***Original Research Article***

***Eco-friendly Biosynthesis of anti-mycobacterium Silver Nanoparticles Using Ficus ingens for potential use in tuberculosis and leprosy***

**Abstract**

Treatment of *Mycobacterium* infections including tuberculosis (TB) and leprosy continue to face challenges from drug resistant mycobacterium and bacterial strains. This has hampered efforts to contain the pandemicdue to rampant treatment failures based on current drugs. Therefore, it is imperative to develop novel therapeutics. This study explored the Biosynthesis of silver nanoparticles (AgNPs) using *Ficus ingens* root extract, evaluating their antimicrobial activity. *Ficus ingens*, a large evergreen prevalent in sub-Saharan Africa has been widely used in TB treatments in traditional medicine in Zimbabwe. Preliminary phytochemical screening of the lyophilized root extract was done using various classical techniques, Oral toxicity evaluations were conducted based on OECD technical guideline 425.The anti-microbial tests were conducted using agar-well diffusion methods on *Mycobacterium* *smegmatis* as well *Escherichia coli*, and *Staphylococcus aureus*. with Rifampin ® as a standard. The bio inspired silver nanoparticles were fabricated through green synthesis techniques and characterized using UV, TEM and DLS, The Metabolomics studies confirmed the presence of numerous pharmacologically active secondary metabolites including flavonoids, tannins, and phenols. Acute oral toxicity studies determined the LD50 of the lyophilised *Ficus ingens* root extract to be toxicologically safe above 4000mg/kg body weight. UV-Vis confirmed the formation of the silver nanoparticles, Transmission electron microscopy and dynamic light scattering confirmed the AgNPs to have an average size of 38 nm, with various shapes including spherical and cubic morphologies. Antimicrobial assays demonstrated that the *Ficus ingens* extract and the AgNPs exhibited activity against *Mycobacterium* smegmatis, *Escherichia coli,* and *Staphylococcus aureus* comparable to the standard. Based on the foregoing , it was concluded that the biosynthesis of AgNPs mediated by *Ficus ingens* was feasible, efficacious, safe and presents a potential adjunct treatment for mycobacterium treatments in neglected tropical diseases.

Keywords: *Ficus ingens*, silver nanoparticles (AgNPs), green synthesis, antimicrobial activity, *Escherichia coli*, *Staphylococcus aureus*, phytochemicals, drug resistance

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# INTRODUCTION

## *Ficus ingens*

*Ficus ingens* (Fig tree), is a semi-evergreen tree with a spreading crown, found across sub-Saharan Africa [1]. The large tree is widespread because it easily adapts to various habitats and soils [2]. Its dull green leaves are heart-shaped or lanceolate with yellow veins, turning from coppery/reddish when young. It bears stalked figs that change from white to pink/red/purple when ripe (figure 1) [3, 4]. In Zimbabwean traditional medicine*, F. ingens* has been used totreat anemia, pile, and diarrhea [5]. The Shonas employ the bark to boost milk production in cows and the South African Zulus use the same tree bark to treat anemia, while in Nigerian, the tree bark, roots and leaves are used to relieve for pile, diarrhea, and as a laxative/diuretic [6] [7]. The plant leaves of *Ficus ingens* are reported to be toxic to cattle, and other reports confirm that the leaf extracts demonstrate anti-inflammatory, analgesic, hypotensive, laxative, and antirheumatic effects [8]. The fruit and leaves from the plant are food and nutraceuticals used by both humans and animals [4]. The weeping bark produces latex which is used locally as a disinfectant that aids in wound healing, the leaves have widely been used in treating malaria, dysentery, STIs, chest issues, convulsions, pain, and wound healing [9] [10] [11]. It is however the plant’s cardinal use in mycobacterium treatments including tuberculosis and leprosy that has motivated this study due to the increase in drug resistant microbes responsible for these conditions to conventional medicines [12] [13]

 

 Fig 1. Images showing wild *Ficus ingens* tree, leaves and fruits

## Antibacterial resistance

Antibacterial resistance is a global health issue, affecting every country worldwide [14]. Since its recognition over 50 years ago, resistance to antimicrobials has led to a significant increase in illness, death, and healthcare costs [15]. Bacteria have continually adapted and developed strategies to evade antibiotic effects, rendering many antimicrobial agents ineffective. The growing presence of multidrug-resistant bacterial strains, including those from the Pseudomonas, Klebsiella, and Staphylococcus species, is particularly alarming. The emergence of extensively drug-resistant strains, which are resistant to most available antibiotics, poses a significant threat [16]. If left unchecked, antimicrobial resistance is projected to claim over 10 million lives within the next two and a half decades, highlighting the urgent need for effective intervention [17].

