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Research Article

Microbiological Assessment of *Oreochromis niloticus* Treated with *Calotropis procera* - Silver Nanoparticles

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Abstract

Oreochromis niloticus is a highly nutritious aquatic food with limited shelf life. The mode of preservation of *O. niloticus* has raised concerns about its safety and public health due to food borne illnesses. Studies have shown that chemical preservatives are toxic and harmful to humans thereby leading to growing interest in *Calotropis procera* for its antibacterial properties, and there is limited information on the use of *C. procera* silver nanoparticles (CP-AgNPs) to preserve *O. niloticus*. Hence, this study aimed at evaluating its preservative effect of CP-AgNPs on *O. niloticus*. A total of one hundred and fifty-six samples of adult wild *O. niloticus* (97.41 ± 0.95 g) were sourced from a local river using simple random sampling. The *O. niloticus* fishes were subjected to four different treatments which included: dipping into sterile distilled water, dipping into NaCl solution, dipping into *Calotropis procera* silver nanoparticles (CP-AgNPs) solution and injecting CP-AgNPs. These were allowed to stand for 30 min, thereafter drained and held in clean basket at ambient conditions for 48 h. Samples were taken at 4 h interval for microbiological analysis according to standard methods. The isolated bacteria were identified using 16S rRNA gene sequencing. All analysis was carried out in triplicates with statistical significance set at $P<0.05$. The microbial count showed that CP-AgNPs exhibited antimicrobial and antifungal activities. The best treatment for preservation was the injected CP-AgNPs. Bacteria identified were *Pseudomonas aeruginosa*, *Streptococcus agalactiae*, *Escherichia coli*, *Enterobacter sichuanensis*, *Enterobacter cloacae*, *Staphylococcus aureus*, *Citrobacter portucalensis*, *Klebsiella pneumoniae*, *Klebsiella variicola*, *Proteus mirabilis* and *Aeromonas caviae*. This study concluded that, injecting CP-AgNPs into *O. niloticus* was the best treatment option; however, CP-AgNPs displayed antibacterial activities and preservative effect on *O. niloticus*.

Keywords

Antibacterial, *Calotropis procera*, Natural Food Preservative, *Oreochromis niloticus*, Silver Nanoparticles

1. Introduction

The Nile tilapia (*Oreochromis niloticus*) is a species of tilapia that is indigenous to much of Africa, especially tropical West Africa. Freshwater habitats with a misty surface, such as rivers, streams, canals, lakes, ponds, and irrigation channels, are where it can be found [1]. It is the perfect species for aquaculture in warm water. It is relatively

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disease-free, grows swiftly, can tolerate a range of dietary practices and water conditions, and yields flesh of excellent quality [2, 3]. *Calotropis procera* is a soft-wooded, evergreen plant that is a member of the Apocynaceae family and the Asclepiadaceae subfamily. Bark and leaf extracts of *C. procera* have shown significant antibacterial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Klebsiella pneumoniae* [4]. Despite the many benefits of eating fish and other fish-related products used in industry for human growth and development; the processing and preservation of fish presented unavoidable challenges that led to microbial contamination and spoilage, thereby making the fish unfit for human consumption. According to Qian et al. [5], the application of nanotechnology in food preservation can potentially provide practical answers to the problems caused by products with limited shelf lives by improving their quality and preventing microbial adhesion. Green synthesized nanoparticles (NPs) are more efficient and environmentally benign than those made chemically. Plants provide an excellent platform for the synthesis of nanoparticles because they can create natural capping agents and don't contain any hazardous chemicals. Additionally, the cost-effectiveness of nanoparticle synthesis is increased by the use of plant extracts, which lowers the cost of microorganism isolation and growth [6]. According to Kemala et al. [7], silver nanoparticles (AgNPs) are employed as antibacterial agents since they are the most reactive metal among other metals. Research has also shown that, in comparison to other compounds, AgNPs can effectively interact with a variety of pathogens and regulate a number of plant illnesses [8]. AgNPs are nontoxic, inexpensive, easy to process, sustainable, and beneficial to the environment [9]. It has been demonstrated that *C. procera* leaves contain potent antibacterial qualities as well as phytoconstituents. The leaf's ability to produce NPs has been attributed to its phytochemical composition, which includes fatty acids, ethyl ester, linoleic amino acids, phenolic compounds, flavonoids, and terpenoids [10]. Microbial contamination is the main cause of the deterioration in fish quality. With its demonstrated significant antibacterial and antifungal properties, *C. procera* leaf extract has the potential to be a naturally occurring food preservative. The use of AgNPs from *C. procera* leaf extract to preserve fish is not well documented in the literature. Following extensive testing, the US FDA approved the direct addition of AgNPs to drinking water at a concentration of 100 µg/L to neutralise water-borne microorganisms. Thus, its potential for preventing fish surface-borne infections cannot be compromised by its toxicity. Therefore, the objective of this study is to isolate and identify bacteria from *C. procera* treated and untreated *O. niloticus* samples.

2. Materials and Methods

2.1. Fish Sampling Site

One hundred and fifty-six samples of adult wild *O. niloticus* with average weight of 97.41 ± 0.95 g and average body length of 13.38 ± 0.07 cm were sourced from local fishermen in Oriyanrin river bank in Abeokuta and transported live in open plastic troughs containing clean water to Microbiology Laboratory of Department of Food Technology, Moshood Abiola Polytechnic, Abeokuta where they were treated within two hours of purchase. Samples were made sterile by immersion in water solution of sodium hypochlorite (10 mg/L) for 1 h, and then rinsed twice with sterilized distilled water.

