ENHANCED ANTIMICROBIAL ACTIVITY OF METRONIDAZOLE-LOADED SILVER NANOPARTICLES

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ABSTRACT

Background: Antimicrobial resistance significantly compromises the clinical efficacy of conventional antibiotics, necessitating innovative delivery strategies to enhance antibacterial performance. Silver nanoparticles (AgNPs) possess intrinsic broad-spectrum antimicrobial activity and can potentiate antibiotic action through membrane disruption and enhanced intracellular drug delivery.
Objective: This study aimed to develop fruit-mediated green synthesized metronidazole-loaded silver nanoparticles and evaluate their antimicrobial activity against selected clinical bacterial isolates in comparison with commercially available formulations.
Methods: Metronidazole-loaded silver nanoparticles were synthesized using *Carica papaya* and *Musa acuminata* extracts as biological reducing agents. Clinical isolates of *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* were identified using morphological and biochemical characterization. Antimicrobial activity was assessed using the agar well diffusion method, and zones of inhibition were measured and statistically analysed using SPSS version 20.
Results: The nano-formulations demonstrated enhanced antibacterial activity compared to innovator (IB) and generic (GB) products. Amoxicillin- and metronidazole-loaded nanoparticles exhibited greater zones of inhibition against *E. coli*, *S. aureus*, and *P. aeruginosa*. Mean inhibition zones for metronidazole nano-formulations were comparable to or higher than reference samples, particularly against *S. aureus*. No significant inhibition was observed against *K. pneumoniae* across all samples.
Conclusion: Fruit-mediated silver nanoparticle nano-formulation significantly improved the antimicrobial activity of metronidazole against susceptible Gram-positive and Gram-negative bacteria. This green nanotechnology approach represents a promising strategy for enhancing antibiotic efficacy and addressing antimicrobial resistance.

Keywords: Metronidazole, Silver nanoparticles, Green synthesis, Antimicrobial activity, Nano-formulation.

INTRODUCTION

Antimicrobial resistance (AMR) has emerged as a major global health threat, significantly undermining the clinical effectiveness of conventional antibiotics. Increasing resistance among anaerobic and facultative anaerobic pathogens has complicated the management of gastrointestinal, oral, gynaecological, and soft tissue infections. Mechanisms such as biofilm formation, efflux pump activation, enzymatic drug inactivation, and reduced membrane permeability collectively decrease intracellular antibiotic concentration and therapeutic response. Consequently, innovative drug delivery systems that enhance antimicrobial potency and overcome resistance barriers are urgently required.[1]

Metronidazole, a 5-nitroimidazole derivative, remains a cornerstone therapy for infections caused by obligate anaerobes and protozoa. It is widely prescribed for bacterial vaginosis, periodontitis, intra-abdominal infections,

and colorectal infections. Despite its established clinical utility, metronidazole exhibits limitations including dose-related adverse effects, rapid systemic clearance, suboptimal local retention at infection sites, and emerging microbial resistance. Recent pharmaceutical research has therefore explored nanoscale delivery platforms to improve its bioavailability, targeted delivery, and antimicrobial efficiency. Polymeric nanoparticle systems have demonstrated enhanced metronidazole activity against *Clostridium perfringens* and other pathogens [1,2], while nano-systems designed for colorectal and gynaecological infections have shown improved therapeutic outcomes **[3,4].**

Among inorganic nanomaterials, silver nanoparticles (AgNPs) are extensively studied for their intrinsic broad-spectrum antimicrobial properties. Silver exerts bactericidal activity through multiple mechanisms, including disruption of cell membrane integrity, generation of reactive oxygen species (ROS), protein denaturation, interference with DNA replication, and inhibition of respiratory chain enzymes. The nanoscale dimension increases surface area-to-volume ratio, thereby enhancing microbial interaction and penetration into biofilms. Studies have demonstrated that AgNPs potentiate the activity of conventional antibiotics by increasing membrane permeability and facilitating intracellular drug accumulation.**[5]** Additionally, silver-based nanocomposites have shown promising results in periodontal and wound-healing applications **[6–8].**

