In Vitro Antibacterial Activity of Adenia Cissampeloides Plant Extracts on Bacteria Isolated from Fishes Recovered from Uwana River, Ebonyi State, Nigeria

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Abstract: Adenia Cissampeloides, a Pesticidal plant has dynamic active ingredients which cause physiological destruction in fishes including Clarias gariepinus. Majority of these Pesticidal plants contain medicinal values. In this study, the in vitro antibacterial activity of A. Cissampeloides plant extracts on bacteria isolated from fishes recovered from Uwana River, Ebonyi State, Nigeria was bacteriologically investigated. The isolation and identification of bacteria from the fishes recovered from Uwana River was carried out using standard microbiology techniques. The phytochemical properties of the plant extracts were studied using phytochemical analysis while the antimicrobial activities of the A. Cissampeloides plant extracts were evaluated using agar well diffusion technique. Results from physicochemical analysis of the water samples and bacterial count established that Uwana River is polluted. Fourteen species of aerobic heterotrophic Gram-positive and Gram-negative bacteria were isolated and identified from the fishes. The Gram-positive bacteria included, Staphylococcus lugdunensis, S. hominis, S. cohnii, Streptococcus pyogenes, Streptococcus pneumoniae, Kocuria varians and Gram-negative bacteria were Raoutella ornithinolytica, Klebsiella pneumoniae, Aeromonas hydrophila, Aeromonas veronii, Proteus vulgaris, Serratia fonticola, and Enterobacter Gergovia. The most frequently isolated organism from the fishes was Raoutella ornithinolytica (19.04% and 31.82%). Phytochemical analysis of the plant extracts shows that it possesses antimicrobial properties. Extract with petroleum ether showed highest inhibition zone diameter (8.2 mm) with Aeromonas veronii, while the least diameter was observed in Staphylococcus cohnii (4 mm). Methanol extracts of the plant on R. ornithinolytica showed (7mm) while on Aeromonas hydrophila it was (5mm). The results of this study show that the plant extracts of A. Cissampeloides had appreciable antimicrobial activity on bacteria isolated from fishes recovered from Uwana River. Conclusively, the tested extracts of A. Cissampeloides plant showed varying spectra of inhibitions and/or antimicrobial activity against the isolated bacteria.

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1. Introduction:

Adenia Cissampeloides is a naturally occurring Pesticidal plant that has dynamic active ingredients which cause physiological destruction in fishes such as Clarias gariepinus and perhaps other water-loving organisms. Majority of these Pesticidal plants possess numerous medicinal values. Other species of Adenia include: Adenia guineensis, Adenia gracilis Harms and Adenia gummifera (Harv). Colloquial names of the plant include snake climbers, Mkengeti, wild granadilla, Mandali, and monkey rope. Adenia Cissampeloides is a native plant of Senegal East to Somalia; and it is mostly found towards the East, West, South and Central Africa countries (Adekunle and Adekunle, 2009). These medicinal plants have several means of application which include infusions as teas, poultices or tinctures, mixtures of diverse plant combination as soups which might be given in different ways as well as nasal (smoking, snuffing or steaming), oral, nasal or as constituent concoction in porridges as well as topical (creams or lotions), rectal or bathing (Adekunle and Adekunle, 2009; Doughari et al., 2009). Several communities may use a single herbal plant to treat several ailments such as asthma, esophageal cancer, and fever. In some communities, hypertension has been managed by the use of native medicinal plants (Rios and Recio, 2005).

Diverse branches of herbal plants and components such as flowers, essential oil, roots, stems, leaves, barks or the combinations of two or more of the bioactive molecules has been engaged in taking care of infectious diseases of

the urinary tract, biliary system, respiratory system, and gastrointestinal tract as well as some skin infections (Adekunle and Adekunle, 2009; Rıos and Recio, 2005).

Much recognition has been given to medicinal plants among the literate populace in the urban settlements, this may be because of the inefficacy of modern drug that is used for management of some infectious diseases such as gonorrhea, typhoid fever, and tuberculosis, as well as much cases of bacteria resistance to different antibiotics available in the market (Smolinski et al., 2003; Levy, 1998). Bacteria present on the fish are normally indigenous of their natural environment and predisposed by the season and the harvesting conditions (ICMSF, 1998).

