Evaluation of the antibacterial efficacy of seven plant extracts against *Aeromonas* and *Pseudomonas* bacteria of farmed catfish (*Heterobranchus longifilis*)

Albert P. Ekanem,[1]
Aniema P. Inyang-Etoh,[1]
Paul C. Inyang-Etoh[3]

[1]Department of Fisheries and Aquaculture, University of Calabar, Nigeria
[2]Department of Medical Laboratory Sciences, University of Calabar, Nigeria

Abstract

Aeromonas and Pseudomonas diseases are responsible for mortalities of farmed *Heterobranchus longifilis* in Nigeria. The objective of the study is to investigate the efficacies of extracts of some medicinal plants against Aeromonas and Pseudomonas pathogens of *H. longifilis*. Ethanol extracts of *Phyllanthus amarus, Allium sativum, Artemisia annua, Citrus limon, Moringa oleifera, Allium cepa* and *Azadirachta indica* were tested against *Aeromonas hydrophila* and *Pseudomonas flourescens* of *H. longifilis* by disc diffusion assay. Extracts of *P. amarus, A. sativum, A. annua* and *C. limon* more ($P<0.05$) sensitive to *A. hydrophila* and *P. flourescens* than *M. oleifera, A. cepa* and *A. indica* which were effective ($P<0.05$) against *P. flourescens*. Minimum inhibitory concentrations (MIC) of the extracts were 25 mg/mL for *P. amarus* and *A. annua*; 25 and 100 mg/mL for *C. lemon* and *A. cepa* respectively and 50 mg/mL for *A. indica*. Alkaloid was demonstrated in all plants except *A. annua* by qualitative methods. Moderate amount (10%) of cardiac glycosides was demonstrated in *A. sativum, M. oleifera* and *P. amarus*. Saponin (15%) was present in *M. oleifera* and *A. indica*, while tannin (10%) was present in *M. oleifera, P. amarus* and *A. indica*. Phlobatanins and Anthraquinones (10%) were present in *P. amarus* and *M. oleifera* respectively.

Extracts of aforementioned plants have potentials as therapy against Aeromonas hydrophila and *Pseudomonas flourescens* of farmed catfish.

Introduction

Antimicrobial resistance has become a global public health problem impacted both by human and non-human microbial usage.[1-3] There is an increasing demand for medicinal plants and plant products as alternative to orthodox medicines especially in developing countries.

Artemisinin, the main active principle in *A. annua*, is now available as an antimalarial drug against drug-resistant strains of Plasmodium specie.[4] It has recently been reported as being efficacious against monogenean parasites of fish.[5] *A. sativum* has been used throughout recorded history for both culinary and medicinal purposes. Lemon is an excellent preventative medicine. The fruit is rich in vitamin C, which helps the body to fight off infections and also to prevent or treat scurvy.[6] Lemons contain unique flavonoid compounds that have antioxidant and anti-cancer properties.[7] Lemon essential oil in vapour form has been found to reduce stress in mice.[8]

M. oleifera is a rich source of minerals, iron, vitamins A, B, and C, calcium and protein.[9] Every parts of Neem tree have been used as traditional medicine for household remedy against various human ailments. Recently, biological activities and medicinal properties of Neem have been extensively reviewed.[10] *P. amarus* is a member of the Euphorbiaceae family which groups over 6500 species in 300 genera.[11] Bacterial diseases of fish are caused primarily by Gram-negative bacilli. This research is focused on the antibacterial activity of various plant extracts against two important bacterial pathogens (*Aeromona* and *Pseudomonas*) of *H. longifilis* under culture conditions.

Materials and Methods

Collection of fish and plant samples

The fish samples of average weight of 100 g used for this study were collected from a catfish farm in Calabar, Nigeria. Fish with obvious signs of bacterial diseases were selected and acclimated in holding tanks for 24 h before the experiments.

The plants used for this study, *Citrus lemon* (*C. limon*), *Allium cepa* (*A. cepa*), *Allium Sativum* (*A. sativum*), *Moringa oleifera* (*M. oleifera*), *Azadirachta indica* (*A. indica*) leaves, *Artemisia annua* (*A. annua*) and *Phyllanthus amarus* (*P. amarus*) were collected from the botanical garden of the Department of Botany, University of Calabar, Nigeria. The choice of plants selected for the study was informed by their medicinal history in human and veterinary medicine.[12] Herbarium samples of all plants used were deposited in the Herbarium of the department of Botany, University of Calabar after identification and confirmation of species.