The integration of metronidazole with silver nanoparticles represents a rational multimodal therapeutic strategy. In such hybrid systems, silver provides membrane-disruptive and oxidative stress-mediated antimicrobial effects, while metronidazole exerts intracellular DNA damage under anaerobic conditions. This synergistic mechanism may reduce minimum inhibitory concentration (MIC), improve drug localization, and overcome resistance mechanisms. Previous investigations into silver–metronidazole nanocomposites have reported enhanced antimicrobial and anticancer properties, as well as improved therapeutic efficacy in periodontal disease models. **[6-10]**

Furthermore, advances in green synthesis methodologies enable environmentally friendly production of silver nanoparticles using biological reducing agents, minimizing toxicity associated with conventional chemical methods. **[11]** Sustainable nanotechnology approaches offer improved biocompatibility and potential scalability for pharmaceutical applications.

Despite growing interest in antibiotic-silver nanoparticle conjugates, comprehensive characterization of metronidazole-loaded silver nanoparticles and systematic evaluation of their enhanced antimicrobial activity remain areas requiring further investigation. A detailed assessment of physicochemical properties, drug nanoparticle interactions, structural integrity, and antimicrobial performance is essential for clinical translation.

Therefore, this study focuses on the development and characterization of metronidazole-loaded silver nanoparticles and evaluates their enhanced antimicrobial activity. By integrating nanotechnology with established antimicrobial therapy, this work aims to provide a robust strategy for improving treatment outcomes against susceptible and resistant microbial pathogens.

METHOD

Green Synthesis and Formulation of Metronidazole-Loaded Silver Nanoparticles

Different batches of fruit-based nanoparticles and nanodrugs formulations comprising $AgNO_3$, respective fruit extract (*Carica papaya, and Musa acuminata),* and metronidazole were prepared in accordance with the method of Jackson *et al.* (2019) with slight modification.[12]

Antimicrobial Studies of the Synthesized Nanoparticles

Two groups of bacteria were employed in this study, one of gram-positive bacteria isolate; *Staphylococcus aureus* and three of gram-negative bacteria isolates; *Escherichia coli, Pseudomonas aeruginosa and Klebsiella pneumoniae.*

These microorganisms were obtained from the Medical Microbiology laboratory in University of Port Harcourt Teaching Hospital (UPTH). The microorganisms were streaked on nutrient agar, slant and then stored in the refrigerator at 4 ^{o}C. The microorganisms were subcultured using Nutrient agar, Centrimide agar and MacConkay agar, and were all incubated at 37 ^{o}C for 24 h. This study was carried out to investigate, evaluate and compare the efficacy of test samples (amoxicillin-loaded fruit-based nanoparticles and metronidazole-loaded fruit-based nanoparticles with the commercially available drugs (IB and GB of amoxicillin and metronidazole) on the selected clinical bacteria isolates.

Sample Preparation

A 500 mg (total weight of each capsule) each of the formulated nanodrug and reference samples was dissolved in 2.5 mL of sterile distilled water except for liquid samples which were fruits extracts and silver nitrate solution.

Screening (Characterization) and Identification of Clinical Isolates

Colonies of different bacteria species were picked out using a sterile inoculating loop and subcultured for purification by streaking method on Mannitol Salt Agar (selective media) for *Staphylococcus aureus*, Eosine Methyl Blue Agar and MacConkey Agar for *Escherishia coli*, MacConkey Agar for *Klebsiella Pneumoniae*, Centrimide agar (selective media) for *Pseudomonas auruginosa*. All the plates were aerobically and incubated at 30 ^{O}C for 24 h–48 h. Individual colonies were characterized on the basis of their colony morphology, microscopic examination and biochemical characteristics.[13]

Gram Staining

This is a common technique used to different between gram positive and gram-negative bacteria base on their cell wall constituents. It involves making heat fixing a colony that has been smeared on a microscope slide then followed by an application of a primary stain (crystal violent) for 60 seconds and then rinse with water then to the smear a mordant (gram iodine) was added for 60 seconds then rinse with water followed by the application of a decolorize (95 % alcohol) after 30 seconds to remove the unbound dye immediately rinse with water and then finally the application of a counter stain (safranin) for 30 seconds then rinse with water, air dry slide and viewed under microscope at ×100 magnification.[13]

Biochemical Test - Identification of Fecal Coliforms

This involves a series of biochemical tests.