Huss (1995), stated that the type and number of pathogenic bacteria present in fish can be divided into two groups: those bacteria (such as Clostridium botulinum, Listeria monocytogenes, Aeromonas hydrophila and Vibrio species) which are commonly found in the aquatic environment (indigenous pathogenic bacteria of fresh water) which are present on the live fish and their presence in the final product is predictable; and non-indigenous pathogenic bacteria (such as Salmonella spp., Escherichia coli and Staphylococcus aureus), which are normally associated with humans or warm-blood animals and their faeces, and not naturally present in fish or seafood products (Rheinheimer, 1985; Olayemi, 1994).

Pesticidal plants have been employed by fishermen locally to harvest fish in large quantity. Local herbs have been in use for the harvesting/catching of fish from our local rivers. However, the impact of these herbs on the bacterial flora of the freshwater fishes has not been properly documented in recent times. In this study, the in the vitro antibacterial activity of Adenia Cissampeloides plant extracts on bacteria isolated from fishes recovered from Uwana River, Ebonyi State, Nigeria was bacteriologically investigated.

2. Materials and methods:

2.1. Collection of samples:

Live fishes were collected using glass aquarium. Fifty samples of the two fishes (28 of catfish and 22 of tilapia) were examined for physical injuries and disease signs like ulcerations and necrotic lesions on the skin surfaces, gills or fin rot, abdominal dropsy and pop eye. Water samples (fourteen (14) samples from each point) were also collected at different points of the river using sterile bottle (upstream, midstream and downstream) containers.

2.2. Isolation of bacteria:

Bacteria were obtained from different parts of the two fishes and also from water samples. The bacteria growths (colonies) were separated into diverse types according to the colonial appearance (color, elevation, shape, size). The (colony types) were then streaked on TSA plate continually until distinct colonies were obtained. Cultures on TSA slant were further stored in the refrigerator at 4ºC as stock for further use. These bacteria colonies were always transferred to fresh slant to avoid contamination every 6 weeks.

2.3. Determination of physical parameters of the river:

The following parameters: temperature, pH, specific gravity, ascorbic acid, sodium, potassium, chloride, phosphate, calcium, copper, magnesium, nitrates, ammonium, dissolved oxygen concentration (DOC) were determined following the method described by Al-Harbi and Uddin (2004).

2.4. Heterotrophic bacterial count:

The heterotrophic bacteria count of the water samples was done using pour plate method. Ten 10^{-1}-fold serial dilutions of the water samples were carried out and samples were collected from the fourth tube (10^{-4}) and the samples were inoculated on nutrient agar and incubated for 18-24 hrs. at 27ºC. Plates were prepared in triplicates. Quebec colony counters with a built-in grid to simplify counting were used.

2.5. Collection of plant material:

Adenia Cissampeloides plants were collected from Botanical Garden at Nsukka, Enugu State. Authentication of the Pesticidal plant was made by a taxonomist, Prof. S.C Onyekwere of the Department of Applied Biology Science, Faculty of Science, Ebonyi State University.

2.6. Maceration and extraction of plant materials:

The leaves of *A. Cissampeloides* were air-dried at room temperature for 2 weeks, after which it was ground to a uniform powder using an electric blender. The methanol and petroleum ether extracts were prepared by soaking 100 g each of the dry powdered plant material in one liter of methanol and petroleum ether at room temperature respectively for 36 hrs. One hundred gram (100 g) of the powdered extracts was soaked in one liter of hot water for 36 hrs. The mixtures were filtered through a Whatman filter paper Number 42 (125 mm). The extracts were concentrated using a rotary evaporator with the water bath set at 60˚C (Ayoola et al., 2008; Deka et al., 2011). The extract was also tested for purity by plating them on nutrient agar and incubated for 24hrs at 37ºC (NCCLS, 1999; Biswas et al., 2002).

2.7. Preliminary phytochemical analysis:

Determination of phytochemical constituents of the Pesticidal plants was made according to the screening procedures in line with standard procedures (Trease and Evans, 1989; Sofowara, 1993; Harbone, 1998).

2.8. Screening for antimicrobial activity of plant extracts:

Eighteen milliliters (18 ml) each of sterilized molten Mueller Hinton (MH) agar was poured aseptically into

sterile Petri dishes and then allowed to solidify. The surface of the MH agar plates was then streaked with a standardized inoculum of the test bacteria that was adjusted to 0.5 McFarland turbidity standards.