Antimicrobial resistance can be categorized into two types: intrinsic resistance, where microorganisms are naturally resistant to certain drugs, and acquired resistance, which develops as a result of evolutionary pressure to counteract antimicrobial agents [15]. The latter can occur through various mechanisms, including genetic mutations, gene transfer, and horizontal gene transfer between microorganisms [17]. Furthermore, microbes have developed multiple strategies to evade the host immune system and chemotherapy, such as using efflux pumps, modifying membrane permeability, and degrading antimicrobial agent [15]. The complex cell wall structure of certain microorganisms, such as *Mycobacterium* species, also contributes to resistance, making it challenging to treat infections and limiting the availability of effective therapeutic options. The impact of antimicrobial resistance is devastating and widespread, with significant consequences for public health. For example, in the case of tuberculosis, the treatment success rates for drug-resistant strains have been relatively low, ranging from 54-65% [14], although the introduction of new treatments such as bedalaquine, pretomanid, and linezolid (BPaL) has shown promise [18]. However, concerns have already been raised about the emergence of resistance to these new treatments, with cases reported in clinical settings [19]. The development of antimicrobial resistance by bacteria is inevitable and is considered a major problem in the treatment of bacterial infections in hospitals and the community. The overuse of antibiotics and consequent antibiotic selective pressure is thought to be the most important factor contributing to the appearance of different kinds of resistant bacteria, even in the treatment of tuberculosis [16]. On the other hand, the use of bioactive compounds can help in fighting resistance by providing alternative modes of antimicrobial activity that can target and kill bacteria in new and innovative ways [20]. This approach can be used always, as bioactive compounds can be continuously developed to stay ahead of the evolving resistance landscape.

## Mycobacterium infections

Mycobacteria are aerobic, rod-shaped bacteria that do not form spores and that are lipid-rich with long-chain mycolic acids in their cell walls, which are largely responsible for their acid fastness [21]. Modern genomic, phylogenetic, and ecological studies have shed light on the origins of the most important mycobacterial infections affecting humans [22]. For example, leprosy and tuberculosis (TB) have had a profound effect on human suffering for thousands of years [23]. The genus *Mycobacterium* is part of the order Actinomycetales and the phylum Actinobacteria and belongs to a variety of environmental habitats, including natural waters, soils, and drinking water distribution systems [24]. Mycobacterial species reside in a wide variety of environments due to multiple adaptations. Some of the features include the presence of a lipid-rich hydrophobic outer membrane, which is a major determinant of surface adherence, biofilm formation, aerosolization, and antibiotic/disinfectant resistance [23]. Additionally, mycobacteria can replicate at a low rate, providing them with a decreased susceptibility to most antimicrobial agents, and they also possess the ability to grow at low carbon levels, thus making them effective competitors in low-nutrient environments (oligotrophs) [24]. From a large mycobacterial pool, some species have evolved into potential major human pathogens. Genomic events such as genome reduction, critical gene acquisition, gene transfer, mutations, and recombination permitted environmental mycobacteria to evolve into host-associated pathogens [22]. Phylogenetic reconstructions of genomic sequences suggest that *Mycobacterium* *marinum*, *Mycobacterium* *leprae*, *Mycobacterium* *ulcerans*, and M. tuberculosis evolved from a common environmental ancestor [22]. Their gene loss or acquisition reflects fluctuating environmental challenges and host-specific pathoadaptations [22]. The molecular mechanisms by which *M. tuberculosis* and *M. leprae* have evolved to cause disease involve complex interactions between the pathogen and the host. In contrast, the pathogenicity of *M. ulcerans* derives from the acquisition of a plasmid encoding the polyketide toxin mycolactone [24]. Plant-based and marine-derived natural compounds exhibit potential as effective antimycobacterial agents, offering innovative mechanisms to combat tuberculous bacteria [20]. In resource-limited settings, fast-growing, non-pathogenic *Mycobacterium* strains, such as Mycolicibacterium aurum and Mycolicibacterium *smegmatis*, serve as suitable surrogate models for assessing antimycobacterial activities of novel compounds in settings with limited laboratory infrastructure [21]. These strains were chosen for our investigation due to their comparable antimicrobial susceptibility profiles with M. tuberculosis and their safe handling in a laboratory setting.