Correspondence: Dr. Albert P. Ekanem, Department of Fisheries and Aquaculture, University of Calabar, PMB 1115 Eta Agbo Road, Calabar, Nigeria.
Tel: +234.8037928430.
E-mail: albertekanem@yahoo.com

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Contribution: APE, plant extract efficacies against bacterial pathogens, data interpretation, final write up, arrangement and presentation of the manuscript; API, collection, identification and extraction of the medicinal plants, responsible for the isolation and identification of the bacterial pathogens from the fish samples, data analysis; PCI, plants phytochemical screening, data statistical analysis.

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Preparation of extracts and juice

The fresh leaves were washed under running tap water, air dried for 24 h and oven-dried at 60°C for 24 h before they were ground to powder using manual blender. Extraction of the plants was done using soxhlet method with 70% of ethanol as solvent. The filtrates were concentrated in a rotary evaporator at 45°C and the extracts were kept in sterile bottles under refrigerated conditions until use.

Citrus limon, A. cepa and *A. sativum* were washed separately in tap water and rinse in distilled water. *C. limon* fruits were squeezed in sterilized juicer into sterile bottles and kept under refrigerated conditions. *A. cepa* and *A. sativum* bulbs were chopped into pieces and then blend in a manual grinder, extracted in a small quantity of sterile distill water and filtered through 0.45 millipore filter into sterile bottle and also kept in a refrigerator.

Processing of sample

Ten fish samples with obvious signs of dis-

ease (moribund swimming behavior, weakness and ulceration) were sacrificed by cardiac puncture and the body cavity slit opened under aseptic conditions to expose the internal organs. Disinfection of the surfaces of the fish was by swabbing with 70% alcohol. Dissecting instruments were sterilized by dipping in 70% alcohol and flamed before use. An incision was made through the body wall in the mid-ventral line opposite the base of the pectoral fins. Blunt-ended scissors was used to extend the incision anteriorly to the symphysis of the mandible and posterior to the vent, taking care not to puncture the intestine.

Isolation and identification of bacteria

Nutrient agar was used to isolate bacteria for the culture. With the help of a culture loop and a heated blade, samples were taken from the skin, kidney, gall bladder, spleen and liver. Inocula of the internal organs for culture were obtained by scaring the exposed surface with a scalped blade. A sterile inoculating loop was inserted through the sterilized area and the resultant inoculum streaked upon the nutrient agar plate and then the plates incubated for 48 h at 37°C. The bacteria were sub cultured on nutrient agar slant for the isolation of pure culture. Isolates were identified using standard cultural, microscopic and standard biochemical methods such as motility test, gram staining, oxidase test, oxidation fermentation test, indole test, catalase test, gelatin liquefaction test, citrate utilization, esculin hydrolysis, urease activity, decarboxylase reactions and hydrogen sulphide production tests.

Preparation of antimicrobial discs

A 6 mm diameter plunger was used to punch a whatman no.1 absorbent filter paper to obtain 6 mm diameter paper discs. The discs were properly labeled for identification purposes and then sterilized by autoclaving for 15 min at 121°C. The disc were impregnated with the plant extracts (0.5 g/mL), dried and stored off in sterile bottles.

Antimicrobial test by discs diffusion method

Antimicrobial activity was tested using a modified discs diffusion assay (DDA) method;[13] 0.5 g of each plant extract was dissolved in 0.5 mL of DMSO (dimethyl sulfoxide) and 10 mL of treated water was added to make up the stock solution. The inoculums for each microorganism were prepared from broth cultures (10^5 CFU/mL). A loop of culture from the nutrient agar (NA) slant stock was cultured in Mueller Hinton medium overnight and spread with a sterile swab into eight Petri-plates. Each bacterial swab was spread on separate plate. Sterile disc (6 mm in diameter) impregnated with the plant extracts were placed on the cultured plates and incubated for 24 h at 37°C. The solvent loaded disc without extracts served as control in the study. The results were recorded by measuring the zones of growth inhibition. Clear inhibition zones around the discs indicated the presence of antimicrobial activity. All data on antimicrobial activity were average of triplicate.

