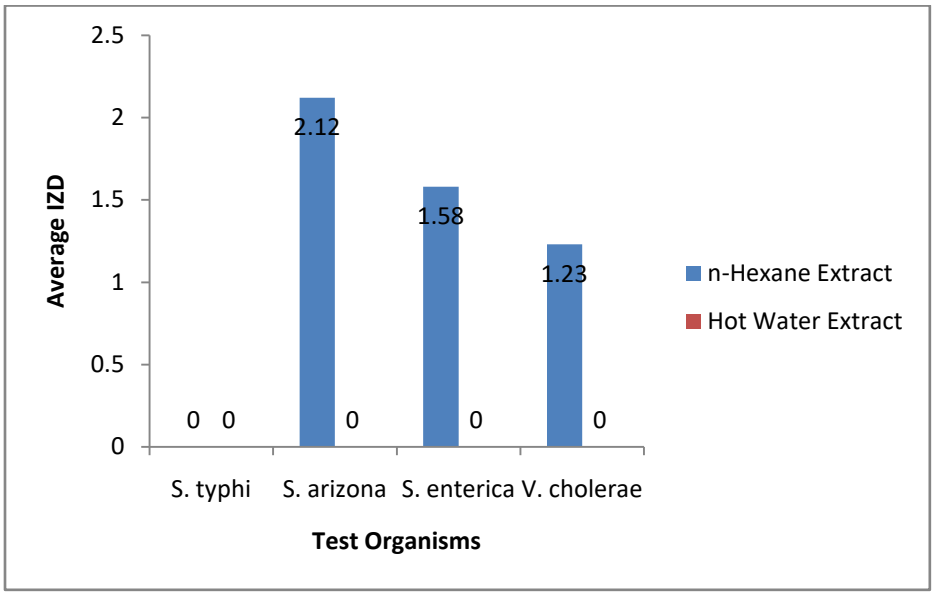

Fig. 1: Average Inhibition Zone Diameters (IZDs) of n-Hexane and Hot Water Extracts of *E. kamerunica.*

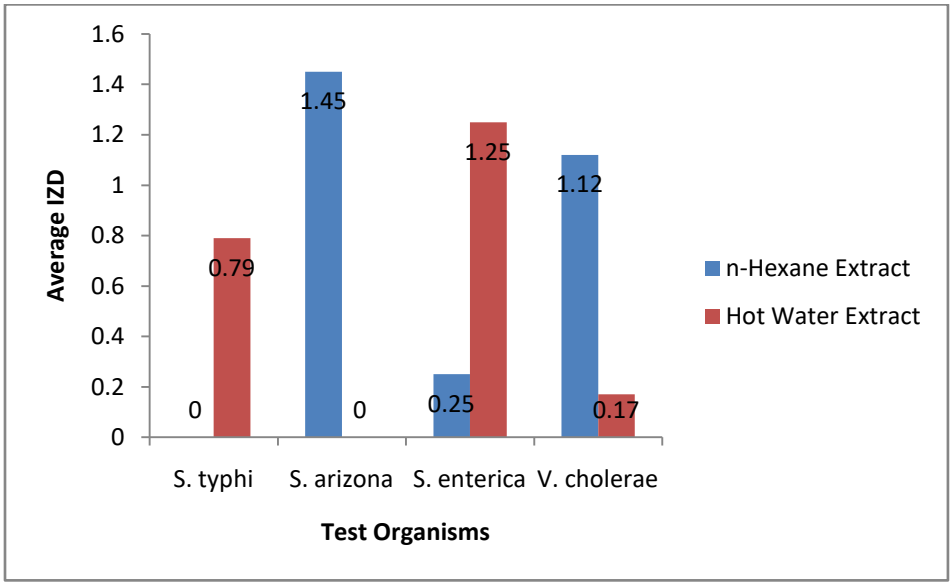

Fig. 2: Average Inhibition Zone Diameters (IZDs) of n-Hexane and Hot Water Extracts of *C. gigantea*

Table 1: Results of Antibiotic Sensitivity.