## Natural antimycobacterial plant polyphenols

The current state of tuberculosis mitigation programs is a pressing concern, as various programs have been put in place since 1882, but their inadequacies are apparent, and global efforts have failed to eliminate TB [25]. This is due to the emergence of MDR and XDR strains. The epidemiology of TB is closely connected with social and economic conditions, which makes TB prevention, care, and control even more challenging. The emergence of MDR and XDR strains of MTB has made it essential to develop newer anti-TB drugs with novel drug targets. Approximately 480,000 newly emerging cases of MDR-TB are estimated to occur every year [14]. Approximately 4% of new TB cases and 20% of previously treated cases are MDR-TB, and only 50% of these cases can be effectively treated [25]. The cost of treating one MDR-TB case is substantial, equivalent to the cost of treating 100 susceptible TB cases [25]. Furthermore, a more severe form of resistance, extensively drug-resistant tuberculosis (XDR-TB), has been identified in 84 countries, highlighting the need for urgent action to address this growing public health threat [25]. Even though pharmacological industries have produced many new antibiotics in the last three decades, resistance to these drugs by microorganisms has increased. In general, bacteria have the genetic ability to transmit and acquire resistance to drugs, which are utilized as therapeutic agents.

Since ancient times, tuberculosis has been a recognized disease, and medicinal plants have traditionally been a primary source of treatment for it [26]. Medicinal plants have been used for centuries to cure TB, and many researchers believe that improved alternative medicines can be developed from plant sources for the treatment of MDR and XDR-TB [19]. Plant species still serve as a rich source of many novel biologically active compounds, yet very few plant species have been thoroughly investigated for their medicinal properties (Amoah, et al., 2014). Approximately 60% of the world's population still relies on medicinal plants for their primary healthcare [27]. A review by Sharma and Yadav cites 72 anti-TB phytochemicals that have been isolated from various plants, including flavonoids and phenolic compounds [28]. The use of plant extracts and phytochemicals, both with known antimicrobial properties, can be of great significance in treatments [5]. Many plants have been used because of their antimicrobial traits, which are due to compounds synthesized in the secondary metabolism of the plant. These products are known by their active substances, for example, the phenolic compounds which are part of the essential oils, as well as tannin [5].

Phytocompounds employ multiple pathways, such as eliciting an inflammatory response, inhibition of metabolic enzymes, and direct killing of *bacilli* [19]. Curcumin and allicin have been shown to have significant antimycobacterial activity against resistant and susceptible *M. tuberculosis* strains [19]. *Ficus ingens* also has been found to possess a diverse range of phytochemicals, including tannins, phenols, glycosides, flavonoids, and saponins [12]. The abundance of these phytochemicals varies among different plant parts, with tannins being the most abundant, followed by phenols, glycosides, flavonoids, and saponins [11]. The medicinal properties and pharmacological effects of *Ficus ingens* can be attributed to the presence of various phytochemicals, which have been shown to support the traditional uses of the genus in fighting against infectious diseases [11]. Tannins are also known for their astringent and antimicrobial properties, making them useful in fighting infectious diseases [29]. Phenols, which are abundant in *Ficus ingens*, play a crucial role in plant defense against pathogens and are effective in controlling human pathogenic infections [30]. The plant is also a good source of glycosides, which have been shown to improve cardiac conditions by reducing blood pressure and inhibiting the accumulation of arteriosclerosis plague and blood clots [13]. Flavonoids, which are present in *Ficus ingens*, are known for their antioxidant and free radical scavenging properties, as well as their wide range of biological activities, including antimicrobial, anti-inflammatory, and anti-allergic effects [29]. Saponins, although present in smaller amounts, have been reported to have inhibitory effects on inflammation, as well as hypolipidemic and anticancer activity [13].