2.2. Collection of Leaf Samples

Fresh *C. procera* leaves was collected from the Botanical garden of Moshood Abiola Polytechnic, Abeokuta, Ogun State. A sample of the plant was authenticated by the Botanists of Enviromental Biology Unit, Science Laboratory Department, School of Science & Technology, Moshood Abiola Polytechnic, Abeokuta, Ogun State.

2.3. Preparation of Aqueous Extract of *C. procera* Leaves

This was carried out according to the modified method of Essien et al. [11]. The leaves were washed with tap water and rinsed with distilled water to remove dust and particle pollutants. They were then size reduced and sun-dried, ground into fine powder and stored in an airtight container for future use. The powder (4.0 g) was mixed with 100 mL of distilled water, sealed, and left for 48 h at room temperature. Afterwards, the mixture was filtered to get the *C. procera* aqueous leaf extract. The resultant extract was then kept at 4 ℃ till further use.

2.4. Synthesis of *C. procera* Silver Nanoparticles (CP-AgNPs)

The synthesis was performed by the method of Dada et al. [12] and Essien et al. [11]. The *C. procera* aqueous leaf extract (1 mL) was added to 9 mL of 10 mM solution of $AgNO_3$. The reaction was incubated at 25^{o}C at 60 rpm for 48 h in the dark condition. A colour change was observed. The solution was centrifuged at 15,000 rpm for 15 min and particles formed washed in deionized water to remove any residual organic compounds present in the AgNPs. The wet particles were dried at 60 ℃ in an oven (NYC-101 oven, FCD-3000 serials, Medical and Scientific, UK) for 12 h, stirred in absolute ethanol to reduce aggregation and then dried at 60 ℃ for 30 min in an oven to expel the solvent.

2.5. Treatment of *O. niloticus*

The fish samples were subjected to four different treatments and allowed to stand for 30 min, thereafter drained and held in clean basket at ambient conditions for 48 h. Samples were taken at 4 h interval for microbiological analysis. Distilled water and NaCl served as controls. All treatments were carried out in triplicates.

Treatment A: Fish samples were dipped into sterile distilled water

Treatment B: Fish samples were dipped into 10 g/L NaCl

Treatment C: Fish samples were dipped into 25 µg/L of CP-AgNPs

Treatment D: Fish samples were injected with CP-AgNPs at 0.1 mL of 2 mg/fish

2.6. Microbiological Studies

2.6.1. Isolation Procedure

Microbiological evaluation of the treated tilapia fish was performed. The Pour plate method was used for the enumeration of colony-forming units. The samples were diluted in 10- fold dilutions following the procedure of Harrigan and McCance [13]. Higher dilutions were plated on already prepared (according to manufacturer's instruction) and sterilized Nutrient agar (NA), Mannitol Salt Agar (MSA), MacConkey Agar (MA), Potato Dextrose Agar (PDA) which was used for total viable aerobic count, *Staphylococci*, coliforms, yeasts and moulds count, respectively. NA. MSA and MA plates were incubated at 35-37^{o}C for 24-48 h while PDA plates were incubated at 25-30^{o}C for 3-5 days. Pure cultures were obtained using streaking method by sub-culturing distinct colonies onto same medium earlier used for isolation. The pure cultures were stored on a slant and preserved at 4^{o}C.

2.6.2. Bacterial Identification Procedure

Identification of the isolates was done using their cultural and morphological characteristics on media, microscopic examination of the isolates under the microscope. Physiological and biochemical tests were also employed with molecular sequencing of DNA to confirm their identification. The shape and motility were observed microscopically.

Gram staining:

A drop of distilled water was placed on a clean grease-free microscopic slide with an already sterilized inoculating loop. A loopful of the colony on the plate was placed on the microscopic slide with the tip of the inoculating loop and smeared. The smear was allowed to air-dry at room temperature after which it was heat-fixed by passing the slide over the flame twice. The smear was flooded with 1% crystal violet for 1 min and washed properly with distilled water. It was stained with Gram's iodine solution for 1 min and washed with distilled water. Ethyl alcohol (75%) was then added and the slide was allowed to stand for 30 sec (the alcohol acted as a decolorizer). The alcohol was washed off with water; the slide was counterstained with safranin for 30 sec, washed off with water and air dried. The slide was observed under the microscope at x 1000 magnification. Grams positive bacteria appeared purple while gram negative bacteria appeared red [14].

Catalase test

The ability of the isolates to decompose hydrogen peroxide to form free oxygen and water was verified. A loopful of the bacteria isolate was placed on a glass slide and few drops of 3% hydrogen peroxide were added according to the method of Seely and Van Demark [15]. Frothing was observed in catalase positive organism.

Citrate utilization test

Simmon's citrate agar was made into slant by dispensing 15.0 ml of the medium into McCartney bottles, autoclaved at 121 ℃ for 20 min and allowed to solidify. The slants were inoculated with the test organism and incubated at 37 ℃ for 96-168 h. Colour change from green to blue indicated positive result while original green colour indicated negative result [16].

Oxidase test

A piece of filter paper was soaked in few drops of oxidase reagent (Tetra-methy-p- phenylenediamine). An inoculum of the test organism was smeared on the impregnated filter paper and observed for colour change. A purple colouration indicated positive result while no colour change indicated oxidase negative results [17].