ISOLATES	ANTIBIOTICS [INHIBITION ZONE DIAMETER (IZD)] (millimeters)										AVERAGE IZD
	SXT	**CH**	**SP**	**CPX**	**AM**	**AU**	**CN**	**PEF**	**OFX**	**S**	
S.typhi	-	-	-	++ (8)	-	-	-	+ (2.5)	-	-	**5.25**
S. arizona	-	++ (8.5)	-	++ (11)	-	-	++ (8)	++ (8)	++(10.5)	++ (8)	**9.0**
S. enterica	-	-	-	++ (10)	-	-	+ (6	+ (2)	-	+ (3)	**5.25**
V. cholerae	-	++ (7.5)	-	++ (9.5)	-	-	+ (2.5)	+ (6)	+ (3)	-	**5.7**

Table 2: Comparison between the Average Inhibition Zone Diameters (IZDs) of Control Antibiotics and n-hexane Extract of *E. kamerunica.*

ORGANISM	AVERAGE IZD OF CONTROL ANTIBIOTICS	AVERAGE IZD OF *E. kamerunica*
S. typhi	5.25	0
S. Arizona	9.0	2.12
S. enteric	5.25	1.58
V. cholera	5.7	1.23
Total Mean	**25.2** **6.3**	**4.93** **1.23**

Table 3: Comparison between the Average Inhibition Zone Diameters (IZDs) of Control Antibiotics and n-hexane Extract of *C. gigantea*

ORGANISM	AVERAGE IZD OF CONTROL ANTIBIOTICS	AVERAGE IZD OF *C. gigantean*
S. typhi	5.25	0
S. Arizona	9.0	1.45
S. enteric	5.25	0.25
V. cholera	5.7	1.12
Total Mean	**25.2** **6.3**	**2.82** **0.705**

Table 4: Comparison between the Inhibition Zone Diameters (IZDs) of Control Antibiotics and Hot Water Extract of *E. kamerunica*

ORGANISM	AVERAGE IZD OF CONTROL ANTIBIOTICS	AVERAGE IZD OF *E. kamerunica*
S. typhi	5.25	0
S. Arizona	9.0	0
S. enteric	5.25	0
V. cholera	5.7	0
Total Mean	**25.2** **6.3**	**0** **0**

Table 5: Comparison between the Inhibition Zone Diameters (IZDs) of Control Antibiotics and Hot Water Extract of *C. gigantea*

ORGANISM	AVERAGE IZD OF CONTROL ANTIBIOTICS	AVERAGE IZD OF *C. gigantean*
S. typhi	5.25	0.79
S. Arizona	9.0	0
S. enteric	5.25	1.25
V. cholera	5.7	0.17
Total Mean	**25.2** **6.3**	**2.21** **0.5525**

Table 6: Minimum Inhibitory Concentration (MIC) of n-hexane and Hot Water Extracts of *E. kamerunica*

TEST ORGANISM	CONTROL	n-HEXANE CONCENTRATIONS OF EXTRACT (mg/ml)							HOT WATER CONCENTRATIONS OF EXTRACT (mg/ml)						
		500	**250**	**125**	**62.5**	**31.25**	**15.6**	**MIC (mg/ml)**	**500**	**250**	**125**	**62.5**	**31.25**	**15.6**	**MIC (mg/ml)**
S. typhi	_	_	_	_	_	_	_		-	-	-	-	-	-	-
S. arizona	_	3.2	3.0	2.0	2.0	1.5	1.0	**15.6**	-	-	-	-	-	-	-
S. enterica	_	2.5	2.0	2.0	1.5	1.0	0.5	**15.6**	-	-	-	-	-	-	-
V. cholerae	_	1.7	1.5	1.5	1.2	1.0	0.5	**15.6**	-	-	-	-	-	-	-

Key: - No Inhibition.

Table 7: Minimum Inhibitory Concentration (MIC) of n-hexane and Hot Water Extracts of *C. gigantea*

TEST ORGANISMS	CONTROL	n-HEXANE CONCENTRATIONS OF EXTRACT (mg/ml)							HOT WATER CONCENTRATIONS OF EXTRACT (mg/ml)						
		500	**250**	**125**	**62.5**	**31.25**	**15.6**	**MIC (mg/ml)**	**500**	**250**	**125**	**62.5**	**31.25**	**15.6**	**MIC (mg/ml)**
S. typhi	_	_	_	_	_	_	_	_	2.0	1.75	1.0	-	-	-	**125**
S. Arizona	_	3.5	2.0	1.5	1.0	0.5	0.2	**15.6**	-	-	-	-	-	-	-
S. enteric	_	1.5	_	_	_	_	_	**500**	3.5	2.5	1.5	-	-	-	**125**
V. cholerae	_	2.5	1.5	1.0	1.0	0.5	0.2	**15.6**	1.0	-	-	-	-	-	**500**

Key: - No Inhibition.