In addition, the bioactivity profiles of natural compounds can be improved through nano-functionalization, such as incorporating plant phytochemicals into nano-formulation [19]. Incorporating plant phytochemicals into nano-formulations could potentially unravel alternative therapies with superior modes of antimicrobial activity, tackling the current antimicrobial resistance challenge [19]. The potential of nano-formulations in tackling antimicrobial resistance is significant, as they can provide a targeted and effective approach to treating TB and other infectious diseases.

## Biosynthesized metallic nanoparticles from polyphenols

Nanotechnology, a rapidly advancing field, utilizes the unique properties that emerge when materials are manipulated at the 1-100 nanometer scale [19]. This capability has spurred diverse applications, particularly within the biomedical domain, encompassing diagnostics and therapeutics. Examples include the use of liposomes, micelles, dendrimers, and both polymeric and metallic nanoparticles [31]. These nanostructures have demonstrated remarkable success in translating laboratory findings into patient care, offering advantages such as improved bioavailability, chemical and therapeutic equivalence, and targeted, controlled, and sustained drug release [31]. These features translate to lower drug dosages and reduced side effects, fueling considerable interest in nanometric drug molecules for enhancing drug efficacy and safety [32].

Similarly, the significant potential of optimized natural bioactive compounds has driven research into drug discovery from natural polyphenols [19]. It has long been recognized that functional groups present in plant secondary metabolites can facilitate the reduction of metallic salts into their elemental nanoscale form [32]. This biological synthesis, also known as green synthesis, presents an opportunity to develop multifunctional biomedical platforms from metallic salts. Existing literature highlights successful biosynthesis of silver, gold, copper, zinc, and iron nanoparticles with applications in treating various diseases, including tuberculosis [31]. This straightforward, one-pot biosynthesis method relies on the inherent bio-reducing, capping, and stabilizing properties of functional groups found in plant secondary metabolites. *Ficus ingens* contains pharmacologically active polyphenolic antimicrobial compounds, making the exploration of synergistic medicinal benefits from both its secondary metabolites and nanometric metallic ions a worthwhile endeavor [6].

Biosynthetically produced metallic nanoparticles are reported to be an effective, safe, and adaptable alternative to chemically synthesized antibacterial nanoplatforms [31]. Furthermore, metallic nanocomposite formulations have shown promise in improving treatment outcomes in animal studies [33]. The therapeutic applicability of metallic nanoformulations is further enhanced by the ability to fine-tune their desired characteristics by adjusting production parameters. The size and shape of the metallic nanoparticles can also be controlled, which significantly impacts their chemical and bioequivalence, as well as their tendency to aggregate [33]. In this study, we detail the biosynthesis of silver nanoparticles using hydroethanolic extracts of *Ficus ingens* secondary metabolites and evaluate the nanoparticles' efficacy against a surrogate *mycobacterium* tuberculosis species, *E. coli* and *S. aureus*.

# METHODOLOGY

This study was conducted with approval from the Joint Research and Ethics Committee of the University of Zimbabwe College of Health Sciences and Parirenyatwa Group of Hospitals. The experiments were performed at the laboratories of the Department of Pharmacy and Pharmaceutical Sciences and the Biochemistry Department. Animals used in the study were handled under ethical guidelines for animal use, and those exhibiting any adverse reactions were humanely euthanized upon completion of the experiments.

### Materials, Equipment and Facilities

Plant extraction, phytochemical characterization, and antimicrobial assays utilized reagents, chemicals, consumables, and equipment sourced from the University of Zimbabwe Pharmacy and Pharmaceutical Sciences Laboratory. In vivo toxicity studies in laboratory animals were performed at the University of Zimbabwe’s Faculty of Medicine and Health Sciences animal laboratory, with all necessary reagents provided by them. The Department of Chemistry and Biochemistry at the University of California, Los Angeles, provided all chemicals, equipment, and facilities for the green synthesis and characterization of silver nanoparticles.

