Biogenic Synthesis of Silver Oxide Nanoparticle from Plants Materials for Control and Management of Microbial Infections in Fishes

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Abstract: Nanoparticles has shown to be effective in drug delivery, though, its synthesis are time and energy consuming while, the process is toxic to the environment, hence, the utilization of green synthesis method with plants extracts. The study focused on the green synthesis of nanoparticles and its application on isolates of diseased fish. Four different isolates namely; *Bacillus infantis, Klebsiella pneumonae, Escherichia coli*, and *Vibrio paraheamoliticus* were from diseased cat fish (*Clarias gariepinus*). The silver nanoparticle synthesis was from *Psidium guajava* L (Guava leaves) and *Azadirachta indica* (Neem plant leaf) extracts. Phytochemical analysis was carried out on the plants leaf extracts to determine its contents using standard methods. The isolates were obtained through culture method and molecular identification. However, the synthesis of plants extract nanoparticles was characterized using LANMAN spectrophometer, while, antimicrobial activities was carried out using agar well diffusion method and minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were also determined. The influence of the microbial activities of the plants extracts silver nanoparticles synthesized shows the level of impact on the isolated pathogenic organisms. The efficacy was dose dependent, 500 um/mg from guava leaf extract had the highest effect on *E. coli* while, the least impact was observed on dosage of 62.5 um/mg of *E. coli*. Neem leaves extract has the highest effect on *V. parahaemolyticus* at 500 um/mg while, the least was on *E. coli* at 250 um/mg. The MBC of neem leaf nanoparticle extracts were more potent as it has more clearance level than the guava 'Therefore, the study revealed that the plant leaf extracts silver nanoparticles synthesized could be used in the treatment of diseases caused by the test organisms
Key word: Silver nanoparticle, *Clarias gariepinus,* Minimum inhibitory concentration, Minimum bactericidal concentration.

INTRODUCTION

The impact of the microbial infection in fish production has been on the increase and this can be linked to high demand of sea foods. This has necessitated the proliferation of fish ponds to meet the market demands. Though, this development is business oriented as it also come with challenges. In Nigeria, Cat fish (*Clarias gariepinus*) is an ideal fish for production due to its tolerance for extreme environmental conditions (FAO, 2022). Moreover, the fish can be stocked in high densities probably, because of their adaptive aggressive and defense mechanism which aids in eliminating physical damage during conflict (Clols-Fuentes *et al*., 2023). During cat fish production, it is imperative that optimal condition is maintained to prevent microbial infection and physiological stress. The emphasis on the availability of heterogenic organisms is necessary as it is beneficial in balancing the biochemical changes that occurs during fish metabolism (Li *et al*., 2021). These organisms must maintain a certain level as its abundance in the ponds can negatively influence the fish health. However, some of these heterotrophic organisms in the fresh water for fish rearing are opportunistic pathogens which can infect the fishes. Examples of pathogens reported in fresh water fishes are *Aeromonas* sp, *Pseudomonas* sp, *Flavobacterium* sp, *Streptococcus* sp, *Shewanella* sp, and *Staphylococcus* sp. Pathogens reported in catfish are *Edwardsiella ictaluri*, *Aeromonas* sp and *Flavobacterium columnare* (Pękala-Safińska, 2018; Wise *et al.*, 2021). Conversely, environmental conditions also contribute to infections experienced by fishes during production (Hennersdorf *et al*., 2016). Although, issues of this type exist in fish production, different approaches have been deployed in ameliorating the situation such as maintaining hygienic environment and utilization of antibiotics for fish farm infections.

The increasing resistance of the pathogenic bacteria to antibiotics has become a one health problem. However, methods like nanotechnology offers an advanced process of mitigating the problem. The concept of synthesizing nanoparticles from biological material has improved the efficiency of the process while being eco-friendly. The application of nanoparticles obtained from biological materials with small size cum large surface area is known as green nanotechnology (Pandit *et al*., 2022). Characteristics which includes thermal, optical and chemical proficiency has expose the relevance of nanomaterials (Al-Dhabi and Valan Arasu, 2018). These nanomaterials can be applied to a variety of industries such as pharmaceuticals, medical, environmental and biosciences due to its antimicrobial action, antiinflammatory and biocompatibility (Khalith *et al*., 2021). There are different metal oxide nanoparticles which includes but, not limited to copper oxide, zinc oxide, iron oxide, titanium oxide, magnesium oxide, cerium oxide, zirconium oxide and so on and so forth (Roy, 2021).