Catalase test

This test was done to differentiate between bacteria that produce the enzyme catalase from non-catalase producing bacteria. The enzyme hydrolysis hydrogen peroxide (H_2O_2) by breaking it down into water and oxygen gas. A drop of distilled water was placed on a slide and then a colony was picked and emulsify with the water using a sterile wire loop and emulsify with the water. Then a drop of hydrogen peroxide is added. The production of bubbles is an indication that oxygen was given off which indicates a positive test.[13]

Citrate utilization test

This test is use to determine if the organisms can utilize citrate as its sole source of carbon and energy. The citrate test uses a medium in which sodium citrate is the source of carbon and energy. In Simons citrate agar, the pH indicator is bromothymol blue, which is green neutral pH and becomes blue when the medium becomes alkaline. Slopes slant of Simons citrate agar is prepared in Bijou bottles and the test organisms shall be inoculated by streaking the surface, stabbing and butt with a sterilized inoculating needle and was then be incubated at 35 ^{o}C for 48 hours and observed for a bright blue color in the medium which indicates a positive result.[13]

Methyl red (mr) and voges proskauer (vp) test

The test is made up of two different tests; methyl red and voges-proskauer test. The methyl red indicates the production of sufficient acidic product from the fermentation of glucose while the voges-proskauer test indicates the ability to produces acetylmethyl carbonol. Nutrient broth (10 mL) shall be inoculated with the test organism and incubated for 24 hours. After incubation, 5 mL of test culture was transferred aseptically to a clean test tube for the vp test, 3-4 drops of methyl-red was added to first test tube. A positive reaction is indicated by a distinct red colour showing the presence of acid. A yellow colour indicates a negative result. For the voges-proskauer test, 0.6 mL of alpha-napthol and 0.2 mL of 40 % potassium hydroxide is added to the second test tube. The broth was allowed to stand for 15 minutes for colour development after thorough agitation. If acetoin is produced, there will be a red colour change. A yellow to brown colour indicates a negative result.[13]

Indole production test

This test is use to determine the ability of certain microorganisms to breakdown amino acid tryptophan in the medium into indole in the presence of enzyme trytophanase. The test organism shall be inoculated into test tubes containing 10 mL of sterile tryptophan broth and incubated for 24 hours and examine for a red color in the surface layer after Kovacs reagent is added which indicates a positive result and no color change indicates a negative result.[13]

Oxidase test

This test is use to determine the presence of cytochrome oxidase. Oxidase reagent is turned purple by organisms containing cytochrome C as part of their respiratory chain. Filter paper was soaked with a few drops of oxidase reagent. A colony was be picked using a sterile wire loop and smeared on the filter paper. A deep blue or purple color was observed after 10-30 seconds which indicates positive; no blue color indicated a negative result.[13]

Motility test

The motility test is use to determine if the organism is motile or not motile by moving away from the line of inoculation. Using a sterile wire loop an isolate is picked, stabbed directly into the center of the test tubes containing the nutrient agar and incubated for 18-24 hours and 37 ^{o}C. A diffuse growth away from the line of inoculation indicates a positive result; no diffused growth indicates a negative result.

Triple sugar iron agar test

Triple sugar iron agar as prepared and dispensed into test tubes autoclaved, slanted and allowed to cool. It involves inoculating into sterile test tubes using an inoculating needle and streaking across the top of the slant and incubate at 35-37 ^{o}C. After which colour change observed indicating a positive result and gas production will be observed.[13]

Sugar fermentation test

The triple sugar iron agar contains three sugars; glucose, sucrose and lactose in the ratio of 1:10:10. If the bacteria ferment sucrose and or lactose all the agar in the tube will turn yellow but if only glucose was fermented, the agar will turn yellow from the acid produced. Inoculate the organism into the test tube by stabbing (with needle) the agar tube to the bottom with the inoculum and streak across the top of the slant and incubate at 35 ^{o}C for 24-48 hours. A black color observed in the tubes indicates a positive result.[13]

