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SUPERSIZING THE INHIBITORY ACTIVITY OF *XYLOPIA AETHIOPICA* EXTRACT AGAINST VIBRIO CHOLERAE USING DOXYCYCLINE

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Abstract

Studies have shown that multi-dung resistant *Vibrio* species is a well-recognized and documented problem worldwide especially in the developing countries, and the gene associated with this resistance are encoded in the plasmid. This study was carried out to evaluate the effect of *Xylopia aethiopica* extract on different strains of *Vibrio* species. Samples were drawn from streams and screened for the presence of test isolates using standard microbiological techniques. The phytochemical constituents of *Xylopia aethiopica* (XA) was quantitatively determined using gravimetric and spectrophotometric methods. The antibacterial activity was carried out using agar-welled diffusion method. *Vibrio cholerae* 01 bio var EI Tor strain C6709 (VCC6), *Vibrio cholerae* 01 bio var EI Tor strain P27459 (VCP2) and *Vibrio cholerae* 01 bio var EI Tor E7946(VCE7) were detected in the studied sample of which VCC6 was significantly ($p < 0.05$) most predominant in the stream samples. The study also showed that XA contain alkaloids, steroids, cardiac glycosides, phenolics, flavonoids, tannins, saponins and was able to significantly ($p < 0.05$) inhibit the strains of *Vibrio* species, of which the inhibition was significantly ($p < 0.05$) increased when combined with Doxycycline (DX), and inhibited VCE7 most. From the above study, VCC6, VCE7 and VCP2 were seen in the studied samples, and the extracts showed pronounced activity the isolates, of which the activity was boosted when combined with DX.

Keywords

Keywords: *Vibrio cholerae, Xylopia aethiopica,* phytochemical, Doxycycline

INTRODUCTION

Vibrio cholerae is the bacterium responsible for the epidemic diarrheal disease cholera, but it is also a natural inhabitant of aquatic environments in many regions of the world (Judy Daboul *et al*., 2021). Due to their widespread existence in the environment, Vibrio species encounter a wide range of unfavorable conditions to which they have to sense and respond appropriately, by up regulating or down regulating specific gene expression (Lutz C *et al.*, 2013). These conditions are also constantly changing due to natural and manmade events. Despite these challenges, due to its unique ability to adapt to and thrive in diverse and changing environments, V. cholerae has been a very successful marine organism and human pathogen. In turn, V. cholerae causes outbreaks of disease that is not always linked to predictable water sources. Therefore, it is critical that we better understand the varied locations where V. cholerae can survive worldwide in order to develop measures to limit human exposure to these ubiquitous aquatic bacteria.

V. cholerae strains are divided into more than 200 different serogroups based on O antigen structure. Historically, the O1 serogroup has been primarily responsible for causing epidemic cholera, and in 1992 another epidemic serogroup, O139, emerged. Pathogenic (toxigenic) V. cholerae strains encode two major virulence factors: cholera toxin (CT) and the toxin-coregulated pilus (TCP), the expression of which leads to the severe diarrhea associated with cholera disease. CT and TCP are encoded by a lysogenic bacteriophage (CTXφ) and a pathogenicity island, respectively. The remaining V. cholerae strains are generally referred to as "non-O1, non-O139" strains and do not cause cholera epidemics. These strains are also often referred to as "non-toxigenic" as they rarely carry the genes for CT and TCP. However, many of these non-O1/non-O139 strains encode other virulence genes, and have caused numerous diseases ranging from gastroenteritis to extraintestinal infections in humans (Trubiano JA, et al.,2014). These accessory virulence factors include the RTX toxin, outer membrane proteins (including OmpU) and hemolysins . In V. cholerae, the rtx gene cluster encodes a multifunctional-autoprocessing RTX (repeats in toxin) toxin that is involved in evasion of the host immune response. OmpU is a porin that is required for bacterial resistance to antimicrobial peptides, among other roles. Hemolysins, including HlyA, are pore-forming toxins that lyse eukaryotic cells . The presence of these virulence factors in non-O1/non-O139 strains are assumed to play some role in their survival in the natural environment (Sakib SN et al 2018). However, due to their likely role in the pathogenesis of non-O1/non-O139 strains, the presence of these accessory virulence factors is often monitored when characterizing V. cholerae strains isolated from environmental and food sources (Ceccarelli D *et al*., 2015)

V. cholerae is typically found in estuarine or coastal environments, with most toxigenic strains residing in subtropical climates such as Southeast Asia. V. cholerae is one of the only Vibrio species that can tolerate the absence of salt, and transmission of cholera from freshwater sources occurs, but typically in regions of the world where pathogenic strains are endemic. In the United States, V. cholera can be isolated from coastal environments, such as the Chesapeake Bay. Typically, these North American strains are non-toxigenic, but studies have shown that there is a high degree of genetic diversity in the isolates and they contain a random assortment of virulence genes that may warrant increased surveillance.