Determination of minimum inhibitory concentration of the extracts on the test organisms by double dilution method

The initial concentration of each of the plant extract (100 mg/mL) was diluted using double fold dilution by transferring 0.5 g of the sterile plant extract (stock solution) into 5 mL of sterile distilled water to obtain 50 mg/mL concentration. The above process was repeated several times to obtain other dilutions: 25 mg/mL, 12.5 mg/mL, 6.25 mg/mL and finally 3.13 mg/mL.[14] Having obtained the different concentrations of the extracts, each concentration was inoculated with 0.1 mL of the standardized bacterial cell suspension and incubation was done at 37°C for 24 h. The growth of the inoculums in the broth was indicated by turbidity or cloudiness of the broth and the lowest concentration of the extract which inhibited the growth of the test organism were taken as the minimum inhibitory concentration (MIC). Negative controls were set up as follows: sterile water only, sterile water and sterile plant extract and finally positive control containing sterile water and the test organism. The process was replicated three times.

The results of the sensitivity tests were expressed as – (0) for no sensitivity, + (5) for low sensitivity, ++ (10) for moderate sensitivity and +++ (15) for high sensitivity.

Phytochemical screening

The extracts were screened for phytochemical constituents using standard procedures of analysis.[15-17]

Statistical analysis

The homogeneity of the three samples of the replicates was checked by the Kruskal-Wallis test before data of the replicates were pooled together and treated as one. The data were further analyzed by the analysis of Variance (ANOVA). Significance was accepted when $P<0.05$.

Results

The results of the sensitivity tests are shown on Table 1. The two isolated fish pathogens (*A. hydrophila* and *P. fluorescens*) were sensitive to *P. amarus*, *A. sativum*, *A. annua* and *C. limon*. However, *P. fluorescens* was weakly sensitive to *A. cepa* and *A.indica*., while *M. oleifera* was weakly sensitive against both fish pathogens.

The MIC results are depicted in Figure 1. There were significant differences ($P<0.05$) in the minimum inhibitory concentrations of the seven plant extracts against both *A. hydrophila* and *P. flourescens*.

Phytochemical screening of plant extracts

The results of phytochemical screening are shown in Figure 2. Phytochemical screening of the ethanolic extract of the seven plants shows that *A. sativum*, *A. cepa, C. limon*, *M. oleifera* and *A. indica* indicated strong (++) alkaloids presence. *A. annua* indicated slight (+) alkaloids presence whereas *P. amarus* indicated very strong (+++) alkaloids presence. *A. sativum*, *M. oleifera* and *P. amarus* indicated strong (++) cardiac glycosides presence while *A. cepa, C. limon*, *A. annua* and *A. indica* indicated slight (+) cardiac glycosides presence. *A. sativum* and *A. annua* showed slight (+) saponins presence, whereas *A. cepa* and *Citrus limon* showed no saponin presence. However, saponin was very strongly (+++) indicated in *M. oleifera* and *A. indica* while *P. amarus* had strong indication of saponin presence in the extract.

Table 1. Sensitivity test result of *A. hydrophila* and *P. flourescens* to seven plant extracts.

Plant extracts	Inhibitory response of extracts on bacterial growth	
	A. hydrophila	*P. fluorescens*
Phyllanthus amarus	++	++
Allium sativum	++	++
Allium cepa	-	+
Artemisia annua	++	++
Citrus limon	++	++
Moringa oleifera	+	+
Azadirachta indica	++	+

>15%, high sensitivity (+++); 10-15%, sensitivity (++); 10-5%, low sensitivity (+); <5%, no sensitivity (-).

There were no indication of the presence of tannin in *A. sativum*, *A. cepa* and *A. annua*, but *C. limon* had slight (+) indication of tannins; *M. oleifera* and *P. amarus* indicated strong (++) tannins presence while *A. indica* showed very strong (+++) tannins presence. Phlobatannins was not indicated in *A. sativum*, *C. limon* and *A. annua*, but was slightly (+) indicated in *A. cepa*, *M. oleifera* and *A. indica* while it presence was strongly (++) indicated in *P. amarus*. Anthraquinones was not indicated in *A. sativum*, *A. cepa, C. limon* and *A. annua*, but it was strongly indicated in *M. oleifera* while *P. amarus* and *A. indica* showed slight indication of its presence in the extract.