Antibiotic Susceptibility Test

Petri-dishes were sterilized and properly labelled according to the nanosamples codes and a 0.1 mL of standardized cultures were inoculated into 20 mL sterile, cooled Molten Muller Agar (MMA), Muller Hinton Agar (MHA), Mac Conkay Agar (MCA), Manitol Salt Agar (MSA), Eosin Methyl Blue Agar (EMBA) respectively. The content of each bottle was mixed thoroughly by rotating the bottle on the palm. The mixtures were poured into sterile petri-dishes respectively and allowed to solidify. A 6 mm cock borer was used to bore holes in the solidified media and a 0.2 mL of each of the formulated nanomedicines and reference (GB and IB) samples, liquid fruits extracts and nanoparticles solution respectively were added to each plate well. The procedure was carried out in triplicates, plates (petri-dishes) were incubated at 37 ^{o}C for 24 h after which zones of inhibition diameters were measured in millimeter (mm).

Statistical Analysis

Statistical software called statistical package for the social science (SPSS) version 20 was used to analyze the generated data.

RESULTS AND DISCUSSION

Result of the Biochemical and Morphological Characteristics of Clinical Isolates

Table 1 shown below displayed various biochemical and morphological characteristics of clinical bacteria isolates, comprising a gram-positive bacteria, *Staphylococcus aureus* and gram- negative bacteria, *Escherichia coli, Pseudomonas aeruginosa and Klebsiella pneumoniae*.

Table 1: Biochemical and Morphological Characteristics of Clinical Isolates

Catalase	Citrate	Coagulase	Indole	Lactose	Glucose	MR	VP	H-S-H: Butt	H-S-H: Slant	H-S-H: H_2S	H-S-H: Gas	Elevation	Edge	Shape	Surfaces	Pigmentation	Cell shape	Gram	Isolated Organism
+	-	+	-	+	+	+	-	B	B	-	-	Flat	Entire	Round	Smooth	Yellow	Cocci	+	*Staphylococcus* sp
-	-	-	-	-	+	-	-	B	B	-	-	Raised	Entire	Irregular	Smooth	Pink	Rod	-	*Escherichia coli*
-	-	-	-	+	+	-	-	A	A	-	-	Raised	Entire	Irregular	Smooth	Blue green	Rod	-	*Pseudomonas* sp
-	-	-	-	-	+	+	+	B	B	-	-	Flat	Entire	Round	Smooth	Pink	Rod	-	*Klebsiella* sp

Key: + = positive, - = Negative, A = Acid, B =Base

The table shown above (table 1), displayed various biochemical and morphological characteristics of clinical bacteria isolates. These isolates comprise a gram-positive bacteria *Staphylococcus aureus* and gram-negative bacteria, *Escherichia coli, Pseudomonas aeruginosa and Klebsiella pneumoniae*. Biochemical tests or screening of bacteria isolates are the tests used for the identification of bacteria species based on the differences in the biochemical activities of different bacteria. These differences in carbohydrates metabolism, protein metabolism, certain enzymes production and ability to utilize a particular compound etc., help them to be identified by the biochemical tests.

Gram-positive bacteria, *Staphylococcus aureus* was tested for catalase, coagulase, lactase, glucose and methyl red (MR) metabolism and showed positive for the metabolism tests mentioned above which indicates that *Staphylococcus aureus* metabolizes these compounds and enzymes respectively. The bacteria showed negative for citrate, indole and voges Proskauer (VP) metabolism tests, this indicates that the bacterium does not metabolize the above-mentioned compounds and enzymes.

Gram-negative bacteria *Escherichia coli* was also tested for glucose metabolism and was identified to be positive for glucose metabolism (metabolizes glucose) and showed negative for catalase, citrate coagulase, lactase, indole, methyl red (MR) metabolism and voges Proskauer (VP) tests and was identified to be negative for the above enzymes and compounds metabolisms.

