www.cenresinpub.org
ISSN 2277-0135

PLANT EXTRACTS AS ALTERNATIVE TREATMENT FOR *Pseudomonas aeruginosa* OCCURRENCE IN *Clarias gariepinus* (BURCHELL, 1822) JUVENILES

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ABSTRACT

The culturing and isolation of *Pseudomonas aeruginosa* from *Clarias gariepinus* juveniles were carried out. The bacteria isolated was tested for sensitivity to three (3) plant extracts (*Garcinia kola, Allium sativum and Allium cepa*) with (8) antibiotics (Tetracycline, Ampiciline, Cotraimozazole, Gentamycin, Naladixic acid, Nitrofurantoin, Colistin, and Streptomycin). The highest and lowest mean value of bacteria count 1.7×10^5 and 0.00 was observed in Lagos state*. Pseudomonas aeruginosa* species was isolated from the gill, skin and buccal cavity. The organism from the different fish farms reacted differently to the concentrations of the plant extracts (onion, garlic and bitter kola). Onion and Garlic were the only plant extracts that showed zone of inhibition at 500mg/ml while there were no inhibition zones at 250mg/ml and 125mg/ml. Bitter kola had no zone of inhibition at 500mg/ml, 250mg/ml and 125mg/ml. The result of the study confirmed that Onion and Garlic were effective on *Pseudomonas aeruginosa* at 500mg/ml. Also, different concentrations of antibiotics were used - only Gentamycin and Colistin had zones of inhibition, but Tetracycline, Ampiciline, Cotraimozazole, Naladixic acid, Nitrofurantoin and Streptomycin had no inhibition zone. Among the plant extracts used for the study, onion and garlic are recommended because they have little or no residual effects on both humans and livestock.

Keywords: *Plant extracts, Pseudomonas aeruginosa, Clarias gariepinus juvenile, Ogun, Lagos, Oyo states*

INTRODUCTION

Fish diseases are one of the major problems in fish farm industry. Even though, vaccines are being developed and marketed, they cannot be used as a universal disease control measure in aquaculture. Disease cause economic losses not only from mortality but also treatment expenses, postponement or loss of the opportunity to sell the fish and contraction of zoonotic diseases by the handler and final consumer of the affected fish. Contamination of hands and surfaces during cleaning and evisceration of fish is a common route of pathogen infection through contamination of other food (Buras, 1993). Fish and Shellfish not only transmit disease to man but are themselves subject to many diseases and capable of transmitting many of the established food borne microbial infections and intoxications (FAO/WHO, 1974). The use of antibiotics to cure bacterial infection and prevent fish mortality in aquaculture is becoming limited as pathogens develop resistance to the drugs (Gonzalez *et al*., 2000; Gomez-Gil *et al*., 2000). The discovery of penicillin in the 1940s and several other antibiotics in subsequent years led to great improvements in the management of infectious diseases particularly in developed countries. However, despite this success, the increased use of antibiotics led to the inevitable development of resistance, with the effect that diseases that

were hitherto thought to have been controlled by antibiotics later re-emerged as resistant infections (Norrby *et al.,* 2005). At present major pathogenic bacteria that contribute the most to the global infectious disease burden such as *Streptococcus pneumoniae, Streptococcus pyogenes, Klebsiella pneumonia, Pseudomonas aeruginosa,* are resistant to standard antibiotic therapies (Styers *et al.,* 2006; Fluit *et al.,* 2001). The characteristic symptom of the disease produced by the bacteria is a remarkable septicemia hemorrhage in the skin of the mouth region, opercula and ventral side of the body (Wakabayashi and Egusa, 1972). *Pseudomonas aeruginosa* is one of the most zoonotic and pathogenic bacteria which infect man and animals and others. The global emergence of multi-drug resistant bacterial strains has limited the effectiveness of current drugs, causing treatment failures (Hancock, 2005). The containment of this drug resistance requires that, new potent antimicrobial compounds be identified as alternatives to existing antibiotics (Overbye and Barrett, 2005). However, the current state of development of new antimicrobial drugs is not encouraging with only a few new ones being licensed in recent years (Levy and Marshall, 2004; Norrby *et al.,* 2005). This mismatch between the slow development of new drugs and the fast emergence of resistant strains makes the future management of infectious diseases look bleak. As an alternative and perhaps a sustainable option, attempts to improve the efficacy of available antibiotics, particularly the older and cheaper ones have been suggested (Lomovskaya and Bostain, 2006). Medicinal plants continue to play a central role in the healthcare systems of large proportions of the world's population, particularly in developing countries, where herbal medicine has a long and uninterrupted history of use (Koduru *et al.,* 2007). According to the World Health Organization (WHO, 2004), up to 80% of the population in Africa depends on traditional herbal medicine for primary health care, accounting for around 20% of the overall drug market (WHO, 2004). The popularity of such plants in these communities owes largely to their local availability and price affordability (Voravuthikunchai and Kitpipit, 2005) and also confirms their effectiveness.

