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Full Length Research Paper

Anti-*Vibrio* and preliminary phytochemical characteristics of crude methanolic extracts of the leaves of *Dialium guineense* (Wild)

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This study investigated the anti-*Vibrio* activities of the leaf extract of *Dialium guineense* on eighteen environmental strains of vibrios. The phytochemical constituents of the extract were also assayed in a preliminary test. The results showed that *D. guineense* crude leaf extract exhibited anti-*Vibrio* activities on fourteen out of eighteen environmental strains of *Vibrio* spp. at a final concentration of 20 mg/ml; the zones of inhibition exhibited by the extract against the test isolates ranged between 12 and 20 mm. Standard antibiotics - ampicillin and streptomycin inhibited the growth of the test isolates with the zones of inhibition ranging between 7 to 35 mm and 10 to 37 mm respectively. *D. guineense* leaf extract exhibited minimum inhibitory concentrations ranging between 0.313 to 5.0 mg/ml against the *Vibrio* isolates while the minimum bactericidal concentrations exhibited ranged between 0.625 and 10 mg/ml by the extract. From the preliminary qualitative phytochemical screening, tannins, alkaloids, flavonoids, saponins, steroids and cardiac glycosides were detected as phytochemicals in the crude extract of the plant. The results obtained from this study may provide a proof of the usefulness of *D. guineense* for traditional uses as folk medicine in the management of various ailments caused by *Vibrio* spp.

Key word: *Vibrio*, *Dialium guineense*, minimum bactericidal concentrations, phytochemical.

INTRODUCTION

Vibrios belong to the class of *Gammaproteobacteria*; members of the family *Vibrionaceae*; are natural inhabitants of sea water but can also be found in fresh water. They are Gram negative, usually motile rods, mesophilic and chemoorganotrophic. They have a facultative fermentative metabolism and are found in aquatic habitats and in association with eukaryotes (Thompson et al., 2004). Some of them are human pathogens and are mainly transmitted to humans through contaminated water or food. They are part of the natural flora of bacteria in seawater and in the gut of many seawater organisms. They cause gastrointestinal illnesses in humans which include diarrhoea (Oliver and Kaper, 2001). Vibrios can be broadly grouped into two, namely: the cholera and non-cholera groups (Brooks et al., 1998). Among the *Vibrio* species that can cause infections in humans are *Vibrio cholerae, V. vulnificus, V. parahaemolyticus,* and *V. fluvialis*. *V. vulnificus* and *V. parahaemolyticus* are invasive organisms affecting primarily the human colon (Kothary et al., 2003). In addition, *V. vulnificus* causes bacteremia, skin and soft tissue infections while the watery diarrhoea caused by *V. parahaemolyticus* is often accompanied with abdominal cramping, vomiting, fever and chills. *V. fluvialis* is associated with wound infection, septicemia and

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gastroenteritis (Chiang and Chuang, 2003). The antibiotics currently used for the treatment of *Vibrio* infections are doxycycline, quinolones, tetracycline and cephalosporins (Brooks et al., 1998) which are expensive for most of the African populations.

It was reported in some literatures that *Dialium guineense* leaves and stem bark are used as folklore remedies for the treatment of infections such as diarrhoea, severe cough, bronchitis, wound, stomachaches, malaria fever, jaundice, antiulcer and haemorrhoids (Bero et al., 2009). Lawal et al. (2010) reported in their findings that *D. guineense* is used as antiulcer and as a vitamin supplement among some tribes in the southern part of Nigeria. Among the 85 medicinal plants investigated for their potency as antimalaria, *D. guineense* was found to inhibit the growth of *Plasmodium falciparum,* that is, the malaria parasite responsible for the illness (Hermans et al., 2010). *D. guineense* Wild belongs to the family Fabaceae and the sub-family Caesalpinioideae*,* it is called "Awin" among the Yoruba tribe of southwestern Nigeria, and it is used as part of various decoction in the treatment of various ailments among the tribe. It is a tree of an average height of 30 m with densely leafy crown, smooth greyish bark. Leaves are hairy and the flowers are usually whitish while the fruits are less circular and flattened. The pulp of the fruit is edible and sweet, fairly low levels of ascorbic acid and tannin are present. It is a fairly good source of protein and minerals (Arogba et al., 2006). The fruits of the plant are chewed among some women in southeast Nigeria to improve lactation and check genital infection (Nwosu, 2000). *D. guineense* is used as chewing stick (indigenous tooth brush) among Nigerian populace. Okwu and Ekeke (2003) reported in their studies that the plant contains saponin which is presumed to add to the cleaning effect of teeth and at the same time prevent caries and plaques on the teeth of the users.