Coagulase test

A suspension of the 18-24 h of the organism culture was made on a clean slide and a drop of fresh plasma was added and mixed. A positive result is indicated by an immediate clumping of the suspension [18].

Indole test

Ten milliliter (10.0 ml) of sterile tryptone broth was aseptically inoculated with the test isolate, leaving one tube un-inoculated to serve as control. The tubes were incubated at 37 ℃ for 48 h. After incubation, 1.0 ml of chloroform was added to each broth culture and shaken gently. After this, 1.0 ml of Kovac's reagent was added. The tubes were then allowed to stand for about 20 min. Red colour at the top layer indicated positive result implying production of indole while no change in colour indicated a negative result [19].

Methyl red test

The test organism was inoculated into test tubes containing 5.0 ml of sterile MR-VP broth aseptically. The tubes were incubated at 37 ℃ for 48 h. After incubation, 5 drops of methyl red indicator were added to each test tube and the medium was observed for colour change. Red colour indicates positive reaction while yellow colour indicates negative reaction [18].

Voges-Prokauer test

The test organism was inoculated into a test tube containing 5.0 ml of sterile MR-VP broth. After 48 h of incubation, 5 drops of Barrit's A (alpha-napthol) and Barrit's B (potassium hydroxide) was added. A pink-burgundy colour within 20-30

minutes indicates a positive reaction [20].

Urease test

Urea agar (basal medium) was prepared and dispensed into tubes, then sterilized. Glucose and phenol red were added to the basal medium and steamed for 1 hour. The filtered sterilized urea solution was added and all content mixed well and dispensed into sterile test tubes. The test organisms was then inoculated and incubated at 37 ℃ for 24-48 h. Colour change was observed, with a positive urea test being denoted by the change of medium colour from yellow to pinkish-red [21].

Carbohydrate fermentation test

This was determined according to the method of Aryal [22]. Purple broth (which consists of peptone with the pH indicator bromocresol purple) was used for studying carbohydrate fermentation reaction. The medium is allowed to warm to room temperature prior to inoculation. The purple broth (with carbohydrate of choice) was inoculated with isolated colonies from an 18-24 hour pure culture of the organism. A control tube of Purple Broth Base was inoculated in parallel with the carbohydrate based media. The inoculated media were then incubated aerobically at 35-37 ℃ for 3-5 days. A positive result was indicated by a yellow colour while lack of yellow colour development is indicative of a negative carbohydrate fermentation reaction. Gas formation is indicated by the appearance of gas bubbles in the Durham tube.

2.6.3. Molecular Characterization of Probable Bacteria Isolates Obtained from Treated *O. niloticus* Samples

Deoxyribonucleic Acid (DNA) extraction

One millilitre of an overnight bacterial broth culture was initially transferred to a microcentrifuge tube and centrifuged at 14,000 rpm for 30 s to collect the cell pellets. Cells were resuspended by gentle vortexing. Sodium dodecyl sulfate (250 µL) and 12 µL of protenaise K were added to the cell suspension, vortexed and incubated at 55 ℃ for 30 minutes. Then 500 µL of binding solution was added to lysate and vortexed while 750 µL of the mixture was applied to the spin column assembly and centrifuged for 1 minute at 8,000 rpm. The flow-through was discarded and wash solution (500 µL) was added to the column and centrifuged for 1 min at 14,000 rpm. The column was spinned for 2 min in order to thoroughly dry the column and then the collection tube discarded. The spin column (with DNA bound to the column) was assembled with 1.7 mL elution tube. Elution buffer (200 µL) was added to the center of the column bed and centrifuged for 1 min at 6,000 rpm with an additional centrifuging at 14,000 rpm for 2 min to collect the total elution volume. The washed and air-dried DNA pellet was dissolved in DNA-grade water and stored in -20 ^{o}C until used for PCR. The concentration and purity of the extracted genomic DNA were evaluated by agarose gel electrophoresis [23]. The DNA was electrophoresed on 1% agarose gel containing 0.5 µg/ml ethidium bromide. The DNA was visualised by UV transilluminator and photographed.

Preparation of 1% agarose

One percent agarose gel was prepared by dissolving 1 g of agarose in 100 mL of 1X Tris Acetate EDTA (TAE). The solution was heated in a microwave for 3 min and allowed to cool. Ethidium bromide (30 µL) was added and mixed thoroughly. The agarose was then poured into an electrophoresis chamber and allowed to solidify. The samples and DNA ladder were loaded into the wells and the electrophoresis was run at 80 V for 1 hour. Analysis of the genomic isolates was done.

Amplification of 16SrRNA genes

Amplification of 16SrRNA genes was performed under standard conditions using universal selective primers (5pMol forward and backward primer) for detection of bacteria, depending on the amplification efficiency and success. PCR amplification for 16SrRNA was performed in 10 µl volume of the following reagents: sterile water of 2.9 µl, 1.0 µl dNTP (10 µM), 1.0 µl 10x buffer, 0.5 µl forward primer (10 µM), 0.5 µl reverse primer (10 µM), 0.3 µl Taq (homemade) and 1.0 µl of 10x fold dilutions of DNA template. The PCR protocol was as follows: initial preheating stage at 94 ℃ for 5 min, followed by 36 cycles of denaturation at 94 ℃ for 30 s, annealing from 55 ℃ for 30 s, extension at 72 ℃ for 45 s and final extension period for 7 min at 72 ℃.