Stream water is a body of water with a current confined within a bed and stream banks. Streams are important as, conduits in the water cycle and corridors for aquatic life, microorganisms and wildlife migration (Ethell *et al*., 2013). The biological habitat in the immediate vicinity of a stream is called a riparian zone. Considering the high level of extinction, streams play a vital role in connecting fragmented habitats, and thus in preserving biodiversity. Stream typically derived most of their water from precipitation in the form of rain and snow. Most of this water re-enters the atmosphere by evaporation from soil and water bodies, or by infiltration and ground water.

Sourcing for potable water had been a major challenge to man for decades. This occurs due to contamination of several water bodies as a result human activity such as indiscriminate defecation, indiscriminate disposal of industrial effluents, dumping of refuse and sewage in water bodies and so on. This had led to several cases of water borne diseases majorly cholera, that can kill thousands of people within few days. Adequate examination of water is paramount especially when it is meant for drinking and other domestic purposes. This would enable indicator organisms to be detected and their presence provides vital information on microbial quality of water (Eze *et al.*, 2013).

Water sources that may be declared suitable for consumption are frequently vulnerable to microbial contamination, which could be the source of threat to consumers (Ethell *et al*., 2013). It is worthy to note that using contaminated water for irrigational purposes is detrimental to human, because, the plants absorb the contaminants which are transferred to humans when such plants are consumed. Therefore, water analysis remains the best measure that can be taken in order to secure the health of the public. Detecting indicator organisms in water provides vital information on the source of the contamination, which invariably facilitates in controlling the menace (Eze *et al.*, 2013).

The development of antibiotic resistant bacteria stems from a number of factors, including inappropriate use of antibiotics in human has lead to the use of plant extracts which have antimicrobial agents in conjunction with antibiotics to treat most of the antibiotic resistant strains of bacteria.

MATERIALS AND METHODS

Isolation and Characterization of Test Isolates

Sample Collection, Handling and Transportation: The samples used for this study were drawn from the rivers. A total of 100 freshwater samples were collected from five different streams used in Uli community. Samples were taken from twenty different sites, each site in triplicates. The stream samples were collected with sterile containers. The containers were thoroughly washed with detergent, rinsed with water, and then rinsed with 70% ethanol and final rinsed three times with distilled water. The containers were placed inverted in order to drain the water inside them. The container was inverted and lowered 5 cm below the river water sample, then placed vertically for the water sample to refill the sample container. This sample was covered immediately and kept in a cooler containing ice block, and this transported to the laboratory for immediate analysis.

Isolation of Organisms: One milliliter (1.0 ml) water sample was aseptically transferred into a sterile test tube (Pyrex) containing 9.0 ml of the diluent (sterile

normal saline) and from this; ten-fold serial dilutions were made up to 10^{-3}. One milliliter of the diluted sample (10^{-3}) was plated on Petri dishes (60 mm OD × 55 mm ID × 13mm high) containing Deoxycholate agar medium (DCA/Biotech) using pour plate method. All the plates in triplicates were incubated inverted at 37±2˚C for 24-48 h. as described in the study published by Iheukwumere and Ejike (2016); Iheukwumere *et al* .(2017) and Iheukwumere *et al.* (2020)

Characterization and Identification of the Isolates: The isolates were sub cultured on nutrient agar (Biotech), incubated in inverted position at 37±2˚C for 24 h. The isolates were characterized and identified using their colonial and morphological descriptions as described in the study published by Iheukwumere *et al*. (2018), biochemical reactions as described in the study published by Iheukwumere *et al.* (2020) and molecular characterization as described in the study published by Gabriela *et al.* (2014). The colonial description was carried out to determine the colours of the isolates on agar media plates, their sizes, edges, consistencies and optical properties of the isolates.