Thereafter, a sterilized 6 mm cork borer was used to bore 4 holes on the MH agar plate, and 3 of the holes were filled with equal volumes of the respective plant extracts (Esimone et al., 2008). Sterilized distilled water was used as the negative control and the positive control used was ciprofloxacin (5 µg). The plates were allowed for about 30 minutes for pre-diffusion of the plant extracts, and these were incubated at 37°C for 24 hrs. After incubation, the inhibition zone diameters (IZD) were measured and recorded. The IZD of each plant extracts were evaluated by subtracting the size of the cork borer from the IZD measured (Onyeagba et al., 2004; Esimone et al., 2008).

3. Results:

Table 1 shows the physicochemical properties of the water samples. The properties include the appearance, pH, temperature, specific gravity, aerobic acid, sodium, potassium, chloride, phosphate, calcium, copper, magnesium, nitrate, and ammonium.

The result obtained showed a high content of phosphate, chloride, nitrates and other substances which indicates pollution. The result of the heterotrophic bacteria counts of a water sample collected from upstream, midstream and downstream is shown in Table 2. The result obtained showed colony forming unit of bacteria between 2.4 X 10^4, 3.4 X 10^4 and 5.6 X 10^4 respectively. The colony count was highest at downstream**.**

Table 1: *Average Physicochemical Parameters of the Water Samples.*

Water Sample	A	B	C
Appearance	Brownish & clear	Brownish & clear	Brownish & clear
pH	6.5	6.5	6.5
Temp	26.1 ± 1.16 °C	24.4 ± 0.94 °C	25.3 ± 0.69 °C
Specific gravity	1	1	1
Ascorbic acid (mg/L)	6.03 ± 0.12	5.87 ± 0.12	5.87 ± 0.12
Sodium (mg/L)	184.07 ± 0.34	175.73 ± 0.87	17.53 ± 0.26
Potassium (mg/L)	3.75 ± 0.03	5.40 ± 0.16	5.20 ± 0.16
Chloride (mg/L)	81.33 ± 1.25	75.27 ± 0.34	75.43 ± 0.24
Phosphate (mg/L)	4.23 ± 0.23	10.47 ± 0.21	10.43 ± 0.25
Calcium (mg/L)	25.23 ± 0.29	28.07 ± 0.77	28.57 ± 0.17
Copper (mg/L)	1173.33 ± 20.5	1213.33 ± 14.4	1208.33 ± 6.24
Magnesium (mg/L)	0.38 ± 0.37	0.60 ± 0.08	0.58 ± 0.02
Nitrates (mg/L)	1.93 ± 0.05	2.67 ± 0.12	2.70 ± 0.16
DOC(mg/L)	4.20 ± 0.08	4.50 ± 0.12	4.60 ± 0.16
Ammonium	0.06 ± 0.00	0.11 ± 0.00	0.16 ± 0.01

Key: Water sample A= Upstream, B= Midstream and C= Downstream, a value expressed in Standard mean deviation (±).

2.9. Determination of minimum inhibitory concentration (MIC):

Cultures obtained were adjusted to 0.5 McFarland equivalents and the inoculums were standardized to obtain 1.0 × 10^5 cfu/ml. The MIC of active extracts were evaluated using agar well diffusion method. The MICs of all the extracts were evaluated by diluting the extracts to various concentrations. Decreasing concentrations of herbal extracts were prepared in serial two-fold (10^{-2}) dilutions using sterile distilled water. The standardized inoculums were seeded on a prepared/solidified MH agar plates. Six (6) millimeter hole was bored using cork borer, and the diluted herbal extracts were used to fill the holes. The inoculated MH agar plates were incubated for 18-24 hrs at 37°C. Plates with clear zones were observed and the data recorded.

Table 2: *Average Colony Forming Unit Count (Heterotrophic Bacteria Count) Analysis on Water Samples.*

Sample	Average CFU Count
Upstream	2.4 X10^4 CFU/ml
Midstream	3.4 X10^4CFU/ml
Downstream	5.6 X10^4 CFU/ml

Key: CFU/ml = Colony Forming Unit per milliliter.

Figure 1 shows the frequency of the bacteria isolated from the Clarias gariepinus fish samples used for this study. The results showed that more bacteria were isolated from the intestines than from the gills and skins of C. gariepinus.

Figure 2 shows the pictorial representations of the frequency of the bacteria isolated from Tilapia zillii samples used for this study. The results showed that in Tilapia zillii, bacteria were isolated more from the skin than gills and intestine.