Gel Electrophoresis

The resulting PCR product was confirmed by 1% agarose gel electrophoresis. The concentration and purity of the purified PCR products were evaluated by 1% agarose gel electrophoresis and by spectrophotometry. The purified PCR products, with a concentration of at least 40ng/µL were sequenced using sequence alignment of the 16S rRNA gene sequences.

Sequence editing and database matching

Bi-directional sequences obtained with forward and reverse primers were edited and aligned to generate a consensus sequence using Bio-Edit sequence Alignment Editor (version 7.1.9) [24]. Consensus sequences were then aligned with sequences deposited in the National Centre for Biotechnological Information (NCBI) gene bank by using the Basic Local Alignment Search Tool (BLAST) to establish identities of the bacterial isolates; a neighbor-joining phylogenetic tree was constructed using MegAlign software.

Statistical analysis

One way analysis of variance was applied to data generated using t-tests for comparison of two parameters. Statistical significance was set at $P<0.05$ using ANOVA. The results obtained from the analyzed data was represented on tables and illustrated in figures.

3. Results and Discusion

Microbial product quality or shelf-life indicators are organisms as well as their metabolic items whose presence in given food sources at specific levels might be utilized to evaluate existing quality or, better, to anticipate product shelf life [25]. Silver nanoparticles (AgNPs) have become an im-

portant application in the field of microbiology such as antibacterial and antifungal activities. Comparing the distilled water, NaCl and AgNPs treatment, AgNPs direct injection treatments exhibited higher antimicrobial activity. Total aerobic counts can be used to measure the level of overall bacterial contamination of food [26]. It was observed from the results that the number of count increased as the duration of storage increased though CP-AgNPs treatment showed highest inhibitory activity. This trend was followed for total aerobic count (Table 1), total Staphylococcus count (Table 2), total coliform count (Table 3), and total fungal count (Table 4). For *Staphylococcus* count, no count was observed in all treatment for 12 h; NaCl treatment exhibited inhibition for up to 16 hours, while dipped and injected treatment gave inhibitory activity for 28 and 36 h respectively. There was significant difference ($P<0.05$) among the treatments, though at the 44 h and 48 h, no significant difference occurred in the CP-AgNPS treated *O. niloticus*. For total coliform count, it was observed from the results that distilled water treatment gave highest coliform counts while the least count was observed in AgNPs direct injection treatments. No count was recorded between 0 and 4 hrs in AgNPs direct injection treatments after which coliform growth was observed. This shows that *C. procera* AgNPs exhibited inhibitory activity against coliform for up to 4 hours. There was significant difference ($P<0.05$) among the treatments. The presence of coliforms indicates fecal contamination and the poor level of hygiene. The trend was expected as signs of spoilage intensified as the duration increases. Coliforms were not supposed to be present in food, however the range observed for CP-AgNPs treatment was still in the permissible limit for raw food. The coliform count in spoiled tilapia can vary widely, but generally, a high coliform count (above 10^4 CFU/g) indicates significant microbial contamination and spoilage. The presence of coliforms suggests a higher risk of pathogens like *Escherichia coli*, *Pseudomonas* contributing to fish spoilage, off-flavors, and unpleasant odors and which could pose a health hazard if the fish is consumed.

The fungi count showed the CP-AgNPs injection treatment having no count for up to 4 hour after which growth was observed. Highest number of count was observed in distilled water treatment while least count was observed in CP-AgNPs treatment. The results of the microbial counts was in line with the reports of De-Freitas et al. [27] and Ishnava et al. [28] where they reported that *C. procera* exhibits anti-microbial activities such as antibacterial, antifungal and anti-candidal activity. Mohamed et al. [29] also reported that latex of *C. procera* has successfully synthesized AgNPs which exhibited good antibacterial activity against Gram-negative bacteria such as *E. coli, Serratia sp. and P. aeruginosa* as well as antifungal activity against dermatophytes and phytopathogenic fungi. Several hypotheses have explained the mechanism of action of AgNPs which include AgNPs ability to attach to the surface of the cell membrane leading to disturbance of permeability and respiration functions of the cell. The strong antimicrobial activity depends on the large surface area of the NPs which give more surface area for interaction with the organisms than available with those for large particles. Other reported mechanisms are: uptake of free silver ions followed by disruption of adenosine triphosphate production and DNA replication, formation of reactive oxygen species and direct damage to cell membranes [29]. Values obtained were statistically different at $P<0.05$. Gram reaction is the ability of the isolates to stain violet due to the presence of a thick layer of peptidoglycan in their cell walls, which retains the crystal violet during gram staining [30]. From the results of the biochemical reactions (Table 5), it was observed that 86 % of the isolated bacteria were rod-shaped and negative to gram reaction while 14 % were cocci-shaped and positive to gram reaction. The isolates were motile (64 %) and non-motile (36 %). Catalase positive is indicative of the isolates inability to hydrolyse hydrogen peroxide in producing catalase enzyme. The isolates (93 %) were positive to catalase while 7 % were negative, while 7% were positive to coagulase and 93 % negative to coagulase. For indole test, 86% were indole negative while the remaining 14% were positive. This implies that they were unable to hydrolyze tryptophan in producing indole [31]. 36 % of the isolates produced acid only on reaction with glucose while 64 % produce acid and gas. This suggests that the isolates were able to hydrolysis starch to produce glucose [30]. The isolates had varying reactions to other sugars like lactose, mannitol, sucrose, maltose, arabinose, cellobiose, sorbitol and xylose. Nine major probable organisms were identified which were *Pseudomonas* sp., *Streptococcus* sp, *Escherichia* sp., *Enterobacter* sp., *Staphylococcus* sp., *Citrobacter* sp., *Klebsiella* sp., *Proteus* and *Aeromonas* sp. Based on the percentage frequency of occurrence (Figure 1), *Pseudomonas*, *Escherichia*, *Enterobacter* and *Proteus* sp. showed the highest frequency of occurrence while least occurrence was observed in *Aeromonas* sp. The presence of the isolated organism was not surprising since fish lives in water habitat full of microorganism. The results of this study is consistent with the report/work of Admasu et al. [32], El-Tahtawi et al. [33], Eves et al. [34], Onjong et al. [35], and Sarkar et al. [36] all for *O. niloticus*. The isolated bacteria were confirmed using 16srRNA gene sequencing as *Pseudomonas aeruginosa*, *Streptococcus agalactiae*, *Escherichia coli*, *Enterobacter sichuanensis*, *Staphylococcus aureus*, *Citrobacter portucalensis*, *Klebsiella pneumonia*, *Enterobacter cloacae*, *Proteus mirabilis*, *Klebsiella variicola* and *Aeromonas caviae*.

