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A Trial to Prevent Vibrio Cholerae Infections in Mice using Napoleona Imperialis Leaves Extracts and Tetracycline

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Abstract
This study was carried out to evaluate the protective effects of *Napoleona imperialis* leaves extracts and tetracycline against *Vibrio cholerae* ETOR type isolated from Ubahudara and Aruolah streams in Uli community, Ihiala L.G.A, Anambra State. A total of sixteen (16) water samples were drawn from each stream and screened for the presence of *Vibrio cholerae* using pour plate method. The colonies generated from the primary isolation was sub-cultured, characterized and identified using their colony description, morphological and biochemical characteristics. The pathogenic potential of the organism on mice was investigated by challenging the mice orally using 0.5 ml of the inoculum (10^8cells/ml). The infected mice were kept under observation for 4 weeks for clinical signs, mortalities, gross lesions, histopathological changes and re-isolation from the internal organs after sacrificing the mice. The protective effects of *Napoleona imperialis* leaves extracts and tetracycline were investigated using *in vivo* method. The result revealed that *Vibrio cholerae* ETOR type was significantly ($P< 0.05$) seen more in Ubahudara stream (67.44%) than Aruolah stream (32.56%). There were gross clinical abnormalities: kidney and liver congestion, perihepatits and fluid accumulation in the intestines. The histopathological examination revealed marked mononuclear cell infiltration, disintegration of cartilage surrounding the bronchiole, necrosis of the tubules of kidney and red pulp of the spleen. The mean counts of the organism were significantly ($P< 0.05$) highest in the lungs, followed by the spleen, kidney while the liver was the least. The results of *in vivo* activity showed that *Napoleona imperialis* leaves extracts and tetracycline was effective in reducing pathological changes. Their effects were significant ($P< 0.05$) when compared with the infected non-treated mice. Thus, this study has shown that tetracycline is more effective in preventing the infections caused by *Vibrio cholerae* ETOR type when compared to *Napoleona imperialis* leaves extract.
Keywords: Napoleona imperialis, tetracycline, Vibrio cholerae, histopathological

INTRODUCTION

Vibrio is a genus of Gram-negative bacteria possessing a curved-rod shape (Thompson *et al*. 2005). In 1854, an Italian scientist, Filippopacini first discovered *Vibrio* in Florence, Italy from cholera patients because of their motility (Michael, 2010). According to Center for Disease Control (CDC), it was estimated that 8,028 vibrio infections and 60 deaths occur annually in the United States. Vibrios are natural inhabitants of the marine, fresh water and estuarine environments (McLaughlin *et al*. 1995). They are highly halophilic, which means that they need salt-rich environment in order to thrive. Many *Vibrio* species are zoonotic and they cause disease in fish and shellfish. They are common causes of mortality among domestic and marine life. The common species of Vibrio includes *Vibrio cholerae, Vibrio mimicus, Vibrio parahaemolyticus, Vibrio vulnificus, Vibrio alginolyticus.* Pathogenic Vibrio species can cause foodborne illness (infection), usually associated with eating undercooked seafood. (Joseph *et al*. 2002).The primary environment variables influencing this occurrence of pathogenic Vibrios in streams are temperature and salinity (Lipp *et al*. 2002). The optimum temperature for growth of this organism is 37^{o}C, with possibilities for growth ranging from 16 to 42^{o}C (Borroto *et al*. 2000). Floods and droughts may affect not only the concentration of the bacterium in this environment, but also its survival, through the effect exerted by these environmental changes on salinity, sunlight, pH, and nutrient concentrations (Bouma and Pascual, 2001).

