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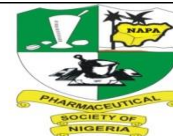

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Antimicrobial Evaluation of Extracts and Fractions of *Daniella oliveri* Leaf on Selected Clinical Enteropathogens

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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

Background: Diarrhoea is one of the major health threats to the populace in the tropics, and also one of the killer diseases in children under 5 years of age. Antimicrobial resistance and its spread pose serious public health threats, hence the need for development of safer and more effective antibacterial agents. *Daniella oliveri* Hutch and Dalz is used in ethnomedicine for the treatment of diarrhoea and other gastrointestinal disturbances.
Objective: To investigate the antimicrobial activity of *Daniella oliveri* leaves on diarrhoeal pathogens.
Methods: Successive plant extraction was carried out with hexane, ethylacetate and methanol using soxhlet apparatus. Methanol extract was fractionated using vacuum liquid chromatography (VLC). Phytochemical screening was done using standard chemical assays. Antibiogram of test isolates was carried out using disc diffusion assay. Antimicrobial activity of plant extract and fractions against strains of diarrhoeagenic *Escherichia coli*, *Salmonella cholerae-suis*, *Shigella dysenteriae, Proteus mirabilis* and *Acinetobacter baumannii* was determined by agar well diffusion and MIC by agar dilution methods. Kill-kinetics study was carried out using viable count technique.
Results: Terpenoids, steroids and anthraquinones were detected in fractions of *D. oliveri*. Antibiogram assay showed that 66% of isolates were MDR. Extract and fractions produced appreciable zones of inhibition on all challenge organisms except *Acinetobacter baumannii*. MICs ranged between 6.25-25 mg/ml. Kill kinetics studies showed total kill on susceptible pathogens after 24 hours.
Conclusion: This research has shown *D. oliveri* is a promising drug candidate for the management and treatment of diarrhoea.
Keywords: *Daniella oliveri,* Antimicrobial, Antidiarrhoeal, Methanol extract, Fractions

INTRODUCTION

Medicinal plants refer to plants that are used to cure diseases or relieve pain and the activities could be due to the phytochemicals they contain (Okigbo and Mmeka*,* 2008; Adesokan *et al*., 2008). Some of these plants contain antimicrobial compounds which are effective in treating bacterial and fungal infections especially in developing countries exemplified, by Nigeria and other African countries and India (UNESCO, 1996; Kubmarawa *et al.,* 2007). It has been reported that about 70% of the entire world population is dependent on plant-based medicines (Pravin, 2006). Recognition of medicinal plants in therapy has become more widespread for several reasons; including increased faith in herbal medicine (Kala, 2005). This belief in medicinal plants is widely linked to properties such as antioxidant, antimicrobial, antipyretic effects conferred by phytochemicals present in them (Adesokan *et al*., 2008); making medicinal plants active against rapidly emerging resistant infections. Indeed several studies in Africa have shown emergence of multi-drug resistant organisms over the last decade, with the general spread of antimicrobial resistance to

commonly used antibiotics (Nascimento *et al*., 2000; Sakagami and Kajimura, 2002; Okeke *et al*., 2005).
Diarrhoea is considered a global killer affecting mostly children and the elderly; being a major health threat to tropical and subtropical populations (Heinrich, 2005). The leading cause of death from diarrhoea is dehydration, resulting in imbalance of body fluids, leading to disruption of blood salt and sugar levels ultimately interfering with proper body functioning. Diarrhoea is the number one killer disease of children under 5 years in Nigeria (Umilabug *et al.,* 2000). The WHO has estimated annual cases to be between 3 to 5 billion, with 1 billion in children below the age of 5 resulting in 5 million deaths (Abdullahi *et al.,* 2000).
Daniella is a genus of legumes in the Fabaceae family. The trees reach heights of 30 to 40 metres. *Daniella oliveri* which produces pea-shaped flowers has been reported to possess anti-malarial, anti-diarrhoeal, anti-inflammatory, antinociceptive, antipyretic, aphrodisiac, antidiabetic and antimicrobial activities (Ahmadu *et al*., 2004; Onwukaeme and Udoh, 1999).

