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Antibacterial activity of silver nanoparticles synthesized from leaf extracts of *Aloe vera* against *Klebsiella pneumoniae* isolated from *Oreochromis niloticus*

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Abstract
The increasing use of natural products for preventing and treating bacterial diseases offers significant benefits, particularly for individuals in low socioeconomic settings within urban and rural communities. This study investigated the antibacterial activity of aqueous *Aloe vera* extract against *Klebsiella pneumoniae* isolated from *Oreochromis niloticus*. *Aloe vera* was collected from a household garden in Keffi Metropolis, and silver nanoparticles (Ag-NPs) were synthesized from its aqueous extract using silver nitrate ($AgNO_3$). Characterization of the Ag-NPs revealed dominant functional groups, including alcohols and alkenes, with an average particle size of 50-80 nm, determined using Fourier-transform infrared spectroscopy (FTIR) and scanning electron microscopy (SEM). The antibacterial efficacy of Ag-NPs, alone and in combination with meropenem, was evaluated against carbapenemase-producing K. pneumoniae isolates using the agar dilution method. Additionally, biofilm formation, inhibition, and dissolution were assessed using the microtiter plate method. At 80% of the minimum inhibitory concentration (MIC), Ag-NPs achieved biofilm inhibition rates ranging from 96.75% to 96.79% and biofilm dissolution rates between 0.0% and 8.7%. The findings demonstrate that synthesized Ag-NPs possess significant antibacterial and antibiofilm activities, which were further enhanced when combined with meropenem, exhibiting synergistic effects against K. pneumoniae.

Keywords: *Aloe vera*, *Klebsiella pneumoniae*, leaf extracts, nanoparticles, *Oreochromis niloticus*

Introduction
Although herbal remedies have been with us for human therapy for millennia, there has been relatively little research on the medicinal plants to be used against fish diseases (Kolkovski and Kolkovski, 2011) [11]. Herbal drugs can be used not only as remedies but even more so, as growth promoters, stress resistance boosters and preventatives of infections. Hence, herbal drugs in disease management are gaining success, because they are cost effective, eco-friendly and have minimal side effects. A large portion of the world population, especially in developing countries, depends on the traditional system of medicine for a variety of diseases. Several hundred genera are used medicinally, and plants are vital sources for potent and powerful drugs. Plants are rich in a wide variety of secondary metabolites of phytochemical constituents such as tannins, alkaloids and flavonoids, which act against different diseases (Harikrishnan *et al.*, 2010; Ravikumar *et al.*, 2011) [7, 16]. Unfortunately, the parasitic outbreak acts as an important limiting factor for aquaculture businesses. Pinkate *et al.* (2013) [13] reported that every tilapia fish (*Oreochromis niloticus*) raised by farmers in Chiang Mai, Thailand has a *Trichodina* parasite infection.
Chitmanat *et al.* (2005) [3] pointed out that the heavy infection of *Trichodina* sp. in small fish has caused gigantic financial losses. Infected fish are lethargic, generate excessive mucus and become off-feed eventually, resulting in considerable deaths (Turker *et al.*, 2019) [18]. There is now a fast-growing interest in screening antiparasitic substances from plants to replace chemical and antibiotic alternatives. Diseases caused by *A. hydrophila* bacterium are some of the most widespread in freshwater fish culture.

Septicemia caused by motile aeromonads is a ubiquitous problem that affects fish found in warm, cool and cold fresh water around the world. A. hydrophila has been associated with diseases in fishes like carp, eels, milkfish, channel catfish, tilapia and ayu. This microorganism can also be an opportunist in stress-related diseases in salmonids. Antibiotics are frequently used to control disease caused by this bacterium, but there is an increasing risk of developing antibiotic resistant (Ravikumar *et al.*, 2019) [15]. *A. hydrophila* is also responsible for skin infections, septicemia and gastroenteritis in human, besides the fish (Castro *et al.*, 2018) [2]. The continuous use of antimicrobial agents in aquaculture has resulted in more resistant bacterial strains. Since ancient times, medicinal plants have been used for the treatment of common infectious diseases and treatments with plants having antibacterial activity are a potentially beneficial alternative in aquaculture. Additionally, phytomedicines provide a cheaper source for treatment and greater accuracy than chemotherapeutic agents in fish (Sharma *et al.*, 2010; Christybapita *et al.*, 2017; Turker *et al.*, 2019) [17, 4, 18].