Molecular characterization of the isolates

Extraction and purification of DNA: All strains were plated on Nutrient Agar (Biotech) and incubated at 37°C for 24 h. Bacterial genomic DNA was then extracted and purified using Zymo Research (ZR) DNA miniprepTM kit (Categor y No. D6005; Irvine, California, USA) as described in the study published by Iheukwumere *et al.* (2018)

Determination of the quality of extracted DNA: This was carried out using mass spectrophotometer (Nanodrop). One micro litre (1µL) was aseptically dropped into a clean aperture in the chamber and the chamber was gently closed. The system was then connected to a computer system which displaced the window that revealed the quality of the sample at 260/280 nm as described in the study published by Iheukwumere *et al* (2018a); Iheukwumere *et al* .(2018b); Iheukwumere *et al.* (2018c)

Amplification of DNA and gel electrophoresis of PCR product: This was carried out using Master cycler Nexus Gradient (Eppendorf). A mixture of primer (20 µl), template DNA (20µl), water (72 µl) and master mix (108 µl), which comprises taq polymerase, DimethylSulfoxide (DMSO), magnesium chloride ($MgCl_2$) and nucleotides triphosphates (NdTPs), was prepared in 1.5 ml tube and homogenized using vortex mixer (Eppendorf). This was then placed in the block chamber of the master cycler and then programmed.The PCR program conditions for amplification the DNA were as follows: initial incubation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 15 sec, annealing at 55°C for 15 sec, elongation at 72°C for 21 sec and final extension period for 10 min at 72°C as described in the study published by Iheukwumere *et al.* (2018a); Iheukwumere *et al*. (2018b); Iheukwumere *et al.* (2018c)

DNA sequencing of 16s rRNA fragment: The amplified PCR products generated from universal primer (16s), was used for the sequencing using ABI DNA sequencer (Applied BiosystemInc) at International Institute of Tropical Agriculture (IITA), Ibadan, and this was further analyzed using Basic Local Alignment Search Tool (BLAST) at National Center for Biotechnology Information (NCBI). as described in the study published by Iheukwumere *et al.* (2018a); Iheukwumere *et al.* (2018b); Iheukwumere *et al*.(2018c)

Preparation, Extraction and Phytochemical Analysis of the Plant Material

Preparations of plant materials: The fresh leaves of *Ocimum gratissimum* was collected from cultivated land at Uli in Ihiala L.G.A of Anambra State, Nigeria. The leaves samples were authenticated appropriately. The fresh leaves were plucked, washed and dried under shade at room temperature for 14 days. The dried leaves were ground to powder form using sterile electric grinder. Twenty grams of the ground leaves each was macerated in two hundred milliliters of distilled water and ethanol respectively for 72 h. The mixture was filtered using what man No.1 filter paper. The extracts were concentrated by evaporation at room temperature in a steady air current as described in the study published by Iheukwumere and Ejike (2016); Iheukwumere *et al*. (2017) and Iheukwumere *et al.* (2020).

Phytochemical analysis of the plant extracts: The phytochemical components (alkaloids, glycosides, flavonoids, phenolics, tannins, steroids and saponins) of the plant extracts were determined quantitatively using the methods described by Iheukwumere and Umedum (2013).

***In Vitro* Antibacterial Activity**

Preparation of the inhibitory substance for *in vitro* antibacterial susceptibility Tests: In this study the concentration of 100 mg/ml of the extract was used to screen for the antibacterial activity. This was carried out using the modified method described in the study published by Iheukwumere *et al*. (2018). Here, 2.5 g of the extract was dissolved in 25.0 ml of peptone water. Similarly, equal concentration of the antibiotic was prepared, and then equal volume of the extract and antibiotic were mixed, and this was used for the study as described in the study published by Iheukwumere and Ejike (2016); Iheukwumere *et al*. (2017) and Iheukwumere *et al.* (2020).