This study reports on the antibacterial potentials of silver oxide nanoparticle synthesized from leaves of *Azadirachta indica* L and *Psidium guajava* L (Neem and Guava) leaves on bacteria isolated from infected fishes.

MATERIALS AND METHODS

Sample collection and preparation of fish samples: A total of 24 diseased *Clarias gariepinus* (African catfish) were aseptically collected from the fish pond at Egbema/Ohaji sites in Owerri Imo State within two months. The fishes were transported in an ice chest to the laboratory for immediate bacteriological analysis within 2hrs.The plants (neem and guava leaves) were obtained from orchards in Federal University of Technology, owerri Imo State, Nigeria and identified according to the methods described by Etim *et al*. (2022). The leaves were washed, un-dried and grind to powder with a laboratory Seward Stomacher 400 circulator blender with serial no. 1120. Ten grams were boiled with 100 ml distilled water for 20 mins and filtered with Whatman filter paper No 1 the filtrates then centrifuged at 4,000 rpm for 5 minutes and the deposits dried and then stored at 4°C in the refrigeratorEtim, 2022).

Qualitative phytochemical analysis of plant leaf extracts: Phytochemicals components such as saponin, alkaloids, tannins, flavonoid, anthraquinone, terpenoids and glycosides were determined the method of Biswan *et al*. (2013).

Biosynthesis of silver nitrate nanoparticles utilizing different plants substrates: Ten milliliters (10 ml) of leaf extract was mixed with 90 ml of 1 mM $AgNO_3$ solution. Each mixture was left under ambient conditions and colour change from yellow to dark green which indicated reduction of Ag^+ to Ag^0 nanoparticles (NPs) was be visually monitored. After 6 hours of incubation, the absorbance of each solution was determined at 400 nm using LANMAN Spectrophotometer at a resolution of 1 nm. The mixture was centrifuged at 4000 rpm for 20 minutes. After discarding the supernatants, the AgNPs was redisposed in distilled water, washed and stored at 4^{o}C in the refrigerator until used (Musimun *et al*., 2022).

Characterization of synthesized silver nanoparticle: Ultraviolet – visible spectrophotometer was used to characterize the silver nanoparticles synthesized at wavelength range of 340 - 820 nm using LABMAN Spectrophotometer at a resolution of 1 nm. Noble metal particles possess strong surface plasmon resonance (SPR) absorption in the visible region and are highly sensitive to the surface modification (Ramkumar *et al*., 2017).

Antimicrobial activity assay of AgNPs from plants extracts:

Minimum inhibitory concentration determination (MIC): The determination of minimum inhibitory concentration was done according to the standard broth dilution method. The antimicrobial efficacy of silver nanoparticles was utilized through the estimation of the noticeable progression of microorganisms in the agar broth. Modified dilution of silver nanoparticles in different concentrations ranging from 500 mg/ml, 250 mg/ml, 125 ml/ml and 62.5 mg/ml with bacterial concentration (1.5×10^8 cfu/ml, 0.5 McFarland'sstandard) were used to determine MIC in Mueller-Hinton broth. The control contained only inoculated broth and incubated for 24 h at 37°C. The MIC endpoint is the lowest concentration of silver nanoparticles where no visible growth is observed in the tubes. The visual turbidity of the tubes was noted, both before and after incubation to confirm the MIC value (Parvekar *et al*., 2020).

Minimum bactericidal concentration (MBC) determination: Aliquots of 50 microliters from all the tubes which showed no visible bacterial growth were dispensed on Brain Heart infusion (BHI) agar plates and incubated for 24 h at 37°C. When 99.9% of the bacterial population was killed at the lowest concentration of an antimicrobial agent, it is designated as MBC endpoint. This was done by observing before and after incubated agar plates for the presence or absence of bacteria (Parvekar *et al*., 2020).

RESULTS

The phytochemical contents of guava and neem leaf extracts were estimated as showed in Table 1. Guava leaf extracts had more tannin content, followed by saponin, while the other estimated quantity of phytochemicals appeared to be available in small quantity. Moreover, neem leaf extracts had more metabolites compared to guava leaf extracts.