4. Conclusions

In conclusion, injecting CP-AgNPs into *O. niloticus* was the best treatment option as it showed high preservative ability against total aerobic, coliform, *Staphylococcus* and fungal count when compared to distilled water and NaCl treatments. This study has demonstrated the potential of *C. procera* AgNPS as a natural food preservative.

Table 1. *Total aerobic count of differently treated O. niloticus.*

Hrs	Distilled water (cfu/g)	NaCl (cfu/g)	CP-AgNPs dipping (cfu/g)	CP-AgNps Injecting (cfu/g)
0	$3.60 \pm 0.17^{a} \times 10^{4}$	$2.44 \pm 0.04^{b} \times 10^{4}$	$2.20 \pm 0.04^{b} \times 10^{4}$	$2.40 \pm 0.03^{b} \times 10^{4}$
4	$9.58 \pm 0.08^{a} \times 10^{4}$	$7.80 \pm 0.10^{b} \times 10^{4}$	$6.20 \pm 0.13^{c} \times 10^{4}$	$6.02 \pm 0.07^{c} \times 10^{4}$
8	$3.29 \pm 0.08^{a} \times 10^{5}$	$2.44 \pm 0.10^{b} \times 10^{5}$	$1.52 \pm 0.04^{c} \times 10^{5}$	$1.42 \pm 0.04^{c} \times 10^{5}$
12	$8.71 \pm 0.18^{a} \times 10^{5}$	$6.56 \pm 0.11^{b} \times 10^{5}$	$2.06 \pm 0.01^{c} \times 10^{5}$	$1.80 \pm 0.04^{c} \times 10^{5}$
16	$1.91 \pm 0.03^{a} \times 10^{6}$	$1.51 \pm 0.05^{b} \times 10^{6}$	$3.34 \pm 0.04^{c} \times 10^{5}$	$2.77 \pm 0.03^{c} \times 10^{5}$
20	$3.28 \pm 0.04^{a} \times 10^{6}$	$3.05 \pm 0.08^{b} \times 10^{6}$	$7.11 \pm 0.06^{c} \times 10^{5}$	$4.89 \pm 0.06^{d} \times 10^{5}$
24	$8.50 \pm 0.06^{a} \times 10^{6}$	$6.40 \pm 0.04^{b} \times 10^{6}$	$1.42 \pm 0.06^{c} \times 10^{6}$	$1.21 \pm 0.04^{d} \times 10^{6}$
28	$1.74 \pm 0.01^{a} \times 10^{7}$	$1.01 \pm 0.01^{b} \times 10^{7}$	$2.77 \pm 0.06^{c} \times 10^{6}$	$2.12 \pm 0.08^{d} \times 10^{6}$
32	$3.84 \pm 0.02^{a} \times 10^{7}$	$3.11 \pm 0.08^{b} \times 10^{7}$	$5.89 \pm 0.07^{c} \times 10^{6}$	$4.38 \pm 0.01^{d} \times 10^{6}$
36	$7.64 \pm 0.01^{a} \times 10^{7}$	$5.42 \pm 0.09^{b} \times 10^{7}$	$1.84 \pm 0.01^{c} \times 10^{7}$	$1.56 \pm 0.01^{d} \times 10^{7}$
40	$1.21 \pm 0.02^{a} \times 10^{8}$	$1.01 \pm 0.03^{b} \times 10^{8}$	$4.57 \pm 0.01^{c} \times 10^{7}$	$3.75 \pm 0.02^{d} \times 10^{7}$
44	$4.42 \pm 0.03^{a} \times 10^{8}$	$4.08 \pm 0.02^{b} \times 10^{8}$	$1.17 \pm 0.02^{c} \times 10^{8}$	$1.00 \pm 0.02^{d} \times 10^{8}$
48	$1.17 \pm 0.02^{a} \times 10^{9}$	$1.01 \pm 0.05^{b} \times 10^{9}$	$7.74 \pm 0.06^{c} \times 10^{8}$	$7.47 \pm 0.05^{d} \times 10^{8}$

Values are represented as means of triplicate readings ± Standard deviation
Values with different superscript letters in the same row are significantly different (P<0.05)
NaCl - Sodium chloride; CP-AgNPs – *C. procera* silver nanoparticle