In vitro antibacterial susceptibility test: This was carried out using the method described in the study published by as described in the study published by Iheukwumere and Ejike (2016); Iheukwumere *et al.* (2017) and Iheukwumere *et al.* (2020). Each labeled plate was uniformly inoculated with the test organism using pour plate method. A sterile cork borer of 5 mm diameter was used to make the wells on the medium. One tenth millilitre of the inhibitory substance was dropped into each labeled wells and then incubated at 37±2ºC for 24 h. Antibacterial activity was determined by measuring the diameter of the zones of inhibition (mm) produced after incubation

Statistical Analysis: The results of the data generated were expressed as mean, percentage and Table; Data were analyzed by two-way Analysis of Variance (ANOVA) to determine the significance of the main effects and interactions at 95 % confidence level. Pair wise comparison of mean was done by Student "t" test as described in the study published by Iheukwumere *et al* .(2018)

RESULTS

The cultural and morphological characteristics of the isolates is shown in Table 1. The study revealed that the isolates had similar appearances on thiosulphate- citrate-bile salts- sucrose agar, similar elevation, edge and surface and also similar morphological characterization on string test, gram reaction, endospore, capsule and motile nature.

The biochemical characteristics of the isolate revealed that the isolates were hydrogen sulphide production, methyl red, urease, arabinose, dulcitol, negative as shown in Table

2. The isolates differ in their variation in utilization of sugars. They were all catalase, citrate, gelatin, oxidase, glucose, and galactose positive but differ in their abilities to utilize inositol, xylose, surbitol and lactose.

The nucleic acid extracted from the isolates showed the ratio of their absorbances at wavelength of 260 nm and 280 nm using Nanodrop was at the range of 1.80-1.90 and this confirmed that the nucleic acids were DNA as shown in Table 3. The molecular identities of the isolates revealed that isolate L,M,and N were Vibrio cholerae 01 bio var EI Tor strain C6709 (VCC6), Vibrio cholerae 01 bio var EI Tor strain P27459 (VCP2) and Vibrio cholerae 01 bio var EI Tor E7946(VCE7) as shown in Table 4.

The quantitative phytochemical constituents of the plant extract revealed the presence of alkaloids, phenolics, flavonoids, saponins, tannins, glycosides and steroids as shown in Table 5. It was also observed that alkaloids were mostly detected whereas steroids were detected least in the study.

The study revealed that the plant extract showed significant activity against the vibrio species and the activity was most against VCP2 as shown in Table 6. It was also observed that the activity of the plant extract was significant ($P<0.05$) boosted with an antibiotic (azithromycin). The study also showed that there was synergistic activity between the extract and the antibiotic as the activity of the combination increased more than the activity of either of the inhibitory substances.

Table 1: Cultural and morphological characteristics of the isolates

Parameter	**L**	**M**	**N**
Appearance on TCBS	Yellow	Yellow	Yellow
Edge	Smooth	Smooth	Smooth
Elevation	Raised	Raised	Raised
Surface	Smooth	Smooth	Smooth
String test	+	+	+
Gram reaction	-	-	-
shape	Rods/comma	Rods/comma	Rods/comma
Endospore	-	-	-
Capsule	-	-	-
Motility	+	+	+

Table 2: Biochemical characteristics of the isolates

Parameter	**L**	**M**	**N**
Catalase	+	+	+
Citrate	+	+	+

Gelatin	+	+	+
H_2S	-	-	-
Methyl red	-	-	-
Oxidase	+	+	+
Urease	-	-	-
Arabinose	-	-	-
Glucose	+	+	+
Galactose	+	+	+
Inositol	-	+/-	-
Dulcitol	-	-	-
Xylose	+/-	-	+/-
Subitol	-	+/-	-
Lactose	+/-	-	+/-

Table 3: Verification of the extracted nucleic acids

Sample ID	**Conc(ug/ml)**	**260 nm**	**289 nm**	**260/280**
L	121.20	3.0120	1.6194	1.86
M	125.70	3.1082	1.6801	1.85
N	132.80	3.2110	1.7643	1.82

Table 4: molecular identities of the isolates

Parameter	**L**	**M**	**N**
Max score	5686	5686	5686
Total score	7295	7295	7295
Query cover(%)	100	100	100
E-value	0.0	0.0	0.00
Identity(%)	100	100	100
Accession length	1070357	10703537	1071008

Accession number	CP047298	CP047300	CP047304
Description	Vibrio cholerae 01 bio var EI Tor strain C6709(VCC6)	Vibrio cholerae 01 bio var EI Tor strain P27459(VCP2)	Vibrio cholerae 01 bio var EI Tor strain E7946(VCE7)