Discussion

Some of the extracts of plants tested in this study were effective against the two pathogenic bacteria (*A. hydrophilla* and *P. fluorescens*) of *H. longifilis* as shown in the results. Although the activities of some of the plants used in this study have been reported by several applications in animals and human models but their application have not been reported in any aquatic animal including *H. longifilis*. The antimicrobial potentials of *P. amarus* in human have already been reported.[18] However, in this study *A. hydrophila* and *P. fluorescens* of *H. longifilis* were sensitive to *P. amarus* with minimum inhibitory concentration of 25 mg/mL.

The present study has shown that both organisms (*A. hydrophila* and *P. fluorescens*) were sensitive to *A. sativum* thereby demonstrating broad-spectrum activities with minimum inhibitory concentration of 12.5 mg/mL. Consistently with our results, Eja *et al*. assessed the antimicrobial effects of *A. sativum* and two known broad-spectrum antibiotics (Ampicillin and ciprofloxacin) against diarreagenic organisms and had MIC of 12.5 mg/mL whereas those of ciprofloxacin and ampicillin were 8.8 and 4.5 mg/mL, respectively.[19]

A. sativum extract has been known to have inhibitory activities against various pathogenic bacteria including multi-drug resistant (MDR) strains such as *Aeromonas, Aerobacter, Citrobacter, Enterobacter, Escherichia, klebsiella, Lactobacillus, Leuconostoc, Micrococcus, Mycobacterium, Proteus, Providencia, Pseudomonas, Salmonella, Serratia, Shigella, Staphylococcus, Streptococcus* and *Vibrio species*.[20]

The present findings are also in line with the results of Muniruzzaman *et al*.,[21] who studied the sensitivity of plant extracts against three important fish (*H. longifilis*) bacterial pathogens (*A. hydrophila, P. fluorescens* and *E. tarda*). Among the eight species of high inhibitory responded plants *A. sativum* showed the highest antibacterial effect against *A. hydrophila* and *P. fluorescens* and MIC of 0.6 mg/mL was reported whereas, in contrast, the present study revealed MIC of 25 mg/mL. The present study is also in agreement with the studi of Indu and colleagues, who reported excellent antibacterial activity of *A. sativum* against 5 species of bacteria including A. *hydrophila* in India.[22] The results obtained from this work are in disagreement with Cellini *et al*., who reported 90% inhibition of their isolates at 5 mg/mL of *A. sativum* extracts.[23] The highest sensitivity of *A. sativum* at a MIC of 3.[23] mm in a study to compare the antibacterial effects of juices of some