Table 2. *Total Staphylococcal count of differently treated O. niloticus.*

Hrs	Distilled water (cfu/g)	Nacl (cfu/g)	CP-AgNPs dipping (cfu/g)	CP-AgNps Injecting (cfu/g)
0	NIL	NIL	NIL	NIL
4	NIL	NIL	NIL	NIL
8	NIL	NIL	NIL	NIL
12	NIL	NIL	NIL	NIL
16	$1.20 \pm 0.03^{a} \times 10^{3}$	NIL	NIL	NIL
20	$2.80 \pm 0.04^{a} \times 10^{3}$	$2.00 \pm 0.07^{b} \times 10^{3}$	NIL	NIL
24	$5.10 \pm 0.10^{a} \times 10^{3}$	$3.20 \pm 0.08^{b} \times 10^{3}$	NIL	NIL
28	$8.30 \pm 0.08^{a} \times 10^{3}$	$6.80 \pm 0.13^{b} \times 10^{3}$	NIL	NIL
32	$1.02 \pm 0.01^{a} \times 10^{4}$	$8.80 \pm 0.04^{b} \times 10^{3}$	$2.40 \pm 0.10^{c} \times 10^{3}$	NIL
36	$1.22 \pm 0.03^{a} \times 10^{4}$	$9.60 \pm 0.11^{b} \times 10^{3}$	$3.20 \pm 0.10^{c} \times 10^{3}$	NIL
40	$1.57 \pm 0.01^{a} \times 10^{4}$	$1.14 \pm 0.01^{b} \times 10^{4}$	$4.00 \pm 0.10^{c} \times 10^{3}$	$3.40 \pm 0.10^{d} \times 10^{3}$
44	$1.74 \pm 0.06^{a} \times 10^{4}$	$1.28 \pm 0.04^{b} \times 10^{4}$	$5.60 \pm 0.16^{c} \times 10^{3}$	$4.80 \pm 0.11^{c} \times 10^{3}$
48	$1.98 \pm 0.11^{a} \times 10^{4}$	$1.51 \pm 0.08^{b} \times 10^{4}$	$6.20 \pm 0.13^{c} \times 10^{3}$	$5.90 \pm 0.13^{c} \times 10^{3}$

Values are represented as means of triplicate readings ± Standard deviation
Values with different superscript letters in the same row are significantly different (P<0.05)
NIL - No growth; NaCl - Sodium chloride; CP-AgNPs - *C. procera* silver nanoparticles

Table 3. *Total coliform count of differently treated O. niloticus.*

Hrs	Distilled water (cfu/g)	Nacl (cfu/g)	CP-AgNPs dipping (cfu/g)	CP-AgNps Injecting (cfu/g)
0	$4.50 \pm 0.13^{a} \times 10^{3}$	$1.50 \pm 0.11^{b} \times 10^{3}$	$1.00 \pm 0.09^{c} \times 10^{2}$	NIL
4	$8.60 \pm 0.13^{a} \times 10^{3}$	$5.60 \pm 0.08^{b} \times 10^{3}$	$2.60 \pm 0.06^{c} \times 10^{2}$	NIL
8	$1.02 \pm 0.03^{a} \times 10^{4}$	$7.30 \pm 0.10^{b} \times 10^{3}$	$3.60 \pm 0.14^{c} \times 10^{2}$	$1.20 \pm 0.09^{d} \times 10^{2}$
12	$1.28 \pm 0.04^{a} \times 10^{4}$	$1.04 \pm 0.02^{b} \times 10^{4}$	$4.00 \pm 0.18^{c} \times 10^{2}$	$2.00 \pm 0.18^{d} \times 10^{2}$
16	$1.84 \pm 0.04^{a} \times 10^{4}$	$1.60 \pm 0.04^{b} \times 10^{4}$	$5.80 \pm 0.04^{c} \times 10^{2}$	$3.00 \pm 0.13^{d} \times 10^{2}$
20	$2.52 \pm 0.11^{a} \times 10^{4}$	$1.88 \pm 0.08^{b} \times 10^{4}$	$7.40 \pm 0.14^{c} \times 10^{2}$	$4.80 \pm 0.13^{d} \times 10^{2}$
24	$2.83 \pm 0.06^{a} \times 10^{4}$	$2.34 \pm 0.10^{b} \times 10^{4}$	$9.10 \pm 0.16^{c} \times 10^{2}$	$6.60 \pm 0.13^{d} \times 10^{2}$
28	$3.18 \pm 0.14^{a} \times 10^{4}$	$2.62 \pm 0.04^{b} \times 10^{4}$	$1.12 \pm 0.02^{c} \times 10^{3}$	$9.20 \pm 0.13^{c} \times 10^{2}$
32	$7.80 \pm 0.10^{a} \times 10^{4}$	$4.01 \pm 0.05^{b} \times 10^{4}$	$1.32 \pm 0.01^{c} \times 10^{3}$	$1.11 \pm 0.02^{d} \times 10^{3}$
36	$1.26 \pm 0.02^{a} \times 10^{5}$	$8.02 \pm 0.11^{b} \times 10^{4}$	$2.05 \pm 0.13^{c} \times 10^{3}$	$1.88 \pm 0.03^{c} \times 10^{3}$
40	$1.43 \pm 0.02^{a} \times 10^{5}$	$1.00 \pm 0.01^{b} \times 10^{5}$	$3.51 \pm 0.02^{c} \times 10^{3}$	$2.78 \pm 0.11^{d} \times 10^{3}$
44	$1.83 \pm 0.03^{a} \times 10^{5}$	$1.40 \pm 0.02^{b} \times 10^{5}$	$5.03 \pm 0.08^{c} \times 10^{3}$	$4.31 \pm 0.09^{d} \times 10^{3}$
48	$2.24 \pm 0.02^{a} \times 10^{5}$	$2.15 \pm 0.03^{b} \times 10^{5}$	$8.05 \pm 0.13^{c} \times 10^{3}$	$6.78 \pm 0.13^{d} \times 10^{3}$