Table 5: phytochemical constituents of Xylopia aethiopica extract

Parameter	Value(g/100g)
Alkaloids	2.14±0.02
Phenolics	1.56±0.01
Flavonoids	0.60±0.01
Tannins	0.88±0.01
Saponins	0.30±0.00
Glycosides	0.28±0.01
Steroids	0.12±0.00

Table 6: Antimicrobial activity

Diameter zone of inhibition [X±SD] mm

Inhibitory substance	VCC6	VCP2	VCE7
EEX	7.20±0.14	8.67±0.33	7.80±0.14
AEX	0.00±0.00	6.00±0.00	0.00±0.00
CPX	14.00±0.17	17.30±0.11	14.50±0.07
DX	19.80±0.14	20.50±0.19	20.10±0.17
EEX+DX	22.00±0.21	24.10±0.41	22.70±0.31
AEX+DX	20.60±0.17	20.80±0.51	20.10±0.17

EEX- Ethanoic Extract of Xylopia aethiopica

AEX- Aqueous Extract of Xylopia aethiopica

CPX- Ciprofloxacin

DX- Doxycycline

DISCUSSION

The occurrences of the studied isolates in the stream water samples supported the findings of many researchers (Tracogna *et al.,* 2013; Li *et al.,* 2014; Bhetwal *et al.,* 2017; Phoon *et al.,* 2015; Ekelozie *et al.,* 2018; Kumar *et al.,* 2018; Nyandjou *et al.,* 2018) but disagrees with the finding of Prakasam *et al.,* (2017) who isolated antibiotics resistant bacteria from hospital dumping sites. The occurrences ofVibrio cholerae 01 bio var EI Tor strain C6709 (VCC6), Vibrio cholerae 01 bio var EI Tor strain P27459 (VCP2) and Vibrio cholerae 01 bio var EI Tor E7946(VCE7) stated that the presence of *Vibrio* species in these samples reaffirms the need for monitoring in order to minimize the risks of infection to exposed persons.

The highest occurrences of VCC6 in the sream water samples observed in the present study could be attributed to the diversity of this strain within the study area. Studies have shown that genetic variation; genomic heterogeneity, antigenic variation, horizontal gene transfer and presence of dispensable (strain-specific) genes which confer fitness advantages to a particular strain, are associated with diverse epidemiological settings among different strains of microorganisms (Yap *et al.,* 2014). Also the occurrence of VCC6 mostly in the river samples could be attributed to poor sanitation, poor handling of fruits within the study area.

The study further revealed the presence of cardiac glycosides, steroids, alkaloids, tannins, flavonoids, phenolics and saponins from the leaves extracts of Xylopia aethiopica and these agree with the findings of many researchers (Osabar *et al.,* 2015; Alexander *et al*., 2016; Ebana *et al.,* 2016; Ajayi *et al*., 2017; Arawande *et al.,* 2018; lawal *et al*., 2018; Shama and Patel, 2018; Jumae, 2019; Owoyale *et al*., 2019; Virshette *et al*., 2019; Iheukwumere *et al.,* 2020). The researchers also pointed that these phytochemical constituents could be responsible for the activities and other ethno medical potentials attributed to the above studied extracts. Also the variations in the amount of the phytochemical constituents associated with the extract could be attributed to source and species of the plant involved. The pronounced activities of X against the resistant strains of VCC6, VCP2 and VCE7 could be attributed to the activities of the phytochemical constituents present in the extracts. The significant reduction in the number of resistant strains observed in this study corroborated with the findings of many researchers (Kumar *et al*., 2013; Shiram *et al*., 2013; Srivastara *et al*., 2014; Mohite *et al.,* 2015; Abduirahman *et al*., 2016; Akinyemi *et al.,* 2017; Orhue *et al.,* 2017; Iheukwumere *et al*., 2020). Several studies have reported the horizontal spread of antibiotic resistant genes among bacteria is driven by bacterial plasmid, which promotes evolution of resistance.

CONCLUSION

The study has revealed the presence of Vibrio cholerae 01 bio var EI Tor strain C6709 (VCC6), Vibrio cholerae 01 bio var EI Tor strain P27459 (VCP2) and Vibrio cholerae 01 bio var EI Tor E7946(VCE7) of which VCC6 was mostly encountered in the stream samples. The Xylopia aethiopica (X) extract showed pronounced activity against the test isolates, of which the activity was boosted when combined with Azithromycin (AZR.)

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