Plants produce a wide variety of secondary metabolites many of which have been reported to be of therapeutic value. Of the more than 250 000 species of higher plants in the world, only about 5 -10% have been chemically investigated (Tshibangu *et al.,* 2001). This raises the prospects of obtaining novel chemotherapeutic compounds if this vastly untapped resource could be adequately explored.The prospect of obtaining drugs from plants has been demonstrated by some notable examples of important pharmaceuticals derived from plant precursors. For instance, the anti-malarial drug Quinine was derived from the quinoline alkaloid of *Cinchona sp.;* the topical analgesic Capsaicin was derived from a phenyl alkyl amine alkaloid of *Capsicum sp*; and the antineoplastic agent Camptothecina was derived from an indol alkaloid of *Camptotheca acuminate* (Raskin *et al.,* 2002). The rich chemical diversity in plants has also been reported to be a promising source of antibacterial compounds (Bylka *et al.,* 2004; Smith *et al.,* 2007; Machado *et al.,* 2002), raising hopes of obtaining novel antibiotics that can aid the fight against drug resistant infections. In addition to plants being potential sources of direct antibacterial drugs, research has also shown that some secondary metabolites of plants with no intrinsic antimicrobial activity are useful in sensitizing bacterial cells to antimicrobial agents (Stermitz *et al.,* 2000; Tegos *et al.,* 2002). These compounds are

believed to play a role in the plant defence against infection by working in synergy with intrinsic antimicrobials. It has therefore been suggested recently, that such compounds can potentially be used to improve the efficacy of antibiotics against bacterial pathogens. Garlic has been used to help prevent heart diseases including atherosclerosis, high cholesterol, high blood pressure, and to improve the immune system as well as protection against cancer (Marryland, 2005). *G. kola* is used in folklore remedies for the treatment of various infections caused by pathogens. Two new chromanols, garcioic and garcinal, together with tocotrienol were reported isolated from *G. kola* (Terashima *et al.,* 2002). The relative pungency of onion has both genetic and environmental components. Sulphur compounds in onions have also been shown to be anti-inflammatory both by inhibiting formation of thromboxanes and by inhibiting the action of platelet-activating factor (PAF) (Wikipedia, 2007). The findings of Shibata *et al.* (2005), Stapleton *et al.* (2004), Marquez *et al.* (2005), Oluwatuyi *et al.* (2004) and Smith *et al.* (2007) have confirmed that indeed plants can be sources of compounds that can potentiate the activity of antibiotics against resistant bacteria pathogens. These compounds have variably been termed resistance modifying, modulating or reversal agents. This study provides information on the use of plant extracts as alternative treatment for bacterial diseases in *Clarias gariepinus* juveniles.

MATERIALS AND METHODS

The Study Area

Fifteen fish farms were visited in three states of Nigeria: Lagos, Ogun and Oyo States. Five farms were visited in each of the states mentioned. Samples were collected from all the farms in the study area. The farms visited were: Feedville, Palm Royal, Quick Link, Palm Wisdom, Glorious (Lagos State); UNAAB, Ultimate, Eweje, Ikililu, and Larry (Ogun State); Hope, Kulturetel, Durante, Zamits and Crams (Oyo State).