Values are represented as means of triplicate readings ± Standard deviation
Values with different superscript letters in the same row are significantly different (P<0.05)
NIL - No growth; NaCl - Sodium chloride; CP-AgNPs – *C. procera* silver nanoparticles

Table 4. *Total fungal count of differently treated O. niloticus.*

Hrs	Distilled water (cfu/g)	Nacl (cfu/g)	CP-AgNPs dipping (cfu/g)	CP-AgNps Injecting (cfu/g)
0	$6.40 \pm 0.13^{a} \times 10^{4}$	$5.20 \pm 0.52^{b} \times 10^{4}$	$3.20 \pm 0.13^{c} \times 10^{4}$	NIL
4	$7.40 \pm 0.16^{a} \times 10^{4}$	$5.60 \pm 0.17^{b} \times 10^{4}$	$3.60 \pm 0.13^{c} \times 10^{4}$	NIL
8	$9.60 \pm 0.33^{a} \times 10^{4}$	$8.50 \pm 0.28^{b} \times 10^{4}$	$3.20 \pm 0.06^{c} \times 10^{4}$	$0.70 \pm 0.09^{d} \times 10^{3}$
12	$1.42 \pm 0.13^{a} \times 10^{5}$	$9.80 \pm 0.35^{b} \times 10^{4}$	$3.00 \pm 0.08^{c} \times 10^{4}$	$1.60 \pm 0.14^{c} \times 10^{4}$
16	$1.64 \pm 0.07^{a} \times 10^{5}$	$1.24 \pm 0.04^{b} \times 10^{5}$	$3.80 \pm 0.09^{c} \times 10^{4}$	$2.00 \pm 0.16^{d} \times 10^{4}$
20	$1.95 \pm 0.08^{a} \times 10^{5}$	$1.42 \pm 0.08^{b} \times 10^{5}$	$4.20 \pm 0.16^{c} \times 10^{4}$	$2.80 \pm 0.13^{c} \times 10^{4}$
24	$2.10 \pm 0.18^{a} \times 10^{5}$	$1.70 \pm 0.09^{b} \times 10^{5}$	$6.60 \pm 0.13^{c} \times 10^{4}$	$3.60 \pm 0.16^{d} \times 10^{4}$
28	$2.28 \pm 0.14^{a} \times 10^{5}$	$1.92 \pm 0.12^{b} \times 10^{5}$	$8.40 \pm 0.42^{c} \times 10^{4}$	$5.20 \pm 0.11^{d} \times 10^{4}$
32	$2.53 \pm 0.08^{a} \times 10^{5}$	$2.28 \pm 0.11^{b} \times 10^{5}$	$9.60 \pm 0.18^{c} \times 10^{4}$	$6.80 \pm 0.57^{d} \times 10^{4}$
36	$2.75 \pm 0.11^{a} \times 10^{5}$	$2.43 \pm 0.08^{b} \times 10^{5}$	$1.13 \pm 0.07^{c} \times 10^{5}$	$8.60 \pm 0.85^{d} \times 10^{4}$
40	$3.28 \pm 0.28^{a} \times 10^{5}$	$2.92 \pm 0.28^{a} \times 10^{5}$	$1.22 \pm 0.09^{b} \times 10^{5}$	$9.4 \pm 0.57^{b} \times 10^{4}$
44	$6.40 \pm 0.25^{a} \times 10^{5}$	$4.40 \pm 0.29^{b} \times 10^{5}$	$1.58 \pm 0.09^{c} \times 10^{5}$	$1.25 \pm 0.11^{c} \times 10^{5}$
48	$8.50 \pm 0.27^{a} \times 10^{5}$	$6.00 \pm 0.18^{b} \times 10^{5}$	$2.35 \pm 0.11^{c} \times 10^{5}$	$1.82 \pm 0.08^{d} \times 10^{5}$

Values are represented as means of triplicate readings ± Standard deviation
Values with different superscript letters in the same row are significantly different (P<0.05)
NIL - No growth; NaCl - Sodium chloride; CP-AgNPs - *C. procera* silver nanoparticles