There are ethnobotanical claims that several parts of *Daniella oliveri* have biological activities (Ahmadu *et al,* 2004). For example, young leaves are used to treat wounds and relieve general body pains (Magallon *et al.*, 2001).
Despite the effective, simple and cheap treatment of oral dehydration therapy, majority of the local populace still rely on herbs to treat diarrhoea especially when it is persistent. Medicinal plants frequently used for treating diarrhoeal infections in Nothern Nigeria include: *Daniella oliveri* Hutch and Dalz (Fabaceae) and *Ficus sycomorus* Linn (Moraceae). In addition to diarrhoea, the leaves of *D. oliveri* are used traditionally in Northern Nigeria to treat diabetes, gastrointestinal disturbances, diarrhoea, as diuretic and aphrodisiac (Hutchinson and Dalziel, 1964; Onwukaema and Udoh, 1999).
As part of our efforts to screen for antidiarrhoeal activity and support the ethnomedicinal claim, the leaves of *D. oliveri* was investigated against selected enteric bacterial pathogens.

METHODOLOGY

Materials and Methods

Collection and Preparation of Plant materials

Leaf samples were collected at Ilorin Balsam, Asa Local Government, Kwara, Nigeria. The plant was identified and authenticated at Forestry Research Institute of Nigeria (FRIN) with voucher number FHI 111057.
The leaves were then air-dried, pulverized and weighed.

Phytochemical screening

The phytochemical analysis of *D. oliveri* was carried out to determine the presence of secondary metabolites using the methods described by (Vinoth *et al*., 2011).

Plant extraction and fractionation

Powdered leaves of *D. oliveri* were successively extracted with organic solvents of increasing polarity: *n*-hexane, ethyl acetate and methanol, using soxhlet apparatus. The extracts were then concentrated to dryness using an electric shaker set at 60°C after which they were weighed and stored in the refrigerator (4°C) for further use.
The methanol extract was subjected to Vacuum Liquid Chromatography (VLC). Thirty-four grams of the extract was weighed and adsorbed with silica gel (60-200 mesh size). Whatmann No 1 filter paper was placed inside a ceramic sieve funnel and silica gel was poured on it. A layer of paper towel was used to cover the silica gel. The absorbed extract was poured inside the funnel. Another layer of paper towel was then placed on top of the absorbed extract. The funnel was fitted into a conical flask with an outlet connected to a vacuum pump. 100 ml of different ratios of the solvents (*n*-hexane, ethyl acetate and methanol) was poured to pass through the extract-silica gel mixture in increasing polarity. At every point each solvent combination was allowed to pass through the extract-silica gel mixture, the fractions were collected in clean collection bottles and labeled appropriately.
A thin layer chromatographic (TLC) plate was spotted with each fraction and this was chromatographed with solvent mixtures of hexane/ethylacetate (4:1), ethylacetate/methanol (1:1) and ethylacetate/methanol (3:2). The fractions were then pooled based on their TLC profiles. A total of four pooled fractions were obtained.

Microorganisms Used

A total of 29 isolates were collected from Molecular Microbiology Laboratory, Faculty of Pharmacy, University of Ibadan. All isolates were of enteric origin; isolated from paediatric faecal samples associated with diarrhoea. They include *Escherichia coli*, *Proteus mirabilis*, *Acinetobacter baumannii, Salmonella choleraesuis*, *Shigella dysenteriae* and *Serratia odorifera.* The isolates which were identified

with Microbact 24E and PCR, were maintained in Luria broth and glycerol at -80°C. Prior to antimicrobial testing, microorganisms were sub-cultured at 37°C for 24 h.

Antibiogram of test isolates

The test was carried out using standard antibiotic multi-discs with agar diffusion method described by Cheesebrough (2000). Isolates were grown in overnight broth cultures. Cultures were diluted and dilutions corresponding to 0.5 McFarland standards were used. 20 mL each of Mueller Hinton Agar was poured in petri dishes and allowed to set. Sterile cotton swab was used to inoculate the plates with microbial cultures. Antibiotic discs were placed firmly on the inoculated plates using sterile forceps to ensure firm contact of disc with agar. Plates were then incubated at 37°C for 24 h. After incubation the diameters of zones of inhibition were measured in millimetres (mm), recorded and interpreted according to the standards of Clinical Laboratory Standards Institute (CLSI, 2016).