Vibrio cholerae has optimum pH of 8.5, salinity of 15%, inhibited at temperature below 15^{o}C and killed at temperature of 65^{o}C and above (Borroto *et al*. 2000). The mainstay of the case management of cholera is treatment of dehydration using Oral Rehydration Therapy (ORS) or Intravenous fluids (Ringer lactate) and electrolytes (Sack *et al*. 2006). Medicinal plants continue to play central roles in the healthcare of large proportion of the world's population. This is particularly true in the developing countries, where herbal medicine has a long and uninterrupted history of use. Recognition and development of medicinal and economic benefits of these plants are on the increase in both developing and industrialized nations (Srinivas *et al*. 2007). Among the use of herbal therapy is in the treatment of infective diseases affecting man all over the world today. The results presently arising from the use of available chemotherapeutic agents are even encouraging factors to use of herbs. Antibacterial resistance among bacterial pathogens in recent time is a critical area of public health concern (Fagbohun *et al*. 2010). There is need for the development of new antibiotics due to acquired resistance more

importantly from natural sources as this delays resistance (Ajaiyeoba, 2000).Measures have been taken to address the root problems of poor sanitation and unsafe water supplies in order to prevent future cholera epidemics. In this regards, perhaps, prevention of the disease is the best way to counter subsequent outbreaks. Necessary measures as boiling the water for drinking, washing and cooking purposes, treatment of infected facilities, sewages and drainage systems, proper disposal of infected materials such as waste products, clothing, and beddings, treatment of infected faecal waste water produced by cholera victims and sterilisation of utensils either by boiling or by using chlorine bleach. Recent studies have also indicated that use of soap and hand washing promotion can achieve a 26 to 62% decrease in the incidence of diarrhoea in developing countries (Bouma and Pascual, 2001).

Many researchers have studied different ways of controlling *Vibrio cholerae* infections using leaves extracts. Onyegbule *et al.* (2011), reported the antimicrobial activity of *Napoleona imperialis* extract against some microorganism. He demonstrated the antibacterial and wound healing properties of this plant extract on albino rats. According to Ayodele (2005), an infusion of the leaves of *Napoleona imperialis* is used to dissolve clotted blood in freshly delivered woman but used as vermifuge for children. He equally stated that the stem is used to cure gonorrhoea while the root is used to cure fever, but not work has been published on the use of *Napoleona imperialis* extract in controlling *Vibrio cholerae.* Therefore this work has been designed to try other measures of controlling *Vibrio cholerae*

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MATERIALS AND METHODS

Study Area: The study was conducted in Aruolah and Ubahudara streams at Uli, Ihiala L.G.A., Anambra State which is located at Latitude 5.78°N and Longitude 6.87°E in Southeast geopolitical zone of Nigeria. Uli Community shares common borders with Ihiala town, Egbu at Imo State, Amorka in Anambra State and Oguisha in Imo State. Within the location of the streams, the major anthropological activities are domestic works

Sample Collection: The containers used for sample collection were washed with detergent and water, and was thoroughly rinsed with water and sterilized with 70% ethanol. The containers were inverted on a swabbed bench, allowing the tiny droplets in the containers to dry up, and were aseptically closed. Water sample were collected by lowering the plastic container inside the water body, 30 cm deep, allowed to overflow before withdrawing the container. The sampling points were approximately 100 mm away from one another. The samplings were done in triplicate. After collection, the sample were covered and placed in a cooler containing ice block to maintain the temperature during transportation for laboratory analysis.

Experimental Mice: A total of twenty (24) mice of mixed sex obtained from animal keeping house at Nnobi, Anambra State were used for this study. The mice were kept in separate, thoroughly cleaned and disinfected cages and provided with feeds and water ad libitum.

Inoculation into the mice: This was carried out using the method of Iheukwumere *et al*. (2012). Broth culture of the isolate was centrifuged at 3000 r.p.m for 10 minutes. The sediment was diluted with sterile phosphate buffer saline (PBS) and adjusted to the 10^8CFu/ml using 0.5 McFarland matching Standard. Then the mice (six in number) were inoculated orally with 0.5 ml of the inoculums while the control group (six in numbers) were only given distilled water.

Examination of infected mice: The infected mice were carefully observed for the clinical manifestation of the organism for a period of 4 weeks. The number of deaths was also observed. After 4 weeks, some of the infected mice sacrificed and gross examination of internal organs morphologies was carried out.