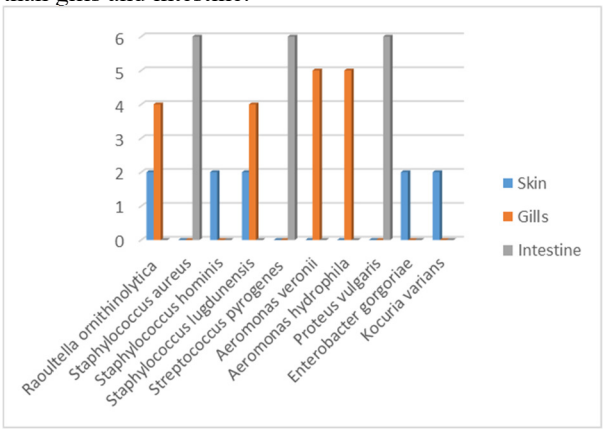

Figure 1. *Frequency of Bacteria Isolated from Clarias gariepinus*

Table 3: *Phytochemical analysis of plant extracts of Adenia Cissampeloides*

Solvent/Extract	Proteins	Carbohydrate	Resins	Saponins	Flavonoids	Alkaloids	Steroids	Phenols	Glycosides	Tannins
Petroleum ether	-	+	+	+	+	+	+	+	-	+
Methanol	+	+	+	+	+	-	+	+	+	+
Water	-	-	-	-	+	+	+	-	+	-

Keynotes: += present, - = Not present.

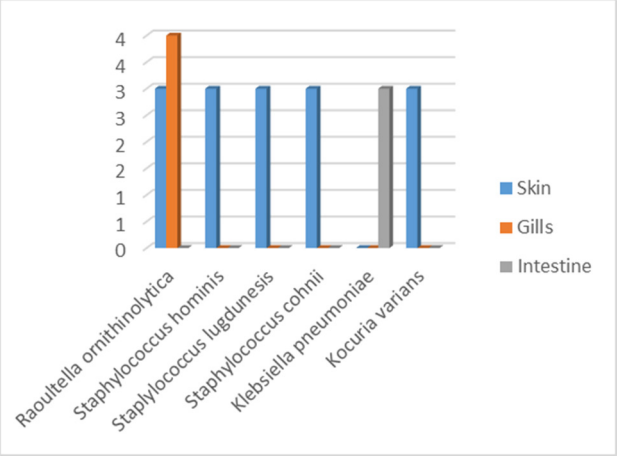

Figure 2. Frequency of Bacteria Isolated from Tilapia zillii

Table 3 shows the phytochemical analysis of petroleum, methanol and aqueous plant extracts of Adenia Cissampeloides. The result obtained showed that the plant contains bioactive molecules such as flavonoid, saponin, tannin, phenol, steroid, rennin, glycoside, carbohydrate, protein and alkaloid. Table 4 shows the inhibition zones diameter (mm) of aqueous, methanol and petroleum ether of Adenia Cissampeloides plant extracts on the selected bacteria. The result obtained showed that extract of petroleum ether had various degrees of activities against the test bacteria thus; (8.2 mm) on Aeromonas veronii, Staphylococcus hominis (8 mm), Streptococcus pyogenes (8 mm) Raoultella ornithinolytica (8 mm) had a highest diameter of inhibition while the least was seen in Staphylococcus cohnii (4 mm). In methanol extracts Raoultella ornithinolytica (7 mm), Staphylococcus hominis (7 mm), Serratia fonticola (7 mm) had the highest diameter of inhibition while Aeromonas hydrophila (5 mm) Staphylococcus hominis (5 mm). had the least. Figure 3 shows the result of the minimum inhibitory concentration (MIC) of the plant extracts on the tested bacterial isolates.

