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

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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 fluorescens 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. fluorescens than M. oleifera, A. cepa and A. indica which were effective (P<0.05) against P. fluorescens. Minimum inhibitory concentrations (MIC) of the extracts were 25 mg/mL for P. amarus and A. annua; 25 and 100 mg/mL for C. limon 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.

Extraction of aforementioned plants have potentials as therapy against Aeromonas hydrophila and Pseudomonas fluorescens of farmed catfish.

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. 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.

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 flame 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 scraping 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, 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) method13 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 plant extract were made through the body wall in the mid-ventral line and 10 mL of treated water was added to make up the stock solution. The inoculums for each plant extract were made through the body wall in the mid-ventral line. The inoculums of the internal organs for culture were obtained by scoring 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, fermentation test, indole test, catalase test, gelatin liquefaction test, citrate utilization, esculin hydrolysis, urease activity, decarboxylase reactions and hydrogen sulphide production tests.

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. 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. fluorescens.

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. fluorescens to seven plant extracts.

<table>
<thead>
<tr>
<th>Plant extracts</th>
<th>Inhibitory response of extracts on bacterial growth</th>
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<tbody>
<tr>
<td></td>
<td>A. hydrophila</td>
</tr>
<tr>
<td>Phylanthus amarus</td>
<td>++</td>
</tr>
<tr>
<td>Allium sativum</td>
<td>++</td>
</tr>
<tr>
<td>Allium cepa</td>
<td>-</td>
</tr>
<tr>
<td>Artemisia annua</td>
<td>++</td>
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<tr>
<td>Citrus limon</td>
<td>++</td>
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<tr>
<td>Moringa oleifera</td>
<td>+</td>
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<tr>
<td>Azadirachta indica</td>
<td>++</td>
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>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. hydrophila* 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. 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 diarrheagenic organisms and had MIC of 12.5 mg/mL whereas those of ciprofloxacin and ampicillin were 8.8 and 4.5 mg/mL, respectively.

*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.

The present findings are also in line with the results of Muniruzzaman et al., 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 study of Indu and colleagues, who reported excellent antibacterial activity of *A. sativum* against 5 species of bacteria including *A. hydrophila* in India. 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. 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
with another study that demonstrated low antibacterial effects of the fresh leaf of *M. oleifera* against *P. aeruginosa* and *S. aureus*, 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. 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*) and the results showed no sensitivity of the extract 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 antibacterial activity against all the organisms tested. 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*. The results of present study also disagree with Odunbaku and colleague, who examined the antibacterial activity of the ethanolic and methanolic extracts 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.

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. 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.

Reference:


References
18. Mazumder A, Mahato A, der Mazum R.