Re-isolation of the organism from the infected organs: The internal organs of the infected mice were harvested and portions were aseptically macerated in peptone water and serial diluted using ten-fold serial dilution. Samples were inoculated into peptone water, incubated at 37^{o}C for 24 h (Iheukwumere *et al*.2017).

Histopathological Study: This was carried out using the modified method of Iheukwumere *et al*.(2013). This study was done in Zoology Department, University of Nigeria, Nsukka. After 4 weeks, the mice were autopsied. The internal organs were removed, portion of these organs were washed with PBS and stored in formalin solution for histopathological examination.

Protection of infected mice: This was carried out using the modified methods of Wafaa *et al.* (2012).*Napoleona imperialis* and Ciprofloxacin were used for this study.

Processing the leaves: The fresh leaves of *Napoleona imperialis* were harvested,washed and dried under shade at room temperature for 14 days. The dried leaves were ground to powdered form using sterile electric grinder. Twenty grams of the ground leaves was macerated with distilled water for 72 h. The mixture was filtered using Whatman No. 1 filter paper (Nwosu *et al.* 2010).The constituent of the extracts were determined quantitatively using spectrophotometric method using the methods of Iheukwumere and Umedum(2013).

Experimental design: The mice were grouped into three (3) groups which include group A, B and C. Each group contained total of six mice. The treatments to the group were as follows: Group A: Blank Control (only distilled water) was given. Group B: Antibiotic (Tetracycline), 0.25g/L for the mice for period of 4 days, Group C: Medicinal Plant (*Napoleona imperialis),* 0.5 ml/ mouse for period of 10days. Group D: Infected without

treatment, 0.5ml/mouse. The experimental mice were then exposed to the isolate via oral route after 7 days. The mice were carefully monitored for a period of 2 weeks.

Examination of protected mice: The protected mice were carefully observed for the clinical manifestation of the inoculated organism for period of 2 weeks, the protection rates of the inhibitory substances were determined, and the mice were sacrificed and gross examination of the morphologies of internal organs and intestine were carried out. Also the internal organs were harvested and some portions of these organs were cultured on TCBS agar, and incubated at 37^{o}C for 48 h. The counts were taken and the colonies were identified morphologically and biochemically (Wafaa *et al*.2012). The remaining portions of the organs were subjected to hispathological examination (Iheukwumere *et al*.,2013).

Statistical Analysis: The data generated from this study were represented as mean ±Standard deviation and then charts. The test for significance at 95% confidence interval was carried out using Student "T" test.

RESULTS

Vibrio cholerae was characterized and identified using its morphology, colony description and biochemical reactions (Table 1). The isolate agglutinates fowl RBCS, haemolyse sheep RBCS and tested positive to catalase test, oxidase test, nitrosoindole test, string test, VP test, glucose test, Sucrose test, Arabinose test, saccharose test, citrate test, and motility test.The prevalence of *Vibrio cholerae* in water samples collected from two major streams used in Uli community is shown in Table 2. The result of the present study revealed that *Vibrio cholerae* was significantly ($P > 0.05$) seen more in water samples collected from Ubahudara stream as compared to water samples from Aruolah streams. The gross morphology of the internal organs and intestines of the infected mice is shown in Table 3. *Vibrio cholerae* caused fluid accumulation in the intestine, hypertrophy of the liver and kidney, liver and kidney congestion, perihepatitis and death of some mice.The mean growth counts of *Vibrio cholerae* from the internal organs of the infected mice is shown in Table 4. *Vibrio cholerae* is significantly ($P > 0.05$) seen most in the lungs, followed by the spleen and liver shows the least count. No *Vibrio cholera* was isolated from the internal organs of the control mice. The results of the histopathological examination of the internal organs of the showed obvious pathological signs. There was severe congestion in the liver especially in the sinusoids, mild disintegration of cartilage surrounding the bronchiole and infilteration of cells with extravasation of blood around the connective tissues in the lungs, diffuse severe necrosis of the tubules with minor blood congestion around the tubules in the kidney and severe necrosis of the red pulp with complete leaching off of venous sinuses in the spleen. No obvious pathological signs were recorded among the control organs.The quantitative phytochemical analysis of the leaves of *Napoleona imperialis* is shown in Table 5. The result revealed the presence of alkaloids, saponnins, flavonoids, phenolics, tannins and glycosides. These phytochemical constituents may be responsible for the activity of the leaves extracts of *Napoleona imperialis.* Gross morphological examination of the internal organs of protected mice against *Vibrio cholera* is shown in Table 6. There was mild fluid accumulation, moderate hypertrophy and congestion of the liver in the intestines of the mice protected with *Napoleona imperialis* leaves extracts and tetracycline. Re-isolation of *Vibrio cholerae* from the internal organs of protected mice is shown in Table 7. *Vibrio cholera* was re-isolated mostly from the organs of mice protected with *Napoleona imperialis* leaves extracts compared to the organs of mice protected with tetracycline. The positive control total count is significantly high. The Percent-organ body weight ratio of the protected mice is shown in Table 8. There is a high Percent-organ body weight ratio of the organs of mice protected with *Napoleona imperialis* leaves extracts compared to the organs of mice protected with tetracycline.