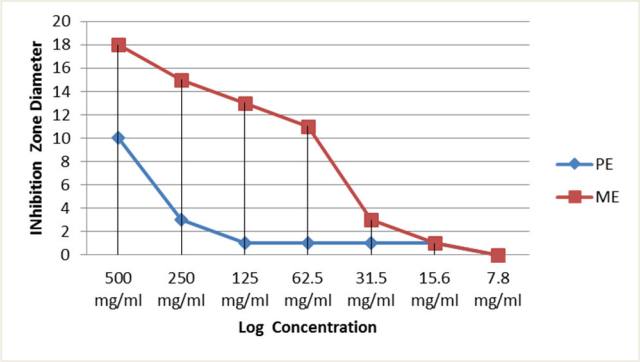

Figure 3. Minimum inhibitory concentration (MIC) gradient of the plant extracts

Key: R =resistant, PE = Petroleum ether extract, ME = Methanol extract, Method: Diffusion technique using Mueller Hinton's agar, mg/ml = milligram per milliliter,

4. Discussion:

The quality of water greatly influences the aquatic environment and is important for the survival of aquatic flora and fauna (Deekae et al., 2010). Human activities and anthropogenic pressures like industrial, community waste disposal, heavy use of insecticides, pesticides, and fertilizers in agricultural practices are major causes of pollution in the aquatic environment. The average temperature of water at the three different points of collection ranged between 25.3 ± 0.69 to 26.1 ± 3°C.

The result agrees with works of Rheinheimer, (1985). This temperature range of water showed appropriate condition for the growth of mesophilic bacteria and optimal temperature for aquatic animals. Average pH of water sample ranged between 6.0 to 6.5 showing slightly acidic condition. This is in conformity with studies by Chakraborty (1998) on fresh water bodies in India which are slightly acidic in nature. The result obtained may be an indicator of pollution of the water body (Patra et al., 2011).

The type of organisms isolated from both water and fishes in this work were similar to those found by Obiajuru and Ogbulie (2006) who isolated Gram-positive bacteria like Staphylococcus spp., Streptococcus spp. and Enterobacter spp. and Gram-negative bacteria like Pseudomonas spp., Aeromonas spp., have been connected with the microflora of fresh water fish (Allen et al., 1983; Rudra and Sudip, 2011). These bacteria were found on the skin, gills or in the intestines of the two fresh water fishes used for this study.

Table 4: *Inhibition zones diameter (mm) of aqueous, methanol and petroleum ether of Adenia Cissampeloides.*

Extract	PE	ME	HW
CG1	0.00	0.00	0.00
CG2	8.33 ± 0.47	5.67 ± 0.47	0.67 ± 0.47
CG3	0.00	4.67 ± 0.47	0.67 ± 0.47
CG4	8.17 ± 0.05	6.17 ± 0.47	0.00
CG5	7.67 ± 0.57	6.67 ± 0.47	0.67 ± 0.47
CS1	4.67 ± 0.47	6.13 ± 0.09	1.67 ± 0.47
CS2	0.00	0.00	0.00
CS3	0.00	0.00	0.67 ± 0.47
CS4	6.33 ± 0.94	5.93 ± 0.09	0.00
C11	8.33 ± 0.47	6.87 ± 0.12	0.00
C12	3.67 ± 0.47	5.87 ± 0.09	0.67± 0.47
C13	7.67 ± 0.47	6.07 ± 0.09	0.00
C14	7.67 ± 0.47	4.93 ± 0.05	0.00
C15	0.00	0.00	0.00
C16	0.00	5.67 ± 0.47	0.00
TG1	6.67 ± 0.47	6.06 ± 0.09	0.00
TG2	4.67 ± 0.47	5.47 ± 0.05	0.00
TG3	6.73 ± 0.52	5.93 ± 0.09	0.00
TG4	6.33 ± 0.94	5.97 ± 0.05	0.00
T11	0	4.07 ± 0.12	1.47 ± 0.05
T12	7.27 ± 0.09	4.33 ± 0.94	0.67 ± 0.47
T13	6.33 ± 0.12	4.93 ± 0.47	0.00
TS1	4.67 ± 0.47	6.97 ± 0.47	0.00
TS2	0.00	0.00	6.5 ± 0.08
TS3	0.00	0.00	0.67 ± 0.47

Key: R = Resistant, CG 1=Staphylococcus lugdunensis, CG 2 =Raoultella ornithinolytica, CG 3 = Aeromonas hydrophila, CG4 =Aeromonas veronii, CG5 = Raoultella ornithinolytica CS1 =Staphylococcus lugdunensis, CS2 = Staphylococcus lugdunensis, CS3 =Enterobacter Gergovia, CS4 =Raoultella ornithinolytica C11 =Proteus vulgaris, C12 = Streptococcus pyogenes, C13 = Staphylococcus hominis, C14 = Staphylococcus aureus, C15 =Aeromonas hydrophila, C16 = Kocuria varians, TG1 = Raoultella ornithinolytica TG2 = Staphylococcus hominis, TG3 = Raoultella ornithinolytica, TG4 = Raoultella ornithinolytica, T11 =Serratia fonticola , T12 = Staphylococcus hominis, T13 = Staphylococcus hominis, TS1 = Raoultella ornithinolytica, TS2 = Staphylococcus lugdunensis, TS3 = Staphylococcus cohnii.