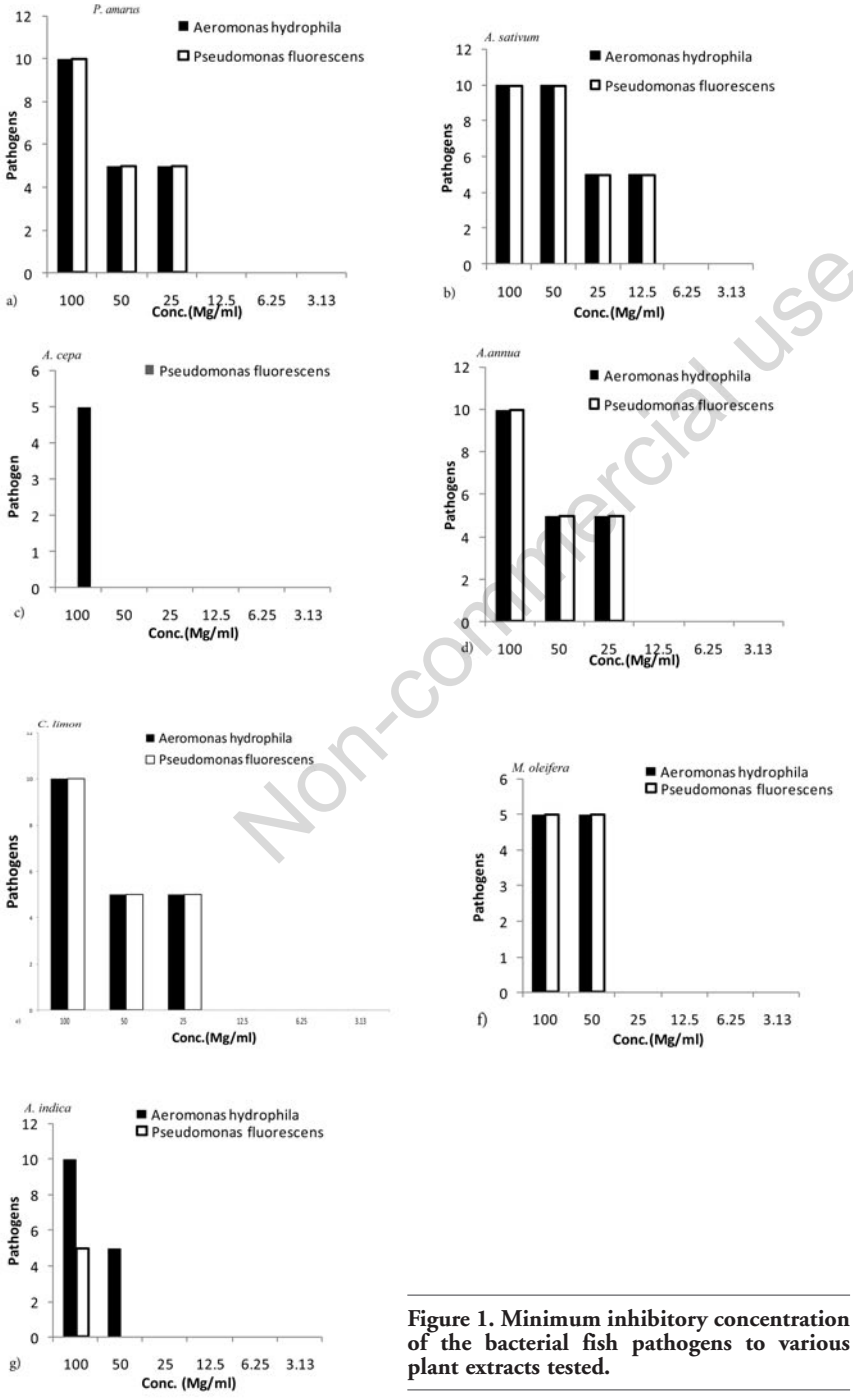

Figure 1. Minimum inhibitory concentration of the bacterial fish pathogens to various plant extracts tested.

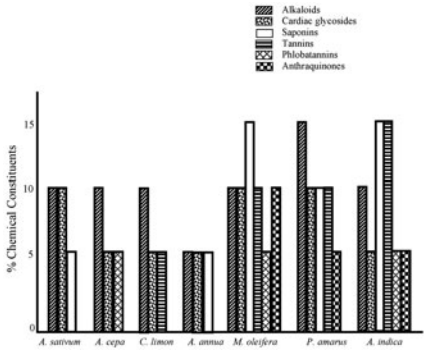

Figure 2. Results of phytochemical screening of plant extracts.

seasonal vegetables and fruits against Gram-positive and Gram-negative bacteria was also reported.[24] These differences might be due, amongst other reasons, to previous exposure of the fish host to antibiotics and the environmental factors affecting the hosts.

The results of the present study shows that *A. cepa* was not effective against *A. hydrophila* but showed very weak activity in the highest test concentration (100 mg/mL) against *P. fluorescens* which in summary presents no activity against the two fish pathogens. This is in strong disagreement with Muniruzzaman *et al*., who reported antibacterial efficacies of *A. cepa* against *A. hydrophila* and *P. flourescens* with MIC of 2.5 mg/mL.[21] It would not have been possible for *A. cepa* to show such wonderful activities against the two pathogens at such low concentration whereas, in our study, the highest concentration (100 mg/mL) did not show any activity against *A. hydrophila* and very low response against *P. flourescens*. Our finding is also supported by Indu *et al.*, who reported lack of sen sitivity of *A. hydophila* to the extract of *A. cepa*.[22]

In this study, the two gram-negative bacteria (*A. hydrophila* and *P. fluorescens*) were sensitive to *A. annua* with MIC of 25 mg/mL. This sensitivity is in line with other studies that reported good microbial activities of *A. annua* against different genera of bacteria.[25,26] This study does not support the results of Verdianrizi *et al*., who reported that Gram-positive bacteria were more sensitive to *A. annua* than Gram-negative, whereas the present study shows that Gram-negative bacteria were very sensitive.[27]