Antimicrobial screening of plant extract and fractions against MDR clinical enteropathogens.

This was conducted on the multi-drug resistant (MDR) isolates using the agar well diffusion method. Twenty milliliters of molten agar was poured into each sterile Petri dish and allowed to set. A few Petri dishes were inverted and incubated overnight to test sterility of media. Sterile cotton swabs were dipped into the standard inoculums (0.5 McFarland equivalents) and the excess pressed out on the side of the test tube to avoid over flooding. Surface of plates were then swabbed. Equidistant wells were bored with the aid of a standard sterile 8mm cork borer and 100 µl of respective extract concentrations and controls were dispensed into corresponding wells. Extracts of the different concentrations were used to fill the bored holes. Methanol served as a negative control while gentamicin (10µg/ml) and ciprofloxacin (5µg/ml) served as drug controls for Enterobacteriacea and *Acinetobacter baumannii* respectively. The plates were thereafter incubated at 37°C for 24h and zones of inhibition were observed and recorded. The screening for antimicrobial activity was carried out on each of the extracts collected and standard antibiotic against the microbial isolates. Agar cup diffusion method was employed in respect of the extracts and standard antibiotic. The same procedure was repeated for the pooled fractions.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extracts

Minimum Inhibitory Concentration (MIC) of extracts on test isolates was determined using agar-dilution method previously described (Adeniyi *et al*., 2009) where extracts were tested at various concentrations. Two milliliters of the extract at different concentrations (50mg/ml, 25mg/ml, 12.5mg/ml, 6.25mg/ml, 3.125mg/ml, 1.5625mg/ml, and 0.78mg/ml) were added to 18ml of sterile molten agar maintained at 45°C. It was properly mixed for even distribution in the bottles and poured into sterile Petri dishes and allowed to set. The surface of the agar was dried in sterile oven at 37°C before streaking with overnight broth cultures of the test organisms. The plates were incubated at 37°C for 24 h and examined for presence and absence of colonies. The least concentration that prevented visible growth of colonies was taken as the minimum inhibitory concentration (MIC). Agar plate without extract was used as negative control.

The MBC for the bioactive extracts was determined by using sterile inoculating loop to swab the MIC plates that did not show any growth. This was used to inoculate freshly prepared nutrient broth. The inoculated broths were then incubated at 37°C for 24 h. The least concentration without growth of microorganism was taken as the minimum bactericidal concentration (MBC).

Time Kill Assay

The time kill assay was carried out following the method of Lajubutu *et al*., 1995. Overnight broth culture of the most susceptible isolate was made in 5mL of nutrient broth. Subsequently, 1ml of the broth culture was inoculated into another 4ml of nutrient broth and incubated for 18 h at 37 °C. This was to ensure that the organism was at the exponential growth phase. Thereafter, 0.1 ml of the culture was inoculated into 2.9 ml of nutrient broth containing 1ml of the extract mixture at a final concentration equal to the MIC. Serial dilutions were made from this mixture containing the microbial culture, broth and extract and 0.1 ml of the 10^{-2} and 10^{-4} dilutions were used to inoculate a freshly prepared nutrient agar at different time intervals beginning from 0 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, 12 h and 24 h. A sterile glass spreader was used to evenly spread the inoculum on the nutrient agar plate. The plates were allowed to dry for few minutes at room temperature before incubation at 37°C for 24 h. This procedure was repeated for extract concentrations containing 2times the MIC and 4times the MIC. Also, a control broth was set up but with no added extract. After the incubation, microbial colony count was done and a graph of log of CFU/ml was plotted against time.

RESULTS AND DISCUSSION

Results summary

Antibiogram results of bacterial enteropathogens showed that 19 of the 29 isolates were MDR. 76% of the total enteric isolates were resistant to amoxicillin/clavulanic acid and 77% to streptomycin while 63% and 53% were resistant to tetracycline and chloramphenicol respectively. 34% were susceptible to ceftazidime and cefotaxime. All isolates of *Acinetobacter baumannii* were resistant to amoxicillin/clavulanic acid, streptomycin, tetracycline, chloramphenicol and cefotaxime. (Figure 1) The yield in grams of the n-hexane, ethyl acetate and methanol extracts of leaves of *Daniella oliveri* are presented in Table 1. The highest yield was obtained from methanol extract.