Significant antioxidant and molluscidal activities of *D. guineense* exhibited have also been reported (Lamien-Meda et al., 2008). Literatures search revealed scanty or no reports on anti-*Vibrio* activities of *D. guineense* crude leaves extracts, hence the need for this research. The objective was to test the antimicrobial potentials of this plant on environmental strains of *Vibrio* species isolated from some rivers in the Eastern Cape Province of South Africa, bearing in mind that, many residents of Eastern Cape depends on rivers for their daily water uses.

MATERIALS AND METHODS

Plant material

Fresh leaves of *Dialium guineense* were collected from Research Farm of Obafemi Awolowo University, Ile Ife, Nigeria. They were authenticated at the Herbarium of Botany Department, Obafemi Awolowo University, Ile Ife, Nigeria. Voucher specimen was deposited at the Herbarium for reference. The leaves were rinsed in clean water and later dried in the hot-air oven at 40°C until the constant weight of the leaves was obtained. The plant sample was later powdered and kept in an air-tight container for further use.

Preparation of the plant extract

Exactly 785 g of the powdered leaf was soaked in a mixture of methanol and sterile distilled water in ratio 3:2 (v/v) for four days and later filtered to obtain the methanolic extract. The mixture was first concentrated *in vacuo* using rotary evaporator to remove the methanol. The aqueous residue was later lyophilized to get the crude extract which was chocolate brown in colour. The yield collected was 293 g.

Preparation of microorganisms for the experiment

The *Vibrio* species used were part of the culture collections of Applied and Environmental Microbiology Research Group (AEMGREG), Department of Biochemistry and Microbiology, University of Fort Hare, South Africa. They were all environmental strains isolated from some rivers in the Eastern Cape Province, South Africa. For the experiment the *Vibrio* isolates were first sub cultured in nutrient broth (Oxoid Ltd.) and incubated at 37°C for 18 h. Mueller-Hinton agar medium (Oxoid Ltd.) was used for the sensitivity testing.

Preliminary phytochemical screening test for the extract

A small portion of the dry extract was subjected to the phytochemical test using Trease and Evans (1983) and Harbourne (1983) methods to test for alkaloids, tannins, flavonoids, steroids, saponins, reducing sugars and cardiac glycoside.

Test for alkaloids

Exactly 0.5 g of the plant extract was dissolved in 5 ml of 1% HCl on steam bath. A millilitre of the filtrate was treated with drops of Dragendorff's reagent. Turbidity or precipitation was taken as indicative of the presence of alkaloids.

Test for tannins

About 1 g of the extract was dissolved in 20 ml of distilled water and filtered. 2 to 3 drops of 10% of $FeCl_3$ was added to 2 ml of the filtrate. The production of a blackish-blue or blackish-green colouration was indicative of tannins. To another, 2 ml of the filtrate was added 1 ml of bromine water. A precipitate was taken as positive for tannins.

Test for flavonoids

A 0.2 g of the extract was dissolved in 2 ml of methanol and heated. A chip of magnesium metal was added to the mixture followed by the addition of a few drops of concentrated HCl. The occurrence of a red or orange colouration was indicative of the flavonoids.