C. limon presented strong sensitivity against *A. hydropilia* and *P. fluorescens* in the present study with an MIC of 25 mg/mL for both. The results are supported by the findings of Hayes *et al*., who reported significant antimicrobial activities of *C. limon* against *Pseudomonas* and other Gram-negative bacteria in their study on the sensitivity of *C. limon* to some bacteria.[28] This study also agrees with Biradar *et al.* report on the effectiveness of plant extracts from *C. limon* against *A. hydrophila* of gold fish by both *in vivo* and *in vitro* studies; the results showed that *C. limon* was effective with 16 mm zones similar to oxytetracyclin.[29] It also confirmed the report of Adedeji *et al*., who demonstrated the efficiency of *C. limon* extracts on *P. aeruginosa* and other bacterial organisms, the results showed that *C. limon* had lethal effect to *P. aeruginosa* and other bacterial organisms with inhibition zones of 7-22 mm in diameter around the colonies.[30]

M. oleifera showed very weak antibacterial activities against *A. hydrophila* and *P. fluorescens* of *H. longifilis* with MIC of 50 mg/mL as shown in the results of the present study This low sensitivity of *M. oleifera* is in conjunction with another study that demonstrated low antibacterial effects of the fresh leaf of *M. oleifera* against *P. aeruginosa* and *S. aureus*,[31] but in disagreement with Vander *et al*., who reported strong antibacterial activity of extract of *M. oleifera* against some organisms including *P. aeruginosa* with zones of inhibition between 9 to 13 mm.[32] However, there is need to confirm their claims by testing the extracts against a wide range of host (animal and fish of different species) under similar environmental situations to justify any conclusions.

The result of this study shows that *A. hydrophila* was moderately sensitive to extract of *A. indica*, while *P. fluorescens* showed low sensitivity with MIC of 100 mg/mL and 50 mg/mL, respectively. This moderate and low sensitivity of *A. indica* leaf extracts disagrees with the study of Muniruzzaman *et al*., who studied the sensitivity of *A. indica* leaf extracts against three fish pathogenic bacteria (*A. hydrophila, P. fluorescens* and *E. tarda*)[21] and the results showed no sensitivity of the extracts against all the organisms tested at various concentrations.

The present study agrees with Rajasekaran *et al*., who evaluated the antimicrobial activity of leaf extracts of *A. indica* against selected Gram -negative and -positive bacterial species and found that the leaf extracts of *A. indica* exhibited significant anti-bacterial activity against all the organisms tested.[33] On the other hand, Chander reported that the water extract of *A. indica* leaves did not only fail to inhibit the growth of *Escherichia coli, Staphylococcus aureus* and *Pseudomonas aeruginosa*, but also enhanced the pigmentation of *P. aeruginosa*.[34] The results of present study also disagree with Odunbaku and colleague, who examined the antibacterial activity of the ethanolic and methanolic extracts of leaves of *A. indica* against 6 human pathogenic bacteria and the results showed that the ethanolic extracts of *A. indica* had more activity at MIC of 300-500 mg/mL than the methanolic extracts of the plants, which was active at a very high MIC of 500-1000 mg/mL.[35]

The various activities shown by the medicinal plants tested against Pseudomonas and Aeromonas of cultured fish in this study may be attributed to some of the biological active substances present in them as indicated by the results of the qualitative screening of the extracts.[36] Alkaloids indicated in reasonable amount in *P. amarus* and in moderate amount in *M. oleifera*, *C. limon, A. cepa, A. indica* and *A. sativum* respectively. This is in support of the study of Houghton *et al*., who isolated alkaloids by column (CC) and thin layer chromatographic (TLC) techniques in some of the plants.[37]

P. amarus, *M. oleifera* and *A. indica* were also indicated as a rich source of saponins, tannins and moderately rich in cardiac glycosides, phlobatanins and anthraquinones. Although, the presence of these secondary metabolites has not been reported by several workers, it can serve as a good source of information for further research in the area.