Phytochemicals detected in *D. oliveri* powdered leaves and fractions include terpenoids, alkaloids, saponins, tannins, flavonoids and anthraquinones. (Tables 2 and 5)

The susceptibility of the isolates is presented in Table 3 while the MIC and MBC values are presented in Table 4. The results of the bactericidal kinetics of methanol extract on some test isolates are represented in Figure 2 showing a concentration dependent kill. .

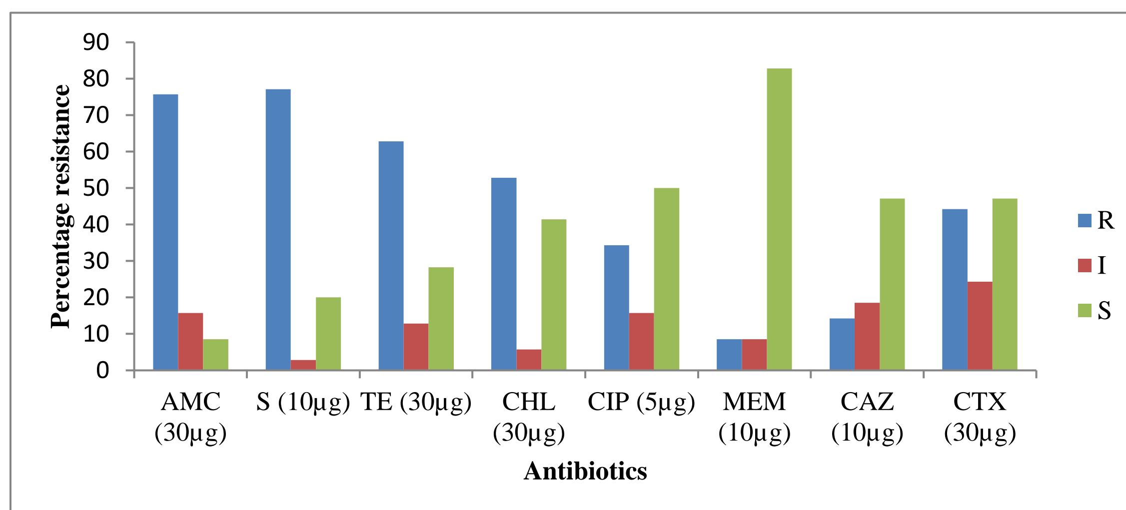

Figure 1: Percentage resistance of test isolates to selected antibiotics

Key: AMC = Amoxicillin/Clavulanic acid, S = Streptomycin, TE = Tetracycline, CHL = Chloramphenicol, CIP = Ciprofloxacin, MEM = Meropenem, CAZ = Ceftazidime, CTX = Cefotaxime, R= Resistant, I= Intermediate, S= Sensitive

Table 1: Percentage Yield of the Crude Extracts of *D. oliveri*

Extract	Weight of pulverized plant sample (g)	Weight of extract (g)	Percentage yield (%)
n-Hexane	1180	30.6	2.59
Ethyl acetate	1180	21.6	1.83
Methanol	1180	138.3	11.72

Table 2: Phytochemical Screening of Powdered Leaves of *D. oliveri*

Secondary metabolite	**Presence/Absence**
Terpenoids	+
Steroids	+
Saponins	+
Tannins	+
Flavonoids	+
Phlobatannins	-
Anthraquinones	+
Alkaloids	+
Cardiac glycosides	-

Key: - = Absent + = present

Table 3: Antimicrobial Screening of Crude Extract and Fractions on Enteric Isolates