Aloe vera is an ornamental and medicinal plant. It is being used therapeutically, since Roman times and perhaps long before (Heggers *et al.*, 2019) [9], different properties being ascribed to the inner colorless leaf gel and to the exudates from the outer layers. Aloe has a history of traditional use by Native Americans for stomach disorders and intestinal disorders including constipation, hemorrhoids, colitis and colon problems. It is said to be a natural cleaner, powerful in penetrating tissues, relieving pain associated with joints and the gel (Heggers *et al.*, 2019) [9] each of which may have a range of mechanisms of actions, acting synergistically or individually to explain more than 200 different constituents notably mucopolysaccharides, enzymes, sterols, prostaglandins, fatty acids, amino acids and a wide variety of vitamins and minerals. It contains several potentially active bioactive compounds including salicylates, magnesium lactate, acemannan, lupeol, campestrol, β-sitosterol, aloin A and anthraquinones. In addition, *Aloe vera* contains at least seven super-oxide dismutases with antioxidant activity (Heggers *et al.*, 2019) [9].

The efficacy of Aloe liquid as an antibacterial agent is shown to have a wide range against Gram positive and Gram-negative bacteria. The antimicrobial agents of *Aloe vera* gel was reported to effectively kill or greatly reduce or eliminate the growth of *Staphylococcus aureus, Klebsiella pneumoniae, Streptococcus pyogenes, Pseudomonas aeruginosa, Escherichia coli, Helicobacter pylori* and *Salmonella typhi* (Fite *et al.*, 2013) [6].

This study was therefore, carried out to determine the antibacterial activity of silver nanoparticles synthesized from leaf extracts of *Aloe vera* against *Klebsiella pneumoniae* isolated from *Oreochromis niloticus.*

Materials and Methods

Plant Collection and Preparation of *Aloe vera* Leaf

Fresh and healthy leaves of *Aloe vera* were collected from a household garden in Keffi Metropolis and transported to Plant Science and Biotechnology Department, Nasarawa State University, Keffi for identification purposes. The collected leaf was washed thoroughly in tap water and rinsed with sterile distilled water and air-dried at room temperature. Finally, the dried material was grounded to coarse powder in a manual grain mill and stored in a plastic container for further use.

Plant Extraction and Preparation of Stock Solutions

Aqueous Extraction

The fine powder leaf of the plants was macerated overnight in distilled water. After maceration, the aqueous solutions of the plant were filtered through No. 1 Whatman filter paper and the resulting solutions dried by pouring in a large beaker and placed in a water bath at 60 °C to allow for evaporation. The recovered dried extracts were placed in sterilized screw-capped bottles and stored at 4 °C.

Phytochemical Screening

The phytochemical analyses of the respective plant extracts, tests for alkaloids, tannins, saponins, flavonoids, steroids, cardiac glycosides, and terpenoids were carried out using standard methods reported by Bankole *et al* (2012).

Preparation of Test Organisms

The inocula of the test organisms were prepared from the stock culture and sub-cultured onto nutrient broth using a sterilized wire loop. It was incubated at 37 °C for 24hrs. About 0.2 ml of the culture was dispensed into 20 ml of a freshly prepared nutrient broth and incubated at 37 °C for 3-5h until bacteria density of 10^6 CFU (colony forming unit) is obtained by comparing it with 0.5 McFarland's turbidity standard (Akpotu *et al.*, 2017) [19].