Diverse opinions of other researchers were also noted in the course of this work from literature regarding activities of some of the plants. These might in addition to other factors be attributed to the extracting solvents used. Some of the workers that reported contrary activities were using water extract and solvents other than ethanol used in the present study. It should be noted that the use of alcohol (ethanol) as an organic solvent for extraction of plant materials provides a higher antimicrobial activities than hexane, ethyl acetate and water.[38] Moreover, some of the fish may have been previously exposed to some antibiotic treatment which may affect subsequent sensitivities of bacteria to other applications. Heavy antibiotic used in aquaculture needs to be reduced and replaced with alternative processes for treating fish diseases to avoid the emergence of antibiotic resistance in pathogenic and environmental bacteria.[39,40]

The results from this study have demonstrated that preparations from medicinal plants have potentials for the control of *A. hydrophila* and *P. fluorescens* in cultured catfish (*H. longifilis*). The high host tolerance to the plant extracts is also an indication that a reliable therapeutic regime could be developed from herbal products for the treatment of aquaculture diseases.

References

1. World Organisation for Animal Heath, WHO, FAO. Joint FAO/OIE/WHO 1st Expert workshop on non-human antimicrobial usage and antimicrobial resistance: scientific assessment. Geneva, Switzerland, 1-5 December 2003. http://www.who.int/foodsafety/publications/micro/en/report.pdf
2. WHO. Monitoring of antimicrobial usage in food animals for the protection of human health. Report of a WHO consultation. 10-13 September 2001, Oslo, Norway. Available from: http://www.who.int/emc/diseases/zoo/antimicrobial.html
3. Henning S. Antimicrobial drug resistance in fish pathogens. In: FM Aarestrup (ed.) Antimicrobial Resistance in Bacteria of Animal origin, pp 213-38. ASM Press, Washington, DC, USA, 2006.
4. Bailey LH, Bailey EZ. Hortus third. Macmillan Publ. Co., New York, 1976.
5. Ekanem AP, Brisibe EA. Effects of Ethanol Extract of Artemisia annua Against Monogenean Parasites of Heterobranchus longifilis. Parasitol. Res. 2010;101:1135-9.