	Methanol extract			Fraction C			Fraction D			Gentamicin	Ciprofloxacin
Concentration	100 mg/mL	50 mg/mL	25 mg/mL	80 mg/mL	40 mg/mL	20 mg/mL	80 mg/mL	40 mg/mL	20 mg/mL	10 ug/mL	5 ug/mL
Isolate											
E. coli CHD001F	19	14	NZI	15	12	11	16	15	14	22	NT
E. coli CHD012C	NT	NT	NT	12	10	NZI	12	10	NZI	16	NT
E. coli CHD001G	15	10	11	16	14	13	20	16	12	18	NT
E. coli CHD001H	17	12	9	NT	NT	NT	NT	NT	NT	13	NT
E. coli CHD102G	19	11	10	NT	NT	NT	NT	NT	NT	13	NT
E. coli CHD003H	20	16	13	18	15	12	22	20	18	16	NT
E. coli CHD076F	22	17	16	14	13	10	19	14	14	13	NT
E. coli CHD076I	21	15.	15	14	12	11	17	16	13	14	NT
E. coli CHD80G	20	14	NZI	22	17	15	15	14	NZI	15	NT
E. coli CHD012C	13	12	9	NT	NT	NT	NT	NT	NT	22	NT
S. dysenteriae CHD043	NZI	13	10	16	13	11	18	15	12	NZI	NT
S. choleraesius CHH003E	12	NZI	10	14	12	11	16	14	13	11	NT
S. odorifera CHH011D	17	16	14	NT	NT	NT	NT	NT	NT	NZI	NT
S. odorifera CHH011E	21	22	17	NT	NT	NT	NT	NT	NT	12	NT
P. mirabilis CHD014D	28	21	12	15	12	12	15	13	11	17	NT
P. mirabilis CHH014D	13	13	11	16	13	NZI	16	15	10	24	NT
P. mirabilis CHD014E	19	17	14	15	12	14	16	14	11	12	NT
A. baumannii CHD075A	NZI	12	13	NT	NT	NT	NT	NT	NT	NT	11
A. baumannii CHD075B	15	14	NZI	13	NZI	NZI	14	11	12	NT	NZI
A. baumannii CHD075C	12	NZI	10	NT	NT	NT	NT	NT	NT	NT	10

Key: '*E. coli*' = *Escherichia coli*; '*S. dysenteriae = Shigella dysenteriae'; 'S. choleraesius = Salmonella choleraesius'*; '*P. mirabilis*' = *Proteus mirabilis*; '*A. baumannii = Acinetobacter baumannii';* 'NZI' = no zone of inhibition*;* 'NT' = not tested.

Table 4: Minimum Inhibitory and Minimum Bactericidal Concentrations of Methanol Extract of *D. oliveri* on Isolates

Organism	MIC (mg/ml)	MBC (mg/ml)
Escherichia coli CHD001F	6.25	25
Escherichia coli CHD001G	12.5	25
Escherichia coli CHD001H	6.25	25
Escherichia coli CHD003H	12.5	25
Escherichia coli CHD076F	6.25	25
Esherichia coli CHD076I	12.5	25
Escherichia coli CHD102G	12.5	25
Esherichia coli CHD012C	6.25	25
Escherichia coli CHD080G	6.25	25
Serratia odorifera CHH011D	6.25	25
Serratia odorifera CHH011E	12.5	50
Proteus mirabilis CHD014D	6.25	25
Proteus mirabilis CHH014D	6.25	25
Proteus mirabilis CHD014E	12.5	25
Shigella dysentriae. CHD043D	25	25
Salmonella choleraesius CHH003E	25	25
Acinetobacter baumannii CHD075A	25	50
Acinetobacter baumannii CHD075B	25	50
Acinetobacter baumannii CHD075C	25	50

Table 5: Phytochemical screening of pooled fractions

Compound	Fraction B	Fraction C	Fraction D
Saponins	-	-	++
Tannins	-	-	++
Flavonoids	-	-	++
Cardiac glycosides	-	-	-
Terpenoids	+	+	+
Steroids	+	++	+
Alkaloids	-	-	+
Anthraquinones	+	+	+