Test for saponins

Freshly prepared 7% blood agar medium was used and wells were made in it. The extract in methanol was applied with distilled water and methanol used as negative control while commercial saponin (BDH) solution was used as positive control. The plates were

incubated at 35°C for 6 h. Complete haemolysis of the blood around the extract was indicative of saponins.

Test for steroids

About 0.5 g of the extract was dissolved in 3 ml of $CHCl_3$ and filtered. To the filtrate was added concentrated H_2SO_4 to form a lower layer. A reddish brown colour was taken as positive for steroid ring.

Test for cardiac glycoside

About 0.5 g of the extract was dissolved in 2 ml of glacial acetic acid containing 1 drop of 1% $FeCl_3$. This was under laid with concentrated H_2SO_4. A brown ring obtained at the interface indicated the presence of a deoxy sugar, characteristic of cardiac glycosides. A violet ring may appear below the brown ring while in the acetic acid layer; a greenish ring may form just above ring and gradually spreads throughout this layer.

Test for reducing sugars

Exactly 1 ml each of Fehling's solutions I and II was added to 2 ml of the aqueous solution of the extract. The mixture was heated in a boiling water bath for about 2 to 5 min. The production of a brick red precipitate indicated the presence of reducing sugars.

Sensitivity testing of *D. guineense* crude extract on *Vibrio* spp.

The sensitivity testing of the crude extract of *D. guineense* was determined using agar-well diffusion method as described by Irobi et al. (1994) with little modifications. The *Vibrio* spp. isolates were first grown in nutrient broth for 18 h before use. The isolates were later subcultured on to Mueller-Hinton agar (Oxoid, Ltd.) and wells were then bored into the agar medium using a sterile 6 mm cork borer. The wells were then filled up with the solution of each of the crude extract and care was taken not to allow the solution to spill to the surface of the medium. The plates were allowed to stand on the laboratory bench for between 1 to 2 h to allow proper inflow of the solution of the fractions into the medium before incubating the plates in an incubator at 37°C for 24 h. The plates were later observed for the zones of inhibition. The effects of the extract on *Vibrio* spp. isolates were compared with those of standard antibiotics, streptomycin and tetracycline at a concentration of 1 mg/ml each.

Minimum inhibitory concentrations (MIC) of the crude extract on *Vibrio* spp.

The MIC of the extract was determined using method of Akinpelu and Kolawole (2004). Two-fold dilutions of the plant extract was prepared and 2 ml of different concentration of the solution was added to 18 ml of pre-sterilized molten nutrient agar at temperature of 40°C to give final concentrations between 0.157 and 10.0 mg/ml. The medium was then poured into sterile Petri dishes and allowed to set. The surface of the medium was allowed to dry before streaking with 18 h old isolates. The plates were later incubated in an incubator at 37°C for up to 72 h after which they were examined for the presence or absence of growth. The MIC was taken as the lowest concentration that will prevent the *Vibrio* growth.

Minimum bactericidal concentrations (MBC) of the crude extract on *Vibrio* spp.

The MBC of the extract was determined using Olorundare et al. (1992) method with little modifications. Samples were taken from plates with no visible growth in the MIC assay and subcultured on to freshly prepared nutrient agar medium and later incubated at 37°C for 48 h. The MBC was taken as the lowest concentration of the extract that did not allow any bacterial growth on the surface of the agar plates.