The minimum inhibitory concentration is a method used to determine the antimicrobial activity of substance such as plant extract leaf silver nanoparticles. The isolates were susceptible to the synthesized nanoparticles. Figure 1 and 2 showed the graphical representation of the nanoparticles activity. Figure 1, demonstrates the efficacy of the silver nanoparticle extracts from guava plant. The nanoparticle concentration of 500 mg/ml appears to have the highest activity on all the test organisms while, concentration 65.2 mg/ml and 125 mg/ml has the least impact. *Bacillus infantis* appears to be less susceptible, followed by *Klebsiella* on guava leaf nanoparticles extract. However, *Vibrio paraheamoliticus* and *E. coli* were more susceptible to the guava extracts compared to the control (Ciprofloxacin 500 mg). In Figure 1, it was observed that the *B.infantis* and *Klebshiella* were more susceptible to the silver nanoparticles extracted from neem leaves. Although, nanoparticles concentration of 500 mg/ml, 250 mg/ml and 62.5 mg/ml had more impact on the test organisms. However, *V. paraheamoliticus* and *E. coli* were less susceptible to neem synthesize silver nanoparticles. Generally, silver nanoparticles synthesize from neem leaf extracts exacted showed more efficacy in affecting the growth of the organisms. All the bacterial strains responded in dose-dependent mode.

This method determines the lowest concentration of the nanoparticles that can eliminate about 99.9% of the test bacterial strains. Tables 2 and 3 showed the influence of different concentrations of plants extracted nanoparticles. Table 2 reveals that concentration 250 mg/ml and 500 mg/ml had the highest effect on *Klebsiella* and *Vibrio paraheamoliticus* compared to the control. However, all other concentration except 62.5 mg/ml either eliminated the organisms or slowed down their growth rate.

Neem leaf extracted with silver nitrate showed stronger activities and the dosage of the concentrations plays important roles as shown in Table 3. Concentration D exacted more effect in eliminating the strains followed by concentration C, athough, concentration A did not show much activities but, it slowed the growth of *B. infantis* and *Vibrio paraheamoliticus* compared to the control.

Table 1: Phytochemical contents of plant leaf extracts

Plants extract	Alkaloid	Flavonoid	Tanin	Saponin	Glyceride	Anthroquinon	Terpenoids
Guava	+	+	+++	++	+	+	+
Neem	++	+++	++	+++	+++	++	++