However, this study cannot ascertain whether these organisms colonized fish or were merely temporary or transient inhabitants. The frequencies of isolated organisms from Clarias gariepinus show the presence of Raoutella ornithinolytica, Staphylococcus aureus, Staphylococcus lugdunensis, Streptococcus pyogenes, Proteus vulgaris, Aeromonas hydrophila, Aeromonas veronii, Enterobacter gorgoviae, Kocuria varians. The following organisms; Raoutella ornithinolytica, Staphylococcus hominis, Staphylococcus lugdunensis, Staphylococcus cohnii, and Klebsiella pneumoniae were isolated from Tilapia zillii.

This result slightly differed from the studies by Adedeji et al. (2011) who isolated more bacteria from the skin of African catfish (Clarias gariepinus) and Nile Tilapia, but similar to the findings of Rudra and Sudip (2011) and Apun et al. (1999) who isolated more bacteria from the intestine (gut), than from skin and gills. Higher bacteria isolated from skin and gills may be as a result of contamination of the aquatic environment. Presence of alkaloids in an appreciable amount in all three extracts used is similar to the studies by Olaifa et al., (1987), Adewunmi (1990). The result of phytochemical analysis of plant (Adenia Cissampeloides) shows that it contains bioactive compounds like saponins; flavonoids; Alkaloids; steroids; phenols; glycosides; resins; tannins which have antimicrobial properties. Extract with petroleum ether showed inhibition zone diameters; (8.17 ± 0.05 mm) on Aeromonas veronii, Staphylococcus hominis (7.67 ± 0.47 mm), Streptococcus pyogenes (3.67 ± 0.47 mm). Raoultella ornithinolytica (6.61 ± 0.47 mm) have the highest diameter of inhibition while the least is seen in Staphylococcus cohnii (0.00 mm). In methanol extracts Raoultella ornithinolytica (6.06 ± 0.09 mm), Staphylococcus hominis (6.07 ± 0.09 mm), and Serratia fonticola (4.07 ± 0.12 mm) had the highest diameter of inhibition while Aeromonas hydrophila (0.00 mm) Staphylococcus hominis (0.00 mm) Staphylococcus cohnii (0.00 mm) showed no activity.

The results show that Adenia Cissampeloides possesses some antimicrobial properties against some of these bacteria isolated from fish. The result obtained from the Hot water extracts of the Pesticidal plant (Adenia Cissampeloides) showed that it had various activities against the test bacteria isolated from fish. The result showed that Raoultella ornithinolytica and Staphylococcus lugdunensis had inhibition zone diameter ranging from 0.67 ± 0.47 and 1.67 ±0.47 respectively. Hot water extracts showed the highest activity against Staphylococcus hominis (6.5 ± 0.08) isolated from Tilapia zillii and had activity against Staphylococcus hominis isolated from Clarias lugdunensis. The result of Minimum Inhibitory Concentration of the plant extracts showed that at a concentration of 500 mg/ml, it had its highest value (18 mm

for methanol and 10 mm for petroleum). The concentration at which plant extract showed least zone of inhibition is 7.8 mg/ml. The MIC gradient obtained showed anti log of intersection point to be at 7.8 mg/ml. Since these plants are usually exploited for fishing activities in this region, the further molecular investigation is needed to investigate the compounds responsible for the antimicrobial activity of the plant as a panacea to developing novel antimicrobial agents from them.

However, additional development of the germ plasma of A. Cissampeloides should be stored for further antimicrobial studies. And efforts ought to be put into developments of novel drugs from these plants owing to the evolving nature of antibacterial resistance, and the notable antimicrobial efficacy that they possess. Conclusively, the results of this study have established the presence of significant pathogenic microorganisms in Clarias gariepinus and Tilapia zillii and also the poor quality of the Unwana River which is a direct reflection of the relationship between the quality of water environment and aquatic animals. Adenia Cissampeloides was found to have an antimicrobial effect on the bacteria isolated. Therefore, further research should be directed on using Adenia Cissampeloides to ascertain its effect on microbial load in fishes.

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