Statistical analysis

Data were expressed as mean ± SD (standard deviation) of six replicates and were statistically analyzed using one way analysis of variance (ANOVA). Means were separated by the Duncan multiple test using SAS (SAS, 2002). Values were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

This study investigated the antimicrobial and phytochemical properties of *D. guineense*. The crude extract of the plant was found to possess bioactivity against fourteen out of eighteen environmental strains of *Vibrio* species tested at a final concentration of 20 mg/ml. On the other hand, the standard antibiotics used – ampicillin inhibited the growth of fifteen out of the eighteen tested strains of the *Vibrio* spp. while streptomycin inhibited the growth of all the tested bacterial isolates (Table 1). The zones of inhibitions exhibited by the extract against the tested bacterial isolates ranged between 12 and 20 mm. The zones of inhibition exhibited by ampicillin against the tested isolates ranged between 7 and 40 mm while streptomycin exhibited between 12 and 32 mm zones of inhibition. *Vibrio* spp. are known to be deadly and can cause gastrointestinal diseases along with other ailments that can lead to death (Pelczar et al*.,* 2006). The growths of *Vibrio* spp. were successfully inhibited by the extract from *D. guineense*. Traditionally, different parts (leaves, roots, stems and barks) of *D. guineense* are used among many tribes in Africa to treat gastrointestinal diseases as well as cholera infections among other diseases caused by bacteria. Thus, the results obtained from this study support the use of *D. guineense* as folklore remedies to treat bacterial infections among many tribes in Africa. The antimicrobial activity of *D. guineense* stem bark extract (though still in crude form) compared favourably with those of the standard antibiotics - ampicillin and streptomycin used in this study. The minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of the extract were also determined. The MIC of the extract against the *Vibrio* isolates ranged between 0.313 and 5.0 mg/ml while the MBC ranged between 0.625 and 10 mg/ml (Table 2).

According to Suffredini et al. (2006), the antibacterial activity of plant extracts is considered significant if the

Table 1. Sensitivity patterns of zones of inhibition exhibited by *Dialium guineense* leaf extract and standard antibiotics on the environmental strains of *Vibrio* species.

Organisms	Zone of inhibition (mm)*		
	***D. guineense* extract (20 mg/ml)**	**Ampicillin (25 µg)**	**Streptomycin (10 µg)**
Vibrio fluvialis (EIS)	19	15	17
Vibrio fluvialis (EIS)	18	17	22
Vibrio parahaemolyticus (EIS)	0	35	37
Vibrio metschnikovii (EIS)	18	15	17
Vibrio fluvialis (EIS)	0	0	12
Vibrio fluvialis (EIS)	20	15	15
Vibrio fluvialis (EIS)	19	14	14
Vibrio Parahaemolyticus (EIS)	12	35	16
Vibrio vulnificus (EIS)	20	15	22
Vibrio vulnificus (EIS)	0	07	10
Vibrio sp. (EIS)	18	17	15
Vibrio parahaemolyticus (EIS)	0	12	15
Vibrio vulnificus (EIS)	18	17	20
Vibrio vulnificus (EIS)	18	0	18
Vibrio sp. (EIS)	16	0	17
Vibrio vulnificus (EIS)	15	18	20
Vibrio fluvialis (EIS)	18	16	24
Vibrio fluvialis (EIS)	20	17	23

Key: mm* = mean diameter of six replicates EIS = Environmental isolated strain.

Table 2. The minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of the stem bark extract of *D. guineense* against the environmental strains of *Vibrio* species.

Organisms	***D. guineense* stem bark extract**	
	MIC (mg/ml)	**MBC (mg/ml)**
Vibrio fluvialis (EIS)	0.625	2.5
Vibrio fluvialis (EIS)	0.625	2.5
Vibrio parahaemolyticus (EIS)	ND	ND
Vibrio metschnikovii (EIS)	0.625	1.25
Vibrio fluvialis (EIS)	ND	ND
Vibrio fluvialis (EIS)	0.313	0.625
Vibrio fluvialis (EIS)	0.313	0.625
Vibrio parahaemolyticus (EIS)	5.0	10
Vibrio vulnificus (EIS)	0.313	1.25
Vibrio vulnificus (EIS)	ND	ND
Vibrio sp. (EIS)	0.625	2.5
Vibrio parahaemolyticus (EIS)	ND	ND
Vibrio vulnificus (EIS)	0.625	1.25
Vibrio vulnificus (EIS)	0.313	0.625
Vibrio fluvialis (EIS)	1.25	2.5
Vibrio vulnificus (EIS)	1.25	2.5
Vibrio fluvialis (EIS)	0.625	1.25
Vibrio fluvialis (EIS)	0.313	0.625

Table 3. The phytochemical compounds present in the leaf extract of *D. guineense*.