Key: - = Absent + = present ++ = present in abundance

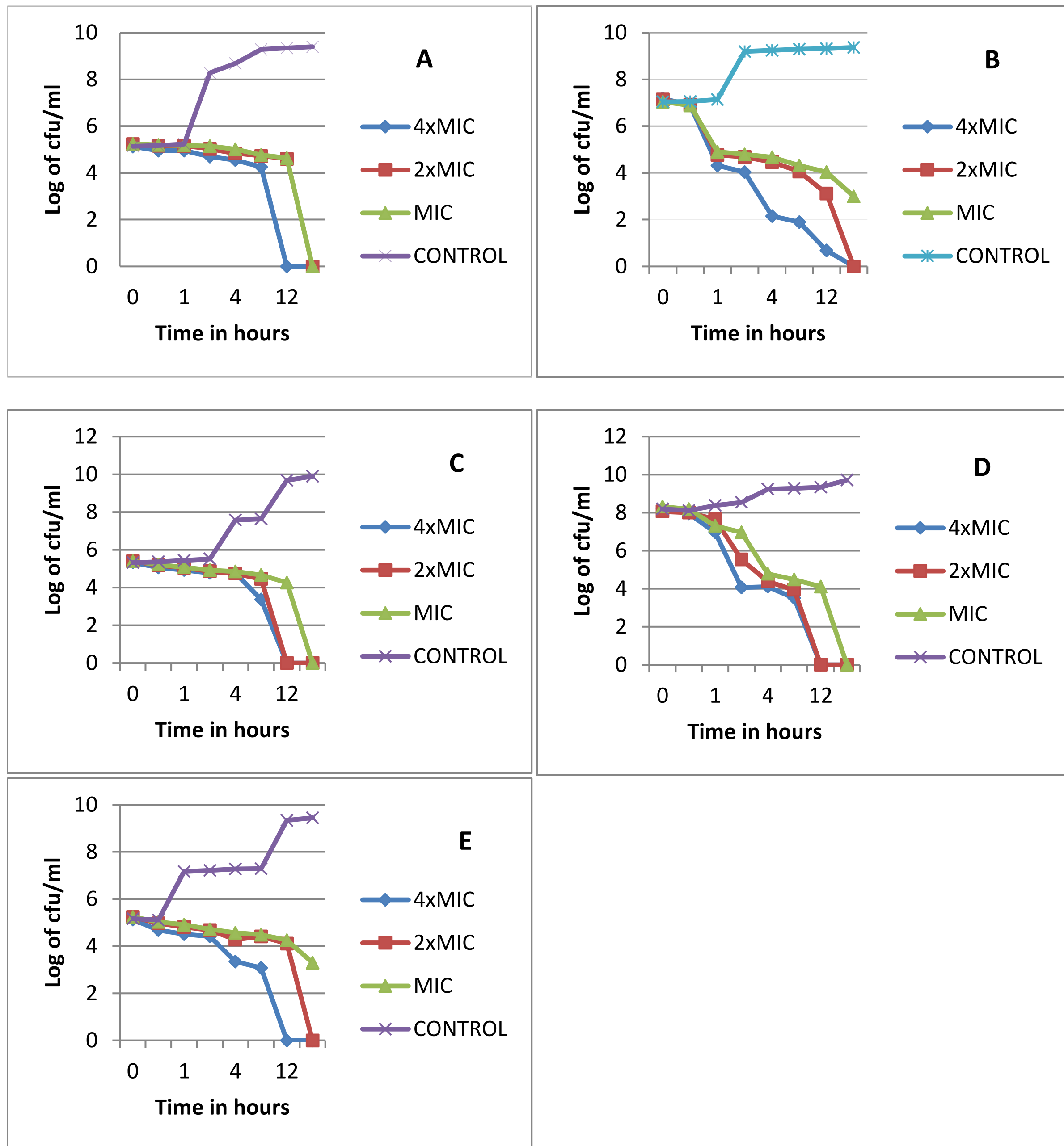

Figure 2: Bactericidal kinetics of methanol extract on *Escherichia coli* CHD001G (A); *Escherichia coli* CHD001F (B); *Escherichia coli* CHD076F (C); *Salmonella cholerae* CHH003E (D); *Shigella dysentriae* CHD043D (E)
MIC: A = 12.5mg/ml; B = 6.25mg/ml; C= 6.25mg/ml; D= 25mg/ml; E=25mg/ml;

DISCUSSION

Plant selection was done based on ethnobotanical survey conducted by several researchers suggesting that the plant has antimicrobial potentials. As reported by Ahmadu *et al*., (2004), all parts of *D. oliveri* have been shown to have biological activities. This research focused on antimicrobial effects of extracts of *Daniella oliveri* against clinical enteropathogens.