6. Chevalier A. The encyclopedia of Medicinal Plants Dorling Kindersley, London, UK, 1996.
7. Huxley A, Griffiths M, Levy M. The new RHS dictionary of gardening. Macmillan Press, London, UK, 1992.
8. Komiya M, Takeuchi T, Harada E. Lemon oil vapor causes an anti-stress effect via modulating the 5-HT and DA activities in mice. Behav. Brain Res. 2006;172:240-9.
9. Abdulkarim SM, Long K, Lai OM, et al. Some physio-chemical properties of Moringa oleifera seed oil extracted using solvent and aqueous enzymatic methods. Food Chem. 2005;93:253-63.
10. Biswas K, Chattopadhyay I, Banerjee RK, Bandyopadhyay U. Properties of neem (Azadirachta indica). Curr. Sci. 2002;82: 1336-45.
11. Chevallier A. Encyclopedia of Herbal Medicine: Natural Health, 2nd ed. Dorling Kindersley Book, London, UK, 2000.
12. Kumar NM, Abdul Khader JBM, Rangaswami P, Irulappan I. Introduction to Spices, Plantation Crops, Medicinal and Aromatic Plants. Oxford and IBH Publishing Co. Pvt. Ltd., New Delhi, India, 1997.
13. Ncube NS, Afolayan AJ, Okoh A. Assessment techniques of antimicrobial properties of natural compounds of plant origin: current methods and future trends. Afr. J. Biotech. 2008;12:1797-806.
14. Ibekwe VI, Ubochi KC, Ezeji EU. Effect of organic nutrient on microbial utilization of hydrocarbons on crude oil contaminated soil. Afr. J. Biotech 2006;5:983-6.
15. Harborne JB Phytochemistry. Academic Press, London, UK, 1993.
16. Sofowora H. Screening Plants for Bioactive Agents In: Medicinal Plants and Traditional Medicine in Africa, 2nd ed. Spectrum Books Ltd., Ibadan. Nigeria, 1993.
17. Trease GE, Evans WC. Pharmacology, 15th edn. Saunders Publishers, London, UK, 2002.
18. Mazumder A, Mahato A, der Mazum R. Antimicrobal potentiality of P. amarus against drug resistant pathogens. Nat. Production Res. 2006;20:323-6.
19. Eja ME, Asikong BE, Abriba C, et al. Comparative Assessment of the Antimicrobial Effects of Garlic (Allium Sativum) and Antibiotics On Diarrheagenic Organisms. Southeast Asian J. Trop. Med. Public Health 2007;38: 343-8.
20. Reuter HD, Koch HP, Lawson DL. Therapeutic Effects and Applications of Garlic and its preparations. In: Garlic: The Science and Therapeutic Applications of Allium Sativum L. and Related species, 2nd ed., pp 135-212. Williams & Wilkins, Baltimore, USA, 1996.
21. Muniruzzaman M, Chowdhury MBR. Sensitivity of fish Pathogenic Bacteria To various Medicinal Herbs. Bangl. J. Vet. Med. 2004 2:75-82.
22. Indu MN, Hatha AAM, Abirosh C, et al. Antimicrobial activity of some of the South Indian spices against Serotypes of Escherichia Coli, Salmonella, Listeria Monocytogenes and Aeromonas hydrophila. Braz. J.Microbiol. 2006;37:153-8.
23. Cellini L, Di Campli E, Masuli M, et al. Inhibition of Helicobacter pylori by garlic extract (Allium Sativum). FEMS Immunol. Med. Microbiol. 1996;13:273-7.
24. Saeed S, Tariq P. Effects of Some Seasonal Vegetables and Fruits on the Growth of Bacteria. Pak. J. Biol. Sci. 2006;9:1547-51.
25. Cha JD, Jeong MR, Choi HJ, et al. Chemical Composition and antimicrobial activity of the essential oil of Artemisia Lavandu laefolia. Planta Med. 2005;71:575-7.
26. Zhang YJ, Yao YL, Yang L, et al. Studies on the Chemical Constituents of the essential Oil of Artemisia dracunculus. Zhongguo Zhong Yao Za Zhi. 2005;30:594-6.
27. Verdian-rizi MR. Chemical Composition and Antimicrobial Activity of the Essential Oil of Artemisia annual L. From Iran. Pharmac. Res. 2009;1:21-4.
28. Hayes AJ, Markovic B. The toxicity of Backhousia Citriodora (Lemon myrthle). Antimicrobial and invitro cytoxicity: Food and Chem. Toxicol. 2002;40:535-43.
29. Biradar SS, Groud N, Rajendra N et al. In vitro and in vivo antibacterial studies of medicinal plant on motile Aeromonad speticemia in fish caused by Aeromonas hydrophila. J. Fish. Aq. Sci. 2007;2:417-21.
30. Adedeji GB, Fagade OE, Oyelade AA. Prevalence of Pseudomonas aeruginosa in clinical samples and its sensitivity to citrus extract. Afr. J. Biom. 2007;10:183-7.
31. Eilert U, Wolters B, Nahrstedt A. The antibiotic Principle of seeds of Moringa Oleifera and Moringa Stenopetata. Planta Med 1981;42:55-61.
32. Vander BDA, Vlietnck. Screening methods for antibacterial and antiviral agents from higher plants. In: K. Hostettman (ed.) Assay for Bioactivity, pp 47-69. Academic Press, London, UK, 1991.
33. Rajasekaran C, Meignanam E, Vijayakumar V et al. Investigations on antibacterial activity of leaf extracts of Azadirachta indica A. Juss (Meliacea): a traditional medicinal plant of India. Ethnobotanical leaflets 2008;12:1213-7.
34. Chander J. Antimicrobial activity of the neem oil and water extract of neem leaves against the standard strains of bacteria. Res. Bull. Panjab Univ. Sci. 1995;45:105.
35. Odunbaku OA, Ilusanya OA. Antibacterial activity of the ethanolic and methanolic leaf extracts of some tropical plants on some human pathogenic microbes. Res. J. Agr. Biol. Sci. 2008;4:373-6.
36. Waterman PG. Searching for bioactive compounds: various strategies. J. Nat. Prod. 1990;53:13-22.
37. Houghton PJ, Woldemariam TZ, O'Shea S, Thyagarajan SP. Two securinega-type alkaloids from P.amarus. Phytochem. 1996;43: 715-717.
38. Febles CI, Arias A, Gil-Rodrigez MC, et al. In vitro study of antimicrobial activity in algae collected from the coast of Tenerife. Anuario del Instituto de Estudios Canarios 1995;34:191-2.
39. Sørum H, L'Abée-Lund TM. Antibiotic resistance in food-related bacteria - a result of interfering with the global web of bacterial genetics. Int. J. Food. 2002;78:43-56.
40. Cabello FC. Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. Environ. Microbiol. 2006;8:1137-44.