Collection of Samples

Collection of samples was done on three juvenile cat fish (*Clarias gariepinus*) whose weights were between 50-140g. For each fish randomly selected for analysis (preserved and transported to the laboratory), sections were taking as follows using swab sticks: the gills, skin and the gut regions. Each (gill, skin and gut) was aseptically taken and homogenised in 10ml of sterile distilled water under hood. These were taken as the original stock culture of the sections of the fish samples.

Bacteriological Analysis

1ml each of the original stock was suspended into 9ml sterile water aseptically in a MacCartney bottle which was then shaken together. Further dilution of 10^{-1}, 10^{-2} and 10^{-3} were carried out, in which 10^{-2} dilution was later used. A loopful each of the stock culture was inoculated onto sterile Nutrient Agar plates. The Nutrient Agar plate was incubated at 37^{0}C for 24 hours for bacterial growth. Three replicates were prepared for every sample examined.

Identification of Microorganism
The organisms were identified using Grams reaction and biochemical tests such as Catalase, Coagulase, Citrate Utilization, Urease, Nitrate Reduction, Indole Reaction, Oxidase, Sugar Fermentation tests and so on were carried out according to Akinyemi (2009) and Olutiola *et al.* (1991) to identify sample by their reaction to the tests.

Preparation of Cold Water Extract of Onion, Garlic and Bitter Kola
50g each of onion bulb, bitter kola and garlic bulb was weighed and mashed in a sterile mortar and pestle which was then soaked in 100ml of sterile water for 24 hours to extract the juice. After 24 hours, each of the extract was sieved by the use of muslin filter paper into different sterile universal bottle. Different concentrations (i.e. 500mg/ml, 250mg/ml and 125mg/ml) of each of the extract was gotten by taking 50ml each from the extract and diluting it with another prepared 100ml of sterile water to get the 250mg/ml. Also out of the 250mg/ml, another 50ml was also taken and diluted with 100ml of sterile water in another bottle to get the 125mg/ml. This procedure was used for the three plant extracts (onion bulb, bitter kola and garlic bulb).

Preparation of Standard Drugs for Comparism
Antibiotic discs were used as comparison for the antimicrobial activity with plant extracts. 8 antibiotics were used which includes; Ampicilin (25mg), Cotraimozazole (25mg), Gentamycin (10mg), Naladoxic acid (30mg), Natrofuranton (30mg), Colistin (25mg), Streptomycin(25mg), Tetracycline (25mg).

Determination of Antimicrobial Activity
2.8g of nutrient agar was dissolved in 1000ml of distilled water in a conical flask corked with cotton wool and foil paper and allowed to dissolve in 1000ml of distilled water in a conical flask. 30ml of the nutrient agar were poured into each MacCartney bottles and then sterilized in an autoclave at 160^0C for 15minutes. After sterilization, the agar was allowed to cool and 1ml of 1×10^5 cfu/ml dilution of the organism (*Pseudomonas aeruginosa*) was seeded into it. The agar were poured into sterile Petri dishes and allowed to set. A cork borer with diameter of 10mm were used to bore holes on the surface of the agar in 3 places into which 0.3ml of each of 500mg/ml, 250mg/ml and 125mg/ml of each of the extracts was poured.
Also, the commercially available antibiotic disc was placed aseptically with the use of a forceps gently at the centre of another nutrient agar plate that has already been seeded with organism. It was then incubated at 37^0 C for 24 hours.

Determination of Minimal Inhibition Concentration
Cold water extracts of bitter kola (*Garcinia kola*), onion bulb (*Allium cepa*), and garlic (*Allium sativum*) were used for the minimum inhibitory concentration (MIC). Having obtained the different dilution and concentrations, three drops of overnight broth cultures of the test organism was inoculated into the different dilutions i.e. 500mg/ml, 250mg/ml and 125mg/ml and incubated at 37^oC for 24 hours. After incubation, the zones of inhibition were measured with the aid of a transparent ruler and recorded.