Table 1: Characteristic and identity of *Vibrio cholerae*

Parameter	*Vibrio cholerae*
Appearance on TCBS Agar	Yellow colonies
Elevation	Convex
Size (mm)	Entire
Gram Reaction	–
Shape	Comma shaped (Vibrio)
Catalase	+
Oxidase	+
Nitrosoindole test	+
String test	+
Agglutination with Fowl RBCS	+
VP test	+
Glucose	+
Sucrose	+
Arabinose	–
Saccharose	+
Citrate	+
Motility	+

Mm = Millimeter, TCBS= Thiosulphate Citrate Bile Sucrose, VP= Vogesprokaurer, RBCs= Red Blood Cells

Table 2: Prevalence of *Vibrio cholerae* in water samples collected from two major streams use in Uli community

Stream	**Total isolate**	**Percentage (%)**
A	29	67.44
B	14	32.56
Total	43	100

A= Ubahudara stream, B= Aruolah stream.

Table 3: Gross morphologies of the internal organs and intestines of the infected mice

Parameter	**Observation**
Haemorrhage of the liver	Absent
Haemorrhage of the lungs	Absent
Haemorrhage of the kidney	Absent
Haemorrhage of the heart	Absent
Fluid accumulation in the intestine	Present
Hypertrophy of the liver	Present
Hypertrophy of the kidney	Present
Liver congestion	Present
Kidney congestion	Present
Pericarditis	Absent
Perihepatitis	Present
Death	Present

Table 4: Mean growth count of *Vibrio cholerae* from the infected organs

Organs	*Vibrio cholera* counts (CFu/g)	
	Test	Control
Liver	77	—
Lungs	124	—
Kidney	42	—
Spleen	105	—

Table 5: Quantitative phytochemical constituents of *Napoleona imperialis* Leaves extracts

Parameter	Amount (%)
Alkaloids	3.14
Tannins	4.16
Saponnins	1.62
Flavonoids	1.08
Phenolics	3.12
Steroids	0.92
Glycosides	1.04

Table 6: Gross morphological examination of the internal organs of protected Mice against *Vibrio cholerae*

Parameter	Observation			
	F_2	A	B	F_1
Haemorrhage of the liver	—	—	—	—
Haemorrhage of the lungs	—	—	—	—
Haemorrhage of the kidney	—	—	—	—
Haemorrhage of the heart	—	—	—	—
Fluid accumulation in the intestine	—	+	+	+++
Hypertrophy of the liver	—	++	++	+++
Hypertrophy of the kidney	—	—	—	—
Kidney congestion	—	—	—	+
Liver congestion	—	++	++	+++
Pericarditis	—	—	—	—
Perihepatitis	—	—	—	+++
Death	—	—	—	++