Primary Screening of Extracts for Antibacterial Activity

The agar well diffusion technique as described by Akpotu *et al.* (2017) [19] was used to determine the antibacterial activity of the extracts. Dilutions of 500, 250, 125, 62.5, 31.25 and 15.6 mg/ml were prepared from the 1000 mg/ml stock solutions of the respective extracts. Exactly 20 ml of molten MHA were poured into sterile Petri dishes (90 mm) and allowed to set. Standardized concentrations (McFarland 0.5) of overnight cultures of test isolates were swabbed aseptically on the agar plates and holes of diameter 8 mm made in the agar plates using a sterile metal cork-borer. A volume of 100 µl of the various dilutions of each extract and control was dispensed in each hole under aseptic condition and left at room temperature for 1 h to allow the agents to diffuse into the agar medium. Ciprofloxacin (5 µg/ml) was used as positive control, while DMSO was used as the negative controls. The plates were incubated at 37ºC for 24 h and the zones of inhibition measured. Extracts that give significant activity against test organisms were further tested against test organisms to determine their MICs.

Determination of Minimum Inhibitory Concentration (MIC) of the Extracts using the Agar Dilution Method

Minimum inhibitory concentration (MIC) of the extract was determined using agar dilution method by modification of the method described by Akpotu *et al.* (2017) [19]. Briefly, 2-fold dilution of the extract in MHA plates to obtain the following concentrations: 50, 25, 12.5 and 6.25 mg/ml were made 10 µl of the standardized MDR isolates were inoculated in the MHA plates containing different concentrations of the extract was incubated at 37 °C for 24 h. The lowest concentration of the extract that inhibited the visible growth was read as the MICs.

Synthesis of Silver Nanoparticles

0.25g of $AgNO_3$ powder was dissolved in 500ml of distilled water to prepare 10 mM $AgNO_3$ stock solution from which a series of 1 mM, 2 mM, 3 mM, 4 mM, and 5 mM $AgNO_3$

solutions were prepared. The $AgNO_3$ solutions were mixed with the aqueous and ethanol extract of *Aloe vera* fresh leaf at a ratio of 1: 1 (v/v) to a volume of 1000 mL in a flask. The flask was wrapped with an aluminum foil and heated in a water bath at 60°C for 5 hours. Furthermore, the mixture was stored in the refrigerator for the antibacterial activity test and further analyzed by using UV-visible spectrophotometer.

Screening of Synthesized Silver Nanoparticle for Antibacterial Activity

The agar well diffusion technique as described by Akpotu *et al.* (2017) was used to determine the antibacterial activity of the silver nanoparticle. Dilutions of 500, 250, 125, 62.5, 31.25 and 15.6 mg/ml were prepared from the 1000 mg/ml stock solutions of the respective silver nanoparticle. Exactly 20 ml of molten MHA was poured into sterile Petri dishes (90 mm) and allowed to set. Standardized concentrations (McFarland 0.5) of overnight cultures of test isolates were swabbed aseptically on the agar plates and holes of diameter 8 mm made in the agar plates using a sterile metal cork-borer. A volume of 100 µl of the various dilutions of each silver nanoparticle and control was dispensed in each hole under aseptic condition and left at room temperature for 1 h to allow the agents to diffuse into the agar medium. Ciprofloxacin (5 µg/ml) was used as positive control, while DMSO was used as the negative controls. The plates were incubated at 37 °C for 24 h and the zones of inhibition measured. Silver nanoparticles that give significant activity against test organisms were further tested against test organisms to determine their MICs.

Results and Discussion

Table 1: MIC of Ag-NPs and MIC of Meropenem on Carbapenemase Resistant Isolates

Isolates	Ag-NPs MIC (µg/ml)	Meropenem MIC (µg/ml)
K. pneumoniae ATCC BAA 1705	1000	4.0
KP1	250	4.0
KP2	250	16.0

Kp1= *K. pneumoniae* 1 isolated *O. niloticus*, Kp2= *K. pneumoniae* 2 isolated from *O. niloticus*

Table 2: Fractional Inhibitory Concentrations of combination of the Ag-NPs and Meropenem

Isolates	FIC	Inference
K. pneumoniae ATCC BAA 1705	0.5	Synergistic effect
KP1	0.5	Synergistic effect
KP2	0.25	Synergistic effect

Kp1= *K. pneumoniae* 1 isolated Cat fish, Kp2= *K. pneumoniae* 2 isolated Cat fish

The indiscriminate use of antimicrobials has led to the development of antimicrobial resistance to drugs. Several researchers have reported the cost-effective use of medicinal plants remedies. Reported synergy of herbal remedies with convectional antimicrobials. The current study supports the continued intensive study of traditional remedies, conservation and value addition of *A. Vera* in ethno medicinal use. Noted that the emergence of bacterial resistance threatens to return us to the era before the development of antibiotics due to increase in antimicrobial resistance in health care associated pathogens. The rapid development of resistance including the emergence of multi-drug-resistant pneumonia (MDR-P) shows that the potency of prevalent antibiotics is decreasing steadily. This situation calls for urgent need for new and safe antimicrobials for replacement of invalidated antimicrobials or use antibiotic in a rotation programs (Hoiby *et al.*, 2010) [10].