RESULTS

Table 1 indicates that the organism (*Pseudomonas aeruginosa)* isolated from all the farms reacted negatively to gram reaction; positively to citrate utilization test; positively to Catalase test; negatively to coagulase test; positively to motility test and negatively to indole test, while for sugar fermentation test using glucose, lactose and sucrose, acid was produced. Table 2 indicates the mean bacteria count recovered from the gill, mouth, and skin. In Ogun state, the highest mean bacteria count was recorded as 6.133×10^3cfu/ml while the lowest count was recorded as 3×10^2cfu/ml, Oyo state recorded the highest mean bacteria count of 5.35×10^3cfu/ml, while the lowest count was 1.6×10^2cfu/ml and Lagos state recorded the highest mean bacteria count as 1.7×10^5cfu/ml while the lowest count was recorded as 1×10^2cfu/ml. In this study*, Pseudomonas aeruginosa,* gram negative bacteria was recovered from the gills, buccal cavity and skin of juvenile *Clarias gariepinus.* This shows that the bacteria count vary from each state. Lagos state was observed to have the highest bacteria count of 3.89×10^4cfu/ml and 1.0×10^2cfu/ml as the lowest count in the gill. From the buccal cavity, the highest bacteria count was recorded as 6.9×10^3cfu/ml in Lagos state and the lowest bacteria count was recorded as 1.0×10^2cfu/ml in Oyo state. In the skin, the highest bacteria count was recorded as 7.5×10^3cfu/ml from Lagos state, while the lowest bacteria count in the skin was recorded as 9×10^2cfu/ml in Ogun state. Table 3 indicates the zones of inhibition of the plant extracts. In all the farms, Onion and Garlic were the most effective plant extracts with the highest zone of inhibition at 500mg/ml, less size of the cork borer (10mm) for the gram negative bacteria (*Pseudomonas aeruginosa*). Table 4 indicates the zones of inhibition of the antibiotics. Only Gentamycin at 10mg/ml and Colistin at 25mg/ml had inhibition zone while Tetracycline, Ampiciline, Cotraimozazole Nitrofurantoin and Streptomycin had no zone of inhibition.

Table 1: Morphological, biochemical and identification of (*Pseudomonas aeruginosa*) isolated from juvenile *Clarias gariepinus*

States	Farms	Gram	Citrate	Catalase	Coagulase	Motility	Indole	Sugar fermentation tests		
								Glucose	Lactose	Sucrose
Ogun	Eweje	-ve	+ve	+ve	-ve	+ve	-ve	A	A	A
	UNAAB	-ve	+ve	+ve	-ve	+ve	-ve	A	A	A
	Ultimate	-ve	+ve	+ve	-ve	+ve	-ve	A	A	A
	Ikililu	-ve	+ve	+ve	-ve	+ve	-ve	A	A	A
	Larry	-ve	+ve	+ve	-ve	+ve	-ve	A	A	A
Oyo	Hope	-ve	+ve	+ve	-ve	+ve	-ve	A	A	A
	Kulturetek	-ve	+ve	+ve	-ve	+ve	-ve	A	A	A
	Durante	-ve	+ve	+ve	-ve	+ve	-ve	A	A	A
	Zamits	-ve	+ve	+ve	-ve	+ve	-ve	A	A	A
	Crams	-ve	+ve	+ve	-ve	+ve	-ve	A	A	A

	Glorious	-ve	+ve	+ve	-ve	+ve	-ve	A	A	A
	Feedvine	-ve	+ve	+ve	-ve	+ve	-ve	A	A	A
Lagos	Palmroyal	-ve	+ve	+ve	-ve	+ve	-ve	A	A	A
	Quicklink	-ve	+ve	+ve	-ve	+ve	-ve	A	A	A
	Palmwisdom	-ve	+ve	+ve	-ve	+ve	-ve	A	A	A