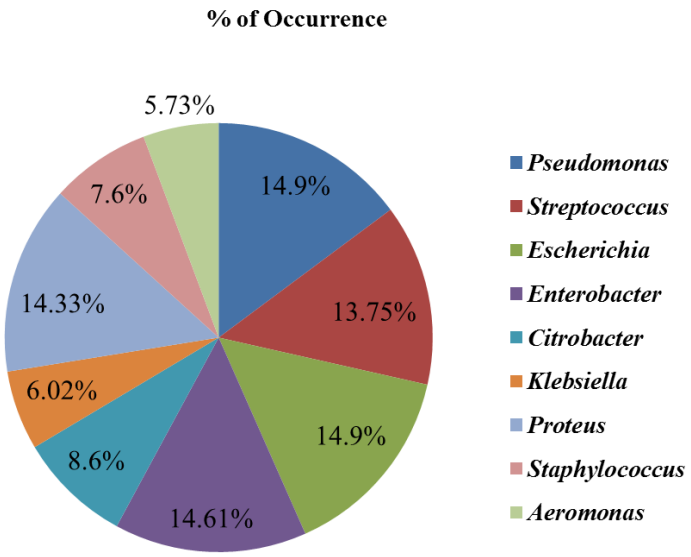

Figure 1. *Frequency of occurrence of isolated bacteria.*

Table 5. *Biochemical characteristics of bacteria isolated from fish samples.*

Isolate Code	Gram Reaction	Shape	Motility	Catalase	Co-agulase	Oxidase	Citrate	H_2S Production	Urease	Indole	Methyl Red	VP	Glucose	Lactose	Manitol	Sucrose	Maltose	Arabinose	Cellobiose	Sorbitol	Xylose	Probable Identity
F1	-	Rod	+	+	-	+	+	-	-	-	-	-	A	-	-	-	-	-	-	-	-	*Pseudomonas* sp.
F2	+	Cocci	-	-	-	-	-	-	-	-	-	-	A	A	-	A	A	-	-	-	-	*Streptococcus* sp
F3	-	Rod	+	+	-	-	-	-	-	+	+	-	A/G	A/G	A/G	-	A/G	A/G	-	A/G	A/G	*Escherichia* sp.
F4	-	Rod	+	+	-	-	+	-	-	-	-	+	A/G	A/G	A/G	A/G	A/G	-	A	A/G	A/G	*Enterobacter* sp.
F5	-	Rod	+	+	-	+	+	-	-	-	-	-	A	-	A	-	-	-	-	-	-	*Pseudomonas* sp.
F6	+	Cocci	-	+	+	-	-	-	+	-	-	-	A	-	A	-	-	-	-	-	-	*Staphylococcus* sp.
F7	-	Rod	+	+	-	-	+	+	+	-	+	+	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	*Citrobacter* sp.
F8	-	Rod	-	+	-	-	+	-	+	-	-	+	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	*Klebsiella* sp.
F9	-	Rod	+	+	-	-	+	-	-	-	-	+	A/G	A/G	A/G	A/G	A/G	-	-	A	A	*Enterobacter* sp.

Isolate Code	Gram Reaction	Shape	Motility	Catalase	Coagulase	Oxidase	Citrate	H_2S Production	Urease	Indole	Methyl Red	VP	Glucose	Lactose	Manitol	Sucrose	Maltose	Arabinose	Cellobiose	Sorbitol	Xylose	Probable Identity
F10	-	Rod	+	+	-	+	+	-	-	-	-	-	A	-	A	-	-	-	-	-	-	*Pseudomonas* sp.
F11	-	Rod	+	+	-	+	+	+	+	-	+	+	A/G	A/G	A/G	-	A/G	A/G	A/G	A/G	A/G	*Proteus* sp.
F12	-	Rod	-	+	-	-	+	-	+	-	-	+	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	*Klebsiella* sp.
F13	-	Rod	+	+	-	+	-	-	-	+	+	-	A/G	-	A	-	A	A	A	-	A	*Aeromonas* sp
F14	-	Rod	-	+	-	-	+	-	+	-	-	+	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	*Klebsiella* sp.

VP - Voges-Proskauer; + Positive; - Negative; A - Acid production only; A/G - Acid production with gas

Table 6. *Blast prediction of 16srRNA of isolated organisms.*

Sample ID	Predicted Organism	% Identity	Gene bank Accession No	Alignment Score	Highest Query Coverage (%)
F1	*Pseudomonas aeruginosa*	98.20	JF513146.1	≥200	99
F2	*Streptococcus agalactiae*	98.47	CP050455.1	≥200	99
F3	*Escherichia coli*	97.82	KF917161.1	≥200	99
F4	*Enterobacter sichuanensis*	99.87	CP027986.1	≥200	99
F5	*Pseudomonas aeruginosa*	93.80	MF943159.1	≥200	99
F6	*Staphylococcus aureus*	99.00%	CP045560.1	≥200	99
F7	*Citrobacter portucalensis*	94.05	CP089316.1	≥200	99
F8	*Klebsiella pneumoniae*	96.81	CP030320.1	≥200	99
F9	*Enterobacter cloacae*	92.58	MZ157016.1	≥200	97
F10	*Pseudomonas aeruginosa*	98.14	JF513146.1	≥200	99
F11	*Proteus mirabilis*	90.12	MF977359.1	≥200	99
F12	*Klebsiella variicola*	97.71	KU359261.1	≥200	99
F13	*Aeromonas caviae*	91.95	OM842836.1	≥200	99
F14	*Klebsiella pneumoniae*	98.80	CP121133.1	≥200	99

Abbreviations

CP-AgNPs	*Calotropis procera* Silver Nanoparticles
AgNPs	Silver Nanoparticles
NPs	Nanoparticles
$AgNO_3$	Silver Nitrate
NaCl	Sodiun Chloride
MR-VP	Methyl Red – Voges Proskauer
DNA	Deoxyribonucleic Acid
PCR	Polymerase Chain Reaction

UV	Ultraviolet
EDTA	Ethylenediaminetetraacetic Acid

Author Contributions

Oluwatoyin Animashaun: Conceptualization, Methodology, Writing - Original Draft Preparation, Project Administration, Funding Acquisition

Daniel Aina: Writing - Review & Editing, Validation, Supervision

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Data Availability Statement

The data is available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare no conflicts of interest.

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Research Fields

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