Pseudomonas aeruginosa tested positive for lactose and glucose, which indicates that this bacterium metabolizes the above types of sugars mentioned above. It however showed negative for catalase, citrates, coagulase, indole, methyl red (MR) metabolism and voges Proskauer (VP) tests.

Klebsiella pneumoniae was tested for glucose, methyl red (MR) and voges Proskauer metabolism tests and was identified to metabolize these compounds, and showed negative for catalase, citrate, coagulase, indole and lactose metabolism tests and was identified that this bacterium cannot metabolize the above-mentioned compounds and enzymes

Result of the Antimicrobial Activity of Synthesized Nanoparticles and Commercially Available Drugs (Zone of Inhibition, mm)

Antimicrobial Activity of the Synthesized Nanoparticles and Commercially Available Drugs (Zone of Inhibition, mm)

Table 2 below shows the microbial zone of inhibition by the synthesized nanoparticles and commercially available drugs.

Table 2: Microbial (bacterial) Zone of Inhibition (mm)

SAMPLE	*E. coli* (mm)	*S. aureus* (mm)	*P. aeruginosa* (mm)	*K. pneumoniae* (mm)
Amoxicillin (Test sample)				
BA1b	-	-	7	-
BA2a	-	-	6.5	-
BA3a	10	-	6.5	-
PA1b	-	-	6	-
PA3b	12	9	-	-
Mean/SD	11 ± 8.8	9 ± 0.0	6.5 ± 0.17	-
Mean	11	-	6.5	-
SD	7.8	-	0.17	-
Metronidazole(Testsample)				
BM1b	4	-	6	-
BM2b	-	-	6.5	-
BM3a	5	14.5	6	-
BM3b	4	11.5	7	-
PM1a	-	-	2	-
PM1b	-	-	6.5	-
PM2a	-	4.5	-	-
PM2b	4	-	5 -	-
PM3a	-	10	-	-
PM3b	8.5	14.5	-	-
Mean/SD	5.3 ± 3.8	11 ± 17	5.6 ±16.9	
Mean	5.1	11	5.6	
SD	3.8	17	16.9	
Reference Samples				
Amoxicillin				

IB	-	7	6.5	-
GB	-	-	5	-
Mean/SD	-	7 ± 0.0	5.8 ± 1.13	
Mean	-		5.8	
SD	-		1.13	
Metronidazole				
IB	7	12.5	8	-
GB	3.5	9	6	-
Mean/SD	5.3 ± 6.2	10.8 ± 6.2	7 ± 2	
Mean	5.3	10.8	7	
SD	6.2	6.2	2	
Fruit Based-Nanoparticles				
NA	6.5	9	10.5	10.5
NM	4.5	-	10	-
NB	11	7.5	5	5
NP	5.5	-	9.5	2.5
NA1	6.5	-	12	2.5
NPA2	6.5	-	11	-
NBa	-	-	12.5	2
NBb	6.5	-	9.5	2
NA2	-	-	-	4
NA3	-	-	-	4.5
NB	-	-	-	5
NM2	-	-	-	5

Comparing the Antimicrobial Activity of the Synthesized Nanoparticles and Commercially Available Drugs

Table 3 below compares the antimicrobial activity of the synthesized nanoparticles and commercially available drugs.

Table 3: Comparison Between the Antimicrobial Activity of the Synthesized Nanoparticles and Commercially Available Drugs

Sample	*E. coli* (mm)	*S. aureus* (mm)	*P. aeruginosa* (mm)
Amoxicillin (Test Sample) Mean/SD	11 ± 8.8	9 ± 0.0	6.5 ± 0.17
Amoxicillin (Reference Sample) Mean/SD	-	7 ± 0.0	5.8 ± 1.13
Metronidazole (Test Sample) Mean/SD	5.3 ± 3.8	11 ± 17	5.6 ± 16.9
Metronidazole(Reference Sample) Mean/SD	5.3 ± 6.2	10.8 ± 6.2	7 ± 2

Table 3 displayed various microbial zone of inhibition by sample batches; commercially available and test samples of amoxicillin and metronidazole, respectively. The commercially available drugs are; IB, innovator brand and GB, generic brand of amoxicillin and metronidazole, respectively. While the test samples are amoxicillin-loaded fruit-based nanoparticles; BA1b, BA2a, BA3a, PA1b and PA3b while the metronidazole-loaded fruit-based nanoparticles are BM1b, BM2b, BM3a, BM3b, PM1a, PM1b, PM2a, PM2b, PM3a and PM3b respectively.