### Test Microorganisms

For the evaluation of potential anti-tubercular activity, a panel of microorganisms was selected, including *Mycobacterium* *smegmatis* as a model organism for *Mycobacterium* tuberculosis. Additionally, *Escherichia coli* and *Staphylococcus aureus* were included to assess the general antibacterial spectrum of the tested compounds. These well-characterized cultures were kindly provided by Prof. Mukanganyama from the Biochemistry Unit at the University of Zimbabwe, with further contributions from the lab technicians within the Department of Pharmacy and Pharmaceutical Sciences. To ensure the reliability of subsequent experiments, the purity and identity of each culture were rigorously confirmed through established microbiological techniques, namely Gram staining and a suite of biochemical identification tests.

### Plant collection and preparation

*Ficus ingens* samples were collected from Mudzingwa village in Buhera, Zimbabwe, situated within agro-ecological regions III-V, characterized by low to moderate rainfall, high summer temperatures, and mild winters. Specifically, Buhera is located at -19° 29' 9.59" S latitude and 31° 49' 23.99" E longitude. Sustainable harvesting practices were employed during sample collection to ensure minimal environmental impact. The *Ficus ingens* samples were subsequently authenticated at the National Herbarium of Zimbabwe. To prepare the plant material for extraction, it was first washed with distilled water to remove any dirt or debris. The samples were then air-dried at room temperature for three weeks to achieve a constant dry weight. Finally, the dried bark of *Ficus ingens* was pulverized into a powder.

### Hydro-ethanolic extract preparation and characterization

*Ficus ingens* root powder was weighed and transferred to a sterile amber bottle. The plant powder was macerated at room temperature for 5 days with shaking in 2.1 L of 70% ethanol. The extract solution was filtered through cotton wool and then under vacuum in a Büchner funnel using Whatman No.1 filter paper. The solvent was removed using a rotary vapor, followed by lyophilization under low pressure and ultra-low temperature. The dried extract was stored in an airtight, sterile amber bottle at 4°C.

### Qualitative phytochemical screening

Qualitative tests for selected phytochemicals known for their antimycobacterial activity were performed using standard screening tests (table 1), as described in related studies by Chipato and Chifamba [19].

Table 1. Metabolomics screening tests done on the lyophilized extract

|  |  |  |
| --- | --- | --- |
| Metabolomics | Screening test done | Results interpretation |
| Test for alkaloids  | Dragendroff’s test. To 5 ml of the lyophilized extract liquor in a test tube, two drops of Dragendroff’s reagent were added [34]. | The presence of alkaloids was determined by the development of a reddish-brown precipitate |
| Tests for tannins | Ferric chloride test. To a test tube, 2-3 drops of ferric chloride was added to 5 ml of the prepared extract liquor [35]. | The presence of catechic tannins signaled by the development of a green-blue color, a blue-black color which indicates the presence of Gallic tannins. |
| Test for phlobotanins | HCL Test. 2ml aq. extract + 2ml 1% HCL (boiled) [36]. | The presence of phlobatanins was determined by a red precipitate  |
| Test for flavonoids | Ammonia test. To 5ml of the lyophilized extract, add 5ml of dil ammonia + conc H2SO4 [34]. | The presence of flavonoids was determined by a yellow color.  |
| Test for saponins | The simplified foam tests. In a 100ml measuring cylinder, 5ml of the extract liquor was added to 30ml distilled water, the mixture was shaken for 2 minutes [36]. | The development of at least 1 cm head of form in the test tube that lasts for 15 minutes confirms the presence of saponins. |
| Test for terpenoids | The Salkowski test: 5 ml of chloroform to 5 ml of the extract liquor in a test tube, followed by the addition of 1 ml of concentrated H2SO4 [37]. | The development of a grey colored solution indicates the presence of terpenoids. |
| Test for phenols | Ferric chloride test. To a test tube, 2-3 drops of ferric chloride was added to 5 ml of the prepared extract liquor [19]. | The presence of phenols was determined by the development of a Dark green/bluish black colour.  |
| Test for glycoside | The modified Borntrager’s assay. 5ml of the extract liquor was mixed with 5ml of dilute hydrochloric acid. The mixture was subsequently treated with 3ml ferric chloride solution and immersed in a water bath at 80oC for 10 minutes. After cooling, extraction was done with 10ml of benzene. The resultant benzene layer was decanted and treated with 5ml ammonia solution [35]. | The mixture was observed for the development of a pink colour which signals the presence of anthranol glycosides. |
| Test for anthocyanins | HCL test. 2ml. plant extract + 2ml.2N HCL (+few ml ammonia) [19].  | Pink-red sol which turns blue- violet after addition of ammonia.  |