Phytochemical compound	Results
Tannins	Positive
Alkaloids	Positive
Flavonoids	Positive
Saponins	Positive
Steroids	Positive
Cardiac glycosides	Positive
Reducing sugars	Negative

MIC of the extract is less than or equal to 200 mg/ml. This is an indication that leaf extract of *D. guineense* exhibited significant antimicrobial activity against the tested *Vibrio* spp. and will also be active against many other pathogens if subjected to the activity of this plant extract. Phytochemical analysis of the plant extract revealed some phenolic compounds. Phenolic compounds from medicinal herbs and dietary plants play important roles in health in addition to enhancing antimicrobial activities in these plants. The medicinal value of medicinal plants therefore lies in these chemical substances that produce a definite physiological action in human body. These phenolic compounds include phenolic acids, flavonoids, tannins, saponins and cardiac glycosides among others. Various bioactivities of phenolic compounds are responsible for their chemopreventive properties which include antioxidants, anticarcinogenic and anti-inflammatory effects. Phytochemical compounds identified in *D. guineense* stem bark extract are tannins, alkaloids, saponins, flavonoids, steroids and cardiac glycosides (Table 3). These phytochemical compounds are known to be biologically active and hence enhanced the antimicrobial activities of this extract. In addition to their antimicrobial properties, tannins are used for the treatment of diarrhoea and dysentery, haemorrhoids, inflamed or ulcerated tissues (Dharmanda, 2003).

Vibrios are known to cause gastroenteritis and these organisms were found to be susceptible to the activity of the extract from *D. guineense* which tested positive for tannins. This is an indication of the usefulness of this plant in folklore remedies for the treatment of this disease and thus supports our findings. *D. guineense* is used traditionally for the treatment of heart disease which might be attributed to the presence of tannins in this plant. Lawrence et al. (1997) reported that tannins possess excellent cardio-protective qualities in addition to its antioxidant action. It precipitates lipoprotein which carries cholesterol and thus reduces the level of in-take cholesterol. Mensah et al. (2009) also reported the usefulness of tannins for the management of hypertension among Esan people of Edo State. These facts support the uses of *D. guineense* that contains tannins for the treatment of heart disease in addition to its antimicrobial activities. The results obtained by James and Friday (2010) from their studies on wound healing effects of tannins supported the use of *D. guineense* as folklore remedies for the treatment of wound infections. Astringent properties of tannins served as a powerful medicine and this support the usefulness of herbs containing tannins in folklore remedies of various diseases caused by pathogens as revealed from results obtained from our findings. Clinton (2009) investigated the use of plant tannins to treat ulcerative colitis caused by food allergens and pathogenic microflora. She observed that a diet rich in tannins might aid the prevention of ulcerative colitis. This report serves as a pointer to the fact that a drug for the treatment of ulcerative colitis can also be developed from *D. guineense*.