Key:
Negative = -ve
Positive = +ve
Acid production = A

Table 2: Bacteria count from juvenile *Clarias gariepinus* in cfu/ml

States	Farms	Gill	Buccal Cavity	Skin	Mean and Standard Error
	Eweje	3×10^{2}	Nil	Nil	$3\times10^{2}\pm 0.00$
	UNAAB	6.2×10^{3}	5.8×10^{3}	6.4×10^{3}	$6.133\times10^{3}\pm 1.76$
Ogun	Ultimate	Nil	4.8×10^{3}	9×10^{2}	$2.850\times10^{3}\pm 1.97$
	Ikililu	1.4×10^{3}	Nil	Nil	$1.4\times10^{3}\pm 0.00$
	Laary	Nil	1.8×10^{3}	3.6×10^{3}	$2.7\times10^{3}\pm 9.00$
	Hope	Nil	Nil	Nil	0.00 ± 0.00
	Kulturetek	1.06×10^{4}	1.0×10^{2}	Nil	$5.35\times10^{3}\pm 5.25$
Oyo	Durante	Nil	1.8×10^{4}	Nil	$1.8\times10^{2}\pm 0.00$
	Zamits	Nil	Nil	1.6×10^{3}	$1.6\times10^{2}\pm 0.00$
	Crams	2.44×10^{4}	Nil	Nil	$2.4\times10^{2}\pm 0.00$
	Glorious	3.89×10^{4}	6.9×10^{3}	7.5×10^{3}	$1.7\times10^{5}\pm 1.05$
	Feedvine	1.0×10^{2}	Nil	Nil	$1\times10^{2}\pm 0.00$
Lagos	Palmroyal	Nil	Nil	Nil	0.00 ± 0.00
	Quicklink	5.2×10^{3}	Nil	Nil	$5.2\times10^{2}\pm 0.00$
	Palmwisdom	Nil	Nil	Nil	0.00 ± 0.00

Table: 3 Antimicrobial Susceptibility Test of Garlic, Onion and Bitter Kola on *Pseudomonas aeruginosa* using Different Concentrations (mg/ml)

States	Farms	Onion			Garlic			Bitter kola		
	Concentrations	500	250	125	500	250	125	500	250	125
	Eweje	4	Nil	Nil	4	Nil	Nil	Nil	Nil	Nil
	UNAAB	5	Nil	Nil	5	Nil	Nil	Nil	Nil	Nil
Ogun	Ultimate	5	Nil	Nil	4	Nil	Nil	Nil	Nil	Nil
	Ikililu	4	Nil	Nil	5	Nil	Nil	Nil	Nil	Nil
	Larry	4	Nil	Nil	4	Nil	Nil	Nil	Nil	Nil
	Hope	5	Nil	Nil	4	Nil	Nil	Nil	Nil	Nil
	Kulturetek	5	Nil	Nil	5	Nil	Nil	Nil	Nil	Nil
Oyo	Durante	4	Nil	Nil	4	Nil	Nil	Nil	Nil	Nil
	Zamits	4	Nil	Nil	4	Nil	Nil	Nil	Nil	Nil
	Crams	5	Nil	Nil	5	Nil	Nil	Nil	Nil	Nil
	Glorious	5	Nil	Nil	4	Nil	Nil	Nil	Nil	Nil
	Feed vine	4	Nil	Nil	4	Nil	Nil	Nil	Nil	Nil
Lagos	Palm royal	5	Nil	Nil	5	Nil	Nil	Nil	Nil	Nil
	Quick link	4	Nil	Nil	4	Nil	Nil	Nil	Nil	Nil
	Palm wisdom	5	Nil	Nil	5	Nil	Nil	Nil	Nil	Nil

Table 4: Antimicrobial Susceptibility Test of Antibiotics on *Pseudomonas aeruginosa* using Different concentration