Antibiogram showed that about 66% of tested isolates were multi-drug resistant (CLSI, 2016). The occurrence of MDR isolates in feaces of children is a great concern, especially as there are chances of exchange of mobile genetic elements in the gut. Resistance could then be transferred across bacteria passing through the gut and disseminated in the

environment, potentially leading to widespread antimicrobial resistance.

Methanol produced the highest yield of extract implying that the plant relatively contained more polar constituents. This corresponds with findings of Onwukaeme and Udoh, (1999) who reported the yield of methanol extract of *Daniella oliveri* to be higher compared to those of *n*-hexane, dichloromethane and ethyl acetate.

Phytochemical analysis of the leaf indicated presence of several secondary metabolites including tannins, saponins, flavonoids, alkaloids, terpenoids, steroids but cardiac glycosides and phlobatannins were absent. It is generally believed that medicinal properties of plants are conferred by secondary metabolites they produce. Such medicinal activity is linked to presence of different phytochemicals which usually acts simultaneously on different targets, explaining medicinal plants activity on drug resistant infections (Javed *et al*., 2012). For example, Otshudi (2000) reported that secondary metabolites such as saponins, tannins, phenols, anthaquinones, flavonoids, cardiac glycoside, sterols, alkaloids and terpenoids have been found active on pathogenic microbes. However, the most efficient therapeutically according to Otshudi (2000) are alkaloids.

Phytochemical screening of pooled fractions revealed similar secondary metabolites as dried powdered leaves. Presence of these groups of secondary metabolites could be responsible for the inhibitory potential shown by the extract and fractions since these compounds have been previously studied to possess antimicrobial activities. Though the mechanism of antimicrobial action of the secondary metabolites is not fully understood; many investigations have been conducted. It is believed that single compounds may not be responsible for the bioactivity, but rather combination of compounds interacting in synergistic or additive manner Some persons have proposed that phytochemicals present in plants produce antimicrobial effect by simultaneously acting on different targets, making it difficult for antimicrobial resistance to develop (Javed *et al*., 2012). The mechanism of flavonoids might be through disruption of cytoplasmic membrane and inhibition of DNA gyrase (Cushnie and Lamb, 2005).

After fractionation by vacuum liquid chromatography (VLC) for further analysis, fractions A and B were not screened for antimicrobial activity because they had low yields. Fractions C and D were screened on selected enteropathogens and showed larger zones of inhibition at varying concentrations in relation to the crude extract. This could imply that fractionation concentrated active constituents indirectly by getting rid of inactive constituents which make up bulk of crude extracts. The emergence of new strains of disease-causing bacteria especially those resistant to drugs routinely used for treatment, require that antibiotic use is closely monitored. For example, a system that shows the rate and extent of bacterial killing (kill kinetics) provides more accurate description of antimicrobial activity than does the MIC (Zhanel *et al*., 1991). The minimum inhibitory concentration for *Escherichia coli* was between 6.25 and 12.5mg/ml while that of *Salmonella cholerae* and *Shigella dysenteriae* were both 25mg/ml.

Kill kinetics have been used to demonstrate the bactericidal activity of antimicrobial drugs on target organisms to depict better killing synergism. It has also given better sensitivity trends to physicians than disk diffusion methods (Okemo *et al*., 2001). From the results of bactericidal kinetics, the plant extract showed a concentration dependent kill. Higher multiples of the MIC gave a faster kill time as compared with lower multiples. For all the tested isolates, it can be depicted that any concentration greater than 4MIC will kill the population growth at 12h interval. This proves that the extracts and fractions possess bactericidal rather than bacteriostatic properties.

CONCLUSION

Daniella oliveri exhibited broad spectrum antibacterial activity even on multi-drug resistant pathogens. This indicates that *D. oliveri* could be a promising lead in the development of antimicrobial drugs for treatment of diarrhoea.

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