Table 8: Minimum Bactericidal Concentrations (MBCs) of n-hexane and Hot Water Extracts of *E. kamerunica*

TEST ORGANISMS	CONTROL	n-HEXANE CONCENTRATION OF EXTRACT (mg/ml)						HOT WATER CONCENTRATIONS OF EXTRACT (mg/ml)			
		15.6	**31.2**	**62.4**	**124.8**	**249.6**	**MCC (mg/ml)**	500	1000	2000	**MCC (mg/ml)**
S. typhi	_	_	_	_	_	_	_	-	-	-	_
S. arizona	_	+	+	+	+	+	**15.6**	-	-	_	_
S. enterica	_	+	+	+	+	+	**15.6**	-	-	_	_
V. cholerae	_	+	+	+	+	+	**7.8**	-	-	_	_

Key: (+) Killing, (-) No Killing

Table 9: Minimum Bactericidal Concentrations (MBCs) of n-hexane and Hot Water Extracts of *C. gigantea*

TEST ORGANISMS	CONTROL	n-HEXANE CONCENTRATIONS OF EXTRACT (mg/ml)							HOT WATER CONCENTRATIONS OF EXTRACT (mg/ml)				
		15.6	**31.2**	**62.4**	**124.8**	**249.6**	**500**	**MCC (mg/ml)**	125	500	1000	**2000**	**MCC (mg/ml)**
S. typhi		-	-	-	-	-	-	-	-	-	+	+	**1000**
S. arizona		-	+	+	+	+	+	**31.2**	-	-	-	-	-
S. enterica		-	-	-	-	-	-	-	-	+	+	+	**500**
V. cholerae		-	-	+	+	+	+	**62.4**	-	-	-	+	**2000**

Key: (+) Killing, (-) No Killing

IV. DISCUSSION

The use of hot water and n-hexane as extracting solvents was to compare the traditional hot water extraction with one done with an organic solvent. Evidently, extraction with n-hexane (and probably other organic solvents) was more effective in inhibiting the organisms (Tables 1, 2 &3). *S.typhi* nevertheless, was exception. However, while the aqueous extract of *E. kamerunica* was completely ineffective in all the organisms, that of *C. gigantean* showed appreciable level of efficacy when compared with the former (Fig. 1&2). Also from the result, *E. kamerunica* was shown to be more effective as an antibacterial preparation than *C.gigantea.* It has earlier been reported than among antimicrobial berbs, *E. kamerunica* has shown wider activity beyond bacterial species. On assessing the *in vitro* activities of species of *C. gigantea, Ocimum gratissimum* and *E. kamerunica*, against the fungus *Candida albicans*, it was discovered that even though they all exhibited antifungal activities, the fungus showed increasing resistance in the order: *C. gigantea*, *Ocimum gratissimum* and *E. kamerunica* at MIC of 120mg/ml due to the poisonous sap of the plant *E. kamerunica* (Sonibare *et al.*, 2009). *S. typhi* showed the most resistance when tested with the herbs. It was inhibited by only the hot water extract of *C. gigantea* up till 125mg/ml. Only the extract from this herb can be improved upon for potential anti-*S. typhi* aqeous preparations. The differences in the inhibition are probably due to the differences in the quantity of their phytochemicals and the efficiency of each extracting solvent. The precedence of n-hexane extract of *E. kamerunica* is in consonance with reports of aliphatic alcohols (with up to 3 carbon atoms) or mixtures of alcohol with water possessing more extractive powers for almost all natural substances of low molecular weight like alkaloids, saponins and flavonoids (Taghreed, 2001; Aduol *et al*, 2014).

Both the n-hexane and hot water extracts of the two herbs achieved relatively lower inhibition zone diameters than conventional antibiotics (Tables 2, 3, 4 and 5). However, the n-hexane extract of *E. kamerunica* showed no statistical

significance in their different inhibition abilities thereby presenting it as a potential drug for the test organisms with more refined extraction. The comparative differences in the inhibitive abilities of other preparations were statistically significant.