The leaf extract of *D. guineense* also revealed the presence of alkaloid which is known to possess pharmacological activities which include antihypertensive effects, antiarrhythmic effects and anticancer actions. A number of alkaloids are used as drugs and the best known is quinine used as antimalaria drug (Cordell, 1983). *D. guineense* is used as folklore remedy for the treatment of malaria and this might be due to the presence of alkaloids in this plant. Hermans et al. (2010) revealed from their studies that *D. guineense* inhibited the growth of *Plasmodium falciparum* the causative agent of malaria; alkaloids might have contributed to this effect on the parasite. Malaria parasites are now fast developing resistance against the available antimalaria drugs, potent drugs for the treatment of malaria may be developed from *D. guineense*. Alkaloids possess anti-inflammatory and anti-asthmatic properties and it is one of the largest groups of phytochemicals in plant that has amazing effect on humans which led to the development of powerful pain killer medications (Staerk et al., 2002). These facts can therefore serve as a pointer towards the development of drug of future from *D. guineense* for the treatment of these ailments. Flavonoids are natural products of high pharmacological potency which are widely distributed in plants. They are one of the phytochemical compounds identified in *D. guineense* leaf extract used in this work. They possess antiallergic, anti-inflammatory, antiviral and antioxidant activities in

addition to playing a good role in cardioprotective (Nijveldt et al., 2001).

Schramm and German (1998) suggested in their review article that if the total plasma flavonoid load exceeds a few micromoles per litre *in vivo*, flavonoid will protect humans against vascular disease. Flavonoids in human diet may reduce the risk of various cancers, as well as preventing menopausal symptoms (Hodek et al., 2002). These properties attributed to flavonoids support the uses of *D. guineense* in folk medicine for the treatment of various ailments. *D. guineense* is used as chewing stick and aid the cleaning effect of the teeth as well as preventing caries and plaques of the teeth of the users. Ammar et al. (1990) revealed in their study that flavonoids can be used to inhibit dental plaque formation. This findings support the traditional usage of *D. guineense* in preventing dental plaque due to the presence of flavonoids in this plant. Flavonoids are known to inhibit important viral enzymes such as reverse transcriptase and protease, and destroy some pathogenic protozoans (Havsteen, 2002). This information is an indication that antiviral drug can also be produced from *D. guineense* because of the presence of large amount of flavonoids in this plant. Furthermore, in their review article Carlo et al. (1999) discussed some beneficial effects of flavonoids and these are antihepatotoxic, antiallergic, anti-inflammatory, antiosteoporotic and antitumor. If great attention is paid on *D. guineense* which we believe possess many pharmacological properties, a potent drug of future for the treatment of bacterial and non-bacterial infections can be developed from this plant. Saponins which are responsible for numerous pharmacological properties (Estrada et al., 2000) also tested positive in *D. guineense* leaf extract. Saponins possess biological activities and are used as folk medicines as well as intensively used in food, veterinary and pharmaceutical industries.

Cardiac glycosides one of the phytochemical compounds detected in *D. guineense* leaf extract are known to increase the force of contraction of the heart for most heart failure patients and thus found to be effective in treating congestive heart failure (Shi et al*.,* 2010). Cardiac glycosides have also been found useful in the treatment of cancer (Katarzyna et al., 2006). Steroid, one of the phytochemical compounds in *D. guineense* leaf extract have been found to possess numerous and diversified physiological functions and pharmacological effects on the functional capacities of the cardiovascular system (Singh and Kaushal, 2007). The presence of these phytochemical compounds in this plant further confirmed its medicinal uses in folklore remedies for the treatment of various infections. Further investigations on this plant might lead to the development of antimicrobial drugs of natural origin that may combat the rapid development of multiple resistant to the available antibiotics by pathogens. In conclusion, *D. guineense* leaf extract exhibited significant antimicrobial properties on the environmental strains of *Vibrio* spp. used in this study and it compared favourably with the two standard antibiotics – ampicillin and streptomycin used as positive controls. *D. guineense* forms part of the ingredients used in preparing decoction for the treatment of some ailments and thus it is suppose to be safe in consumption and drugs formulated from this plant may pose no danger to the users. Antimicrobial drugs of natural origin developed from this plant may go a long way in preventing the establishment of an infection caused by vibrios and other pathogens that are now developing resistance to the existing antimicrobial drugs.

Efforts are going on in our laboratory to isolate pure compounds of pharmacological importance from the plant crude extract.

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