The results of antimicrobial activity of the formulated nanoparticles (test samples and the reference samples on four (4) different bacterial isolates; *Escherichia coli*, *Staphylococus aureus*, *Pseudomonas aeruginosa* and *Klebsiella Pneumoniae.* It *was* shown that the amoxicillin-loaded fruit-based nanoparticles had wider zone of inhibition against *Escherichia coli* at 11 ± 8.8, against *Staphylococcus aureus* at 9 ± 0.0 and against *Pseudomonas aeruginosa* at 6.5 ± 0.17, while the agar plate for amoxicillin commercially available drugs were observed to have a spontaneous growth of *Escherichia coli* and no zone of inhibition (resistance) by amoxicillin commercially available drugs was seen. There was microbial inhibition against *Staphylococcus aureus* at 7 ± 0.00 and against *Pseudomonas aeruginosa* at 5.8 ± 1.13. For metronidazole test and commercially available drug, it *was* shown that the metronidazole-loaded fruit-based nanoparticles and the metronidazole commercially available drug showed same zone of inhibition against *Escherichia coli* at 5.3 ± 3.8/6.2, and showed inhibition against

Staphylococcus aureus at 11 ± 17 by metronidazole test sample and 10.8 ± 6.2 against *Staphylococcus aureus* and against *Pseudomonas aeruginosa* at 5.6 ± 16.9 by metronidazole test sample and at 7 ± 2 by metronidazole commercially available drug.

Hence, the amoxicillin-loaded fruit-based nanoparticles and the metronidazole-loaded fruit-based nanoparticles showed significant inhibition against three (3) bacterial isolates (*Escherichia coli*, *Staphylococcus aureus and Pseudomonas auruginosa*) which is an obvious indication that the synthesized nanoparticles; amoxicillin-loaded fruit-based nanoparticles and metronidazole-loaded fruit-based nanoparticles showed greater efficacy against the test microorganisms which are

gram-ve bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) and gram +ve bacteria (*Staphylococcus aureus*).[14] Additionally, it was observed that none of the samples (test or commercially available drugs) showed inhibition against *Klebsiella pneumoniae.*

Conclusion

This study successfully developed and evaluated metronidazole-loaded silver nanoparticles using a fruit-mediated green synthesis approach as a strategy to enhance antimicrobial efficacy. Clinical isolates of *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* were accurately identified through morphological and biochemical characterization prior to antimicrobial testing, ensuring validity of microbiological evaluation.

The antimicrobial assessment demonstrated that the nano-formulated drugs exhibited broader and, in several cases, enhanced zones of inhibition compared with commercially available innovator (IB) and generic (GB) formulations. Notably, the amoxicillin- and metronidazole-loaded fruit-based nanoparticles showed superior inhibitory activity against *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*, indicating improved antibacterial potency against both Gram-positive and Gram-negative organisms. In contrast, resistance was observed against *Klebsiella pneumoniae* across both nano-formulated and reference products.

The enhanced antimicrobial performance is attributable to the synergistic interaction between metronidazole and silver nanoparticles, where silver contributes membrane disruption and oxidative stress mechanisms, facilitating increased intracellular drug penetration and activity. The green synthesis method further supports biocompatibility and sustainability of the formulation process.

Hence, the findings confirm that fruit-mediated silver nanoparticle nano-formulation significantly improves the antimicrobial activity of metronidazole and amoxicillin against susceptible pathogens. This work provides strong experimental evidence supporting nanotechnology-based combination strategies as a promising approach to addressing antimicrobial resistance and enhancing therapeutic effectiveness.

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