Key: + = small ++ =moderate ++++ = maximum

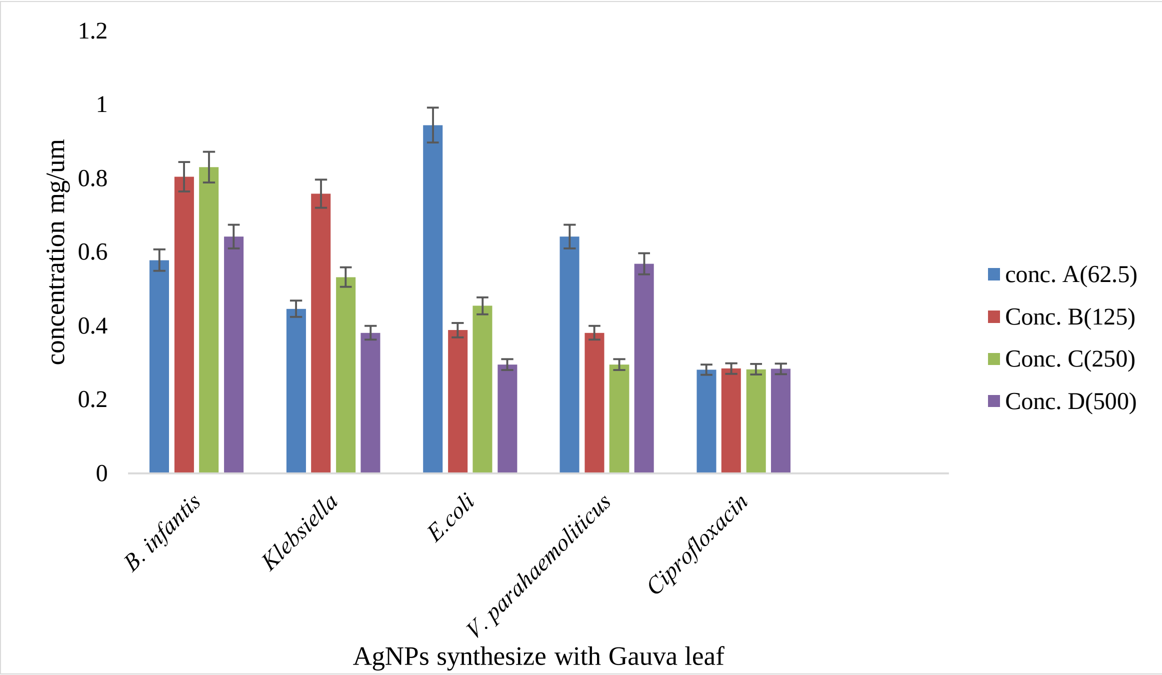

Figure 1: Minimum Inhibitory Concentration of guava leaf extracts of silver nanoparticle

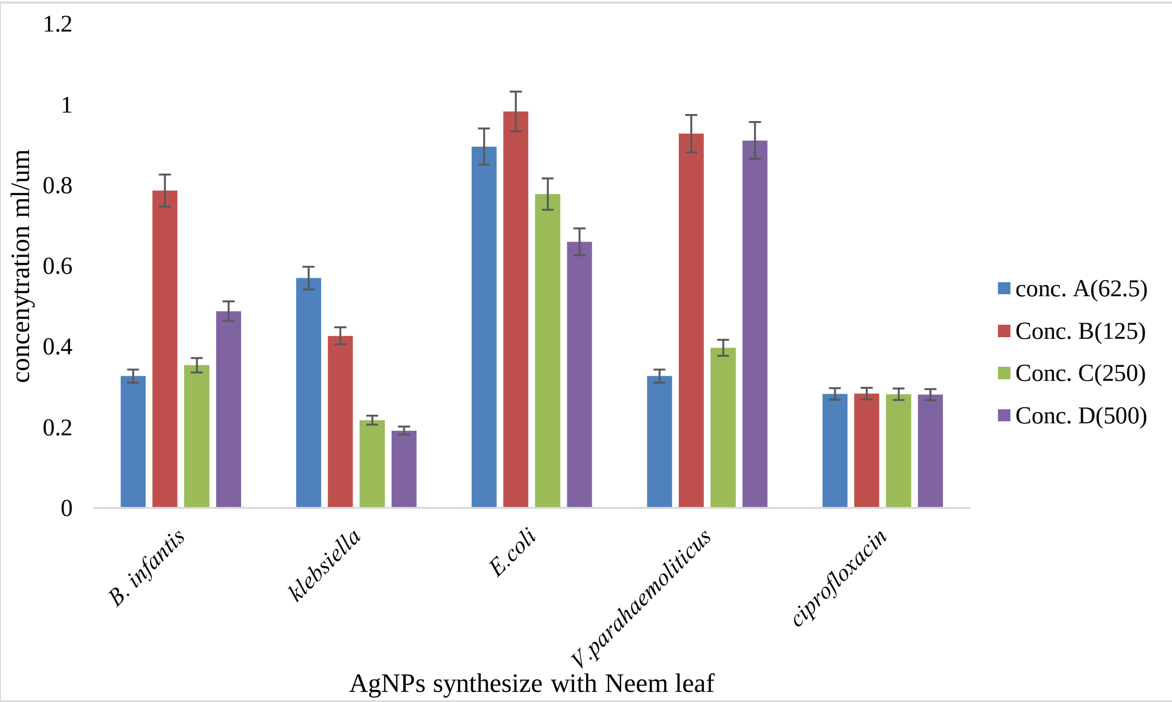

Figure 2: Minimum Inhibitory Concentration of neem leaf extracts of silver nanoparticle

Table 2: Minimum bactericidal concentration AgNPs synthesize with Guava leaf

Isolates	Conc. A	Conc. B	Conc. C	Conc. D
B. infantis	+++	+++	+++	++
Klebsiella	+++	+	-	-
E. coli	+++	+++	+	++
V. paraheamoliticus	+++	+	-	-
Ciprofloxacin	-	-	-	-