The Minimum Inhibitory Concentrations (Tables 6 &7) and Minimum Bactericidal (Tables 8 & 9) highlighted the organisms' susceptibility to the herbs as well as the herbs' potentiality as future drugs. Salmonella typhi not only resisted the most effective herbal extract in this study but also resisted most of the antibiotics used (being sensitive to only ciprofloxacin and pefloxacin). The organism's resistance to most antibiotics has been reported widely especially those of nosocomial origin. When assessing the antimicrobial susceptibility of *S. typhi* in hospitalized cancer patients in a Ukrainian hospital, Hossam and El-Sharif discovered that the organism was resistant to a 10mg/ml concentration of septrin, chloranphenicol, amoxicillin and streptomycin (Ashour and Amany, 2004). *S. arizona* exhibited the highest level of sensitivity being inhibited by n-hexane extracts of both herbs to the largest degree. It also had the highest susceptibility to conventional antibiotics. This could account for its very low prevalence as it clearly cannot thrive in the slightest of antibacterial conditions. The susceptibility of S. enteric was most pronounced with the n-hexane extract of *E. kamerunica*, followed by aqueous extract of *C. gigantea* and resisted completely, the aqueous extract of *E. kamerunica*. This is a considerable departure from the findings of Shi *et al*, (2008). They suggested that *S. enterica* showed a considerable susceptibility to herbs having similar constituents as *C. gigantea* and *E.* kamerunica at all dilutions of the herbs. Further testing with conventional gram negative antibiotics revealed a fair susceptiblity to ciprofloxacin, pefloxacin, gentamycin and streptomycin. Synergistic effect can be achieved when the herbs are used simultaneously with the antibiotics as demonstrated by Nweze and Eze (2009) who suggested a 37.5% synergism when *Ocimum gratissimum* was evaluated for its antibacterial properties against *Escherichia coli*, *Proteus mirabilis*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* and its antifungal properties using a clinical isolate of *Candida albicans*. *V. cholerae* was remarkably susceptible at all concentrations of the n-hexane extracts of both plants, while also being inhibited by five conventional antibiotics but with reduced sensitivity to hot water extracts. Clearly, the organism is relatively fragile but continuously prevalent. This can be stemmed with the use of constituents of such herbs as those used in this work in drug formulation. The relative fragility of the organism was reported by Doel and Segrott (2004) in its characteristic sensitivity to almost all antimicrobial agent but *Syzigium aromaticum*, the clove plant. From their bactericidal profile (Table 8 & 9), n-hexane extracts of herbs achieved bacterial death especially on *S. Arizona*, *S. enteric* and *V. cholera*, with relatively low concentration, (not more than four folds its minimum inhibitory concentration). This demonstrates their good candidature for future drugs against those organisms. Same is applicable to the aqueous extract of *C. gigantea* on *S. typhi*.

Statistical Analysis:

The treatments were compared using t-test. Statistically, the treatments were not significantly different between the n-hexane extract of *E. kamerunica* and the gram negative antibiotics but were significantly different between the antibiotics and the n-hexane extract of *C. gigantean* and aqueous extracs of both herbs. All tests were carried out at 0.05 ($\alpha/2$) level of significance.

V. CONCLUSION

The traditional practices of using herbs for the treatment of gastrointestinal disorders increase the risk of overdosing or mutual inactivation of herbal constituents which would otherwise be medicinal in the situation. The use of water as the only solvent for extracting these medicinal constituents is a factor militating against the full exploitation of the medicinal values of these herbs. As shown in this research, use of organic solvent (n-hexane) has really improved on the traditional practice. Researches and experiments with plants of antimicrobial potentials are therefore useful in formulating and standardizing affordable drugs for the treatment of certain health conditions.

VI. RECOMMENDATIONS

Several trials of different organic solvent are needed until the best extracting solvent is discovered. This is in view of the peculiarities of these solvent in the extraction of different phytochemicals. Further studies should be carried out to unravel the toxicities of these herbs at their therapeutic doses. Efforts should also be geared towards verifying the synergistic effects of these medicinal plants and some common antibiotics as a way of curbing the menace of multidrug resistant organisms as well as their roles in the microbial resistance attributes.

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