States	Farms	Zone of inhibition (mm)							
		Tet (25mg)	Amp (25mg)	Cot (25mg)	Gen (10mg)	Nal (30mg)	Nit (25mg)	Col (25mg)	Str (25mg)
Ogun	Eweje	Nil	Nil	Nil	6	Nil	Nil	6	Nil
	UNAAB	Nil	Nil	Nil	5	Nil	Nil	6	Nil
	Ultimate	Nil	Nil	Nil	5	Nil	Nil	5	Nil
	Ikililu	Nil	Nil	Nil	6	Nil	Nil	6	Nil
	Larry	Nil	Nil	Nil	5	Nil	Nil	7	Nil
Oyo	Hope	Nil	Nil	Nil	7	Nil	Nil	6	Nil
	Kulturetek	Nil	Nil	Nil	6	Nil	Nil	5	Nil
	Durante	Nil	Nil	Nil	6	Nil	Nil	6	Nil
	Zamits	Nil	Nil	Nil	7	Nil	Nil	5	Nil
	Crams	Nil	Nil	Nil	6	Nil	Nil	6	Nil
Lagos	Glorious	Nil	Nil	Nil	5	Nil	Nil	6	Nil
	Feedvine	Nil	Nil	Nil	7	Nil	Nil	6	Nil
	Palmroyal	Nil	Nil	Nil	6	Nil	Nil	6	Nil
	Quicklink	Nil	Nil	Nil	5	Nil	Nil	5	Nil
	Palmwisdom	Nil	Nil	Nil	6	Nil	Nil	5	Nil

Less size of cork borer (10mm)

Tet – Tetracycline
Nit – Nitrofuranton
Cot – Cotraimozazole
Str – Streptomycin
Nal – Naladixic Acid
Amp – Ampiciline
Col – Colistin
Gen – Gentamycin

DISCUSSION

In the study, a bacterium (*Pseudomonas aeruginosa*) was recovered from the gill, buccal cavity and skin of *Clarias gariepinus* juvenile. The highest bacteria count was detected in the gill (3.89×10^4 cfu/ml).This confirmed the findings of Sugita *et al.* (1988) - he confirmed that fish take a large number of bacteria into their gill from the water sediment. The isolated organism was a gram negative *(Pseudomonas aeruginosa).* The biochemical test indicated that *Pseudomonas aeruginosa* reacted negative to gram reaction, positive to citrate utilization test, positive to catalyze test, negative to coagulase test, positive to motility test, negative to indole test and produced acid in sugar fermentation test using glucose, lactose and sucrose sugar. The result of the present study revealed the prevalence of the highest antibiotic and plant extract sensitivity (i.e. the widest inhibition zone in the gram negative bacterium isolated from the gill, skin and buccal cavity of *Clarias gariepinus* juvenile). For the plant

extracts, the highest inhibition zone was observed for onion and garlic at 500mg/ml to be effective. This is because garlic has been used as medicine in many cultures for thousands of years (Marryland, 2005) and also for its inhibitory effect (Banerjee and Maulik, 2002). Onion is also a good antibacterial plant that showed zone of inhibition because of the flavonoids compound produced by onion e.g. quercetin. Flavonoids are chemical compounds active against microorganisms. Flavonoids have been found in-vitro to be effective antimicrobial substances against a wide array of microorganisms Ekwenye *et al.* (2005). Among the antibiotics used, Gentamycin at 10mg had inhibition zone, and Colistin at 25mg while no zone of inhibition was observed using Tetracycline at 25mg, Ampiciline at 25mg, Cotraimozazole at 25mg, Naladixic acid at 300mg, Nitrofurantoin at 25mg and Streptomycin. This supports the findings of Banerjee and Maulik (2002), who confirmed garlic as a medicinal plant which has inhibitory effect on many microbes.

CONCLUSION

This study provided information on the bacteria (*Pseudomonas aeruginosa*) sensitivity to three (3) plant extracts (*Garcinia kola, Allium sativum, Allium cepa*) and eight (8) antibiotics (Tetracycline, Ampiciline, Cotraimozazole, Gentamycin, Naladixic acid Nitrofurantoin, Colistin and Streptomycin). This study confirms the potency of *Allium cepa* and *Allium sativum* because they showed inhibition zone among the three plant extracts used. Also, among the antibiotics used, only Gentamycin and Colistin had inhibition zone. In conclusion, plant extracts such as onion and garlic should be used as alternative treatments for fish diseases not only because of their antibacterial properties, but because they are natural plants that have no residual effects on fish populace, and they are available in the market.

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