Key: Conc. A: 62.5, Conc. B: 125, conc. C: 250, conc. D: 500, -: absence, +: presence, ++: +++

Table 3: Minimum bactericidal Concentration of AgNPs synthesize with neem leaf

Isolates	Conc. A	Conc. B	Conc. C	Conc. D
B. infantis	+	+	-	-
Klebsiella	+++	-	++	-
E. coli	++	++	+	++
V. paraheamoliticus	+	++	++	-
Ciprofloxacin	-	-	-	-

Legend: Conc. A: 62.5, Conc. B: 125, conc. C: 250, conc. D: 500, -: absence, +: presence, ++: +++

DISCUSSION

Silver has been reported to possess antimicrobial properties. The ion of the silver can bind to cellular structural function, generate reactive oxygen species and interfere with the DNA function (Thangaraj *et al.,* 2014). It is biocidal to both Gram positive and negative bacteria. Different plants extract has shown to be medicinal due to inherent phytochemicals components. Phytochemicals are chemicals produced by plants for its protection against diseases. These chemicals are not known to hold any nutritive value though; they have been reported to possess antimicrobial activities (Min *et al*., 2008). The extracts from the leaves of guava and neem plants extracts have been reported to elicit antimicrobials against different microorganisms. Guava leaf contain bioactive components with capability of eliminating pathogens, maintain blood glucose level while, contributing to weight loss. The peaf extract possess essential oil with abundance in flavonoids, tannins, triterpenes, resin, mineral salts, chlorophyll, malic acid, cellulose and so on (Biswas *et al*., 2013). The study shows the quantity of phytochemicals contained in guava and neem leaves extract. The presence of phytochemicals obtained from the guava leaf were minimal compared to the neem leaf. This suggest that the plants extracts can be used as an antimicrobial and also in remedying physiological issues. Sanches *et al*. (2005) reported that tannin and flavonoids can interfere with the protein synthesis through binding on proline rich proteins while forming complexes with bacterial cell wall and soluble proteins. However, saponins have also been found to have inhibitory effects on Gram positive bacteria (Min *et al*., 2008). The four bacterial isolates from fish source used responded differently to silver nanoparticles synthesized from both guava and neem leaves compared to ciprofloxacin. In guava leaf nanoparticles extracts, concentration D and C recorded higher activities with *E. coli* and *V. paraeamoliticus* than *B. Infantis* and *Klebsiella* sp. This demonstrates that its efficacy is dose dependent. However, neem leaf nanoparticle extracts had more effect on *B. infantis* and *Klebsiella* sp. This differences observed in the antimicrobials activities of the plants nanoparticle extracts maybe attributed to the morphological and physiological make-up of the test bacterial strains. All the bacterial strains were Gram negative except *B. infantis.* Silver nanoparticles synthesis from guava were more active against Gram negative strains while, neem leaf nanoparticle extract was active against both Gram positive and Gram negative strain.

The synthesize sliver nanoparticles size and the bacteria cell wall might have also contributed to the variations in their antimicrobial activities. Liao *et al*. (2019) reported the importance of silver nanoparticle size in demonstrating its antimicrobial efficacy. Diameter between 1-15 nm has more potential in eliminating pathogens than 20 nm. Minimum bactericidal concentration revealed the lowest concentration at which the bacteria was killed (*B. infantis*). The MIC and MBC of plant nanoparticle extract demonstrates the differences in dosage by mainly observing the effects on organisms. Some of the organism growth rate were slowed down while, it eliminated some. This reveals the potentials of plant extract silver nanoparticles. Though, the impact were deficient, enhanced method of silver nanoparticle is encouraged for effective binding of the ions.

CONCLUSION

Disease causing pathogens in fish has been linked to environmental hygiene of the ponds and surroundings. Natural remedy for curing the diseases have been encouraged as it constitutes little effect on the environment. The impact of nanoparticle in enhancing the drug delivery through improved surface cannot be over emphasized. The effect of the plant extracts nanoparticle was observed on the different bacterial isolates obtained from the diseased fish. The differences in the efficacy of the silver nanoparticles synthesize from plants leaf extracts in inhibiting or slowing down the growth of the microorganisms indicates it is dependent on dosage.

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