A — *Napoleona imperialis*, B — Tetracycline, +++ = High , ++ = Moderate, += Mild
F_1 = Positive control, F_2 = Negative control

Table 7: Re-isolation of the *Vibrio cholerae* from the internal organs of Protected mice

Organs	Total Mean Counts (Cfu/g) $\times 10^2$			
	A	B	F_1	F_2
Liver	—	—	77	—
Lungs	27	22	124	—
Kidney	31	26	42	—
Spleen	47	33	105	—

A-*Napoleona imperialis* leaves extracts, B- Tetracycline, F_1 - Positive control, F2- Negative control

Table 8: Percent-Organ body weight ratio of the protected mice

Organs	Percent-Organ body weight Ratio (%)			
	A	B	F_1	F_2
Liver	14.02	13.86	14.40	13.27
Lungs	2.98	2.36	3.08	2.11
Kidney	1.34	1.17	1.86	0.93
Spleen	1.31	1.21	1.61	0.91

A- *Napoleona imperialis* leaves extracts, B- Tetracycline, F_1- Positive control, F_2- Negative control

DISCUSSION

The biochemical reactions, morphological tests and colony description of vibro cholera isolated from stream samples collaborates with the report of Sagar (2014), who worked on biochemical tests for *Vibrio cholerae*. The variation in *Vibrio cholerae* loads among the two streams could be attributed to the fact that Ubahudara stream is more close to human settlement and more anthropological activities takes place in this stream than Aruolah stream. Similar conclusions were drawn by Faruque *et al*. (2000). Wenjing (2000) also reported that most drinking water contamination can be attributed to human activities. The accumulation of fluid in the intestines

collaborates with the report of Muanprasat *et al.* (2012). The accumulation of fluid in the intestine could be due to the production of hemolysin-cytolysin toxin as a result of choleragen toxin activation. Similar result was reported by De, (2007). The gross morphological examination of internal organ of infected mice against *Vibrio cholerae* revealed congestion in liver and kidney. This agrees with the report of Nanayakkara *et al.* (2009). Oliver *et al.* (2007) also reported the death of mice associated with systemic spread of infection caused by choleragen toxin released by *Vibrio cholerae.* The significant growth of *Vibrio cholera* observed from the internal organs of infected mice showed that the organism was able to invade and multiply in these organs. Similar result was reported by Basu, (2000). The present study revealed that the significant growth of *Vibrio cholera* observed in the lungs agrees with the findings of Oliver *et al.* (2007).This phytochemical constituent seen in the studied plant extract could be responsible for the pharmacological effect of the extract as reported by Onyegbule *et al*. (2011). Many researchers have shown that plant rich in tannin and phenolic compounds have been shown to poses antimicrobial activities against a number of microorganisms (Bhalodia and Shukla, 2011). The absence of kidney congestion, perihepatitis, death and mild accumulation of fluid in the intestine associated with the protected mice corroborates with the findings of other researchers (Esimine *et al*., 2005; Fagbolun *et al*., 2010). The low count of *Vibrio cholera* associated with internal organs of protected mice after infection shows that these antimicrobial substances were able to protect the organs against invading pathogens (Colwell *et al.,* 2000). Tetracycline offered better protection when compared with the *Napoleona imperialis* leaves extracts. The Percent-organ body weight ratio of the organs of mice protected with *Napoleona imperialis* leaves extracts weighed high when compared to the organs of the mice protected with tetracycline. The liver of the mice protected with medicinal plant has the highest weight, followed by the lungs; the spleen has the least weight. The low weight associated with mice protected with tetracycline could be as a result of the side effect of tetracycline (Wilson *et al*. 2007).

CONCLUSION

The study revealed the effects of *Napoleona imperialis* leaves extracts and tetracycline on *Vibrio cholerae.* It can also be stated that the both can be used in the treatment of *Vibrio cholerae.* However, tetracycline was more effective and can be used in the treatment of human diseases caused by *Vibrio cholerae.*

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