The antibacterial activity of silver nanoparticles synthesized *A. vera* against *K. pneumonia* isolated from against *Oreochromis niloticus* observed in this study justifies the use of *A. vera* for medicinal purposes for treatment of bacteria related diseases. The antimicrobial activity of Ag-NPs synthesized from *A. Vera* observed in this study is in agreement with the study earlier reported by Hoiby *et al.* (2010) [10].

The activity of the Ag-NPs synthesized from *A. vera* against the carbapenemase producers is an indication that the particles may be useful as treatment regimen for carbapenemase producing *K. pneumoniae* causing both pneumonia and UTI. It is well known that carbapeneme producers are serious global threat and may be responsible for high rate of morbidity and mortality.

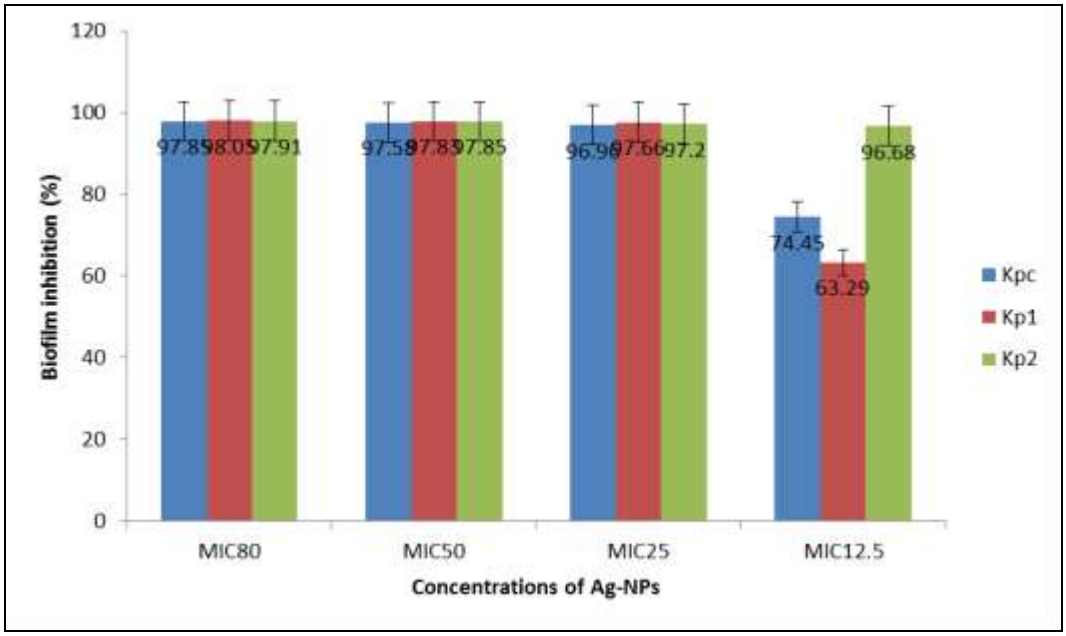

Fig 1: Percentage inhibition of biofilm by different concentration of combination of Ag-NPs and Meropenem

Conclusion

The synthesized silver nanoparticles from crude leaf extracts of *Aloe vera* was of small size and the functional groups; alcohol and alkenes, were predominantly detected in the nanoparticles. This study has revealed that silver nanoparticles of *Aloe vera* extracts has antibacterial and biofilm inhibitory effect, hence, a cheaper and safe method to combat multidrug resistance but the combination of the silver nanoparticles and meropenem had synergistic effect against most of the isolates.

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