### Quantification of total flavonoid and tannins

The total flavonoid content of the lyophilized *Ficus ingens* extract was determined spectrophotometrically at 510 nm. Following established procedures [38]. 1 mg of the extract was dissolved in 2 mL of distilled water. To this solution, 0.5 mL of 1M sodium nitrite and 2 mL of a 1M NaOH solution were added, and the volume was adjusted to 10 mL with distilled water. The mixture was shaken and allowed to stand at room temperature for 15 minutes, after which the absorbance was measured. The total flavonoid content was expressed as milligrams of quercetin equivalent per gram of extract (mg QE/g extract) on a dry weight basis, using a standard curve. The Prussian Blue Assay, employing optical density (OD) values at 700 nm, was used for the quantification of tannins [39].

### Acute oral toxicity testing of lyophilized F. ingens

The acute oral toxicity study of *Ficus ingens* was conducted using the Up-and-Down Procedure (UDP) following OECD Test Guideline, following the main test protocol based on the original procedure described by Bruce [40] and adopted by the ASTM in 1987 with revisions in 1990 [41]. This approach is designed to estimate the median lethal dose (LD₅₀) while using fewer animals than traditional methods. A total of six adult healthy female nulliparous and non-pregnant Wistar albino rats, aged between 8 and 10 weeks and weighing approximately 215 ± 10 grams, were used in the main test. The animals were selected based on health assessments by a certified veterinary officer and housed under standard laboratory conditions. A 10-day acclimatization period was observed, during which the animals were fed a standard rodent diet and provided with water ad libitum. Before dosing, the animals were fasted for 18 hours with free access to water. Each rat was dosed individually via oral gavage using a fixed volume of *Ficus ingens* extract dissolved in distilled water. The initial dose was set at 250 mg/kg body weight, based on prior range-finding and estimation of sub-lethal effects. Following Bruce's original up-and-down approach, dosing continued one animal at a time, with a minimum of 48 hours between animals to allow for adequate observation of clinical signs and mortality. Each subsequent dose was determined by the response of the previously treated animal. If the animal survived, the dose for the next rat was increased by a factor of 3.2; if the animal died, the next dose was reduced. The doses range from 250mg, 800mg, 2560mg, up to 4000mg. This sequence continued until the stopping criteria defined by OECD 425 were met. All animals were closely observed during the first 30 minutes post-dosing, then hourly for the first 12 hours, and subsequently twice daily for a total of 14 days. Observations included behavioral changes, signs of toxicity (such as lethargy, tremors, salivation), and mortality. Body weights were recorded on Day 0 (before dosing), Day 7, and Day 14. This protocol allowed for the ethical assessment of the acute oral toxicity of *Ficus ingens* with a reduced number of animals, while still obtaining scientifically valid and reproducible data.

## Biosynthesis of silver nanoparticles

The biosynthesis of silver nanoparticles using *Ficus ingens* root extract was carried out through a green synthesis approach [38, 19]. Initially, 2 grams of lyophilized *Ficus ingens* root extract was dissolved in 50 ml of distilled water. The solution was stirred for 15 minutes and gently heated to 50°C to facilitate the extraction of phytochemicals, which act as reducing and stabilizing agents. To this mixture, 1 gram of silver nitrate (AgNO₃) was added while maintaining the temperature and continuous stirring for approximately 60 minutes. A gradual color change from pale yellow to dark brown indicated the successful formation of silver nanoparticles due to the reduction of Ag⁺ ions by the plant’s bioactive compounds. Following synthesis, the reaction mixture was left to stand at room temperature overnight to ensure complete nanoparticle formation. The resulting solution was then centrifuged at 4000 rpm for 30 minutes to separate the nanoparticles. The supernatant was carefully decanted, and the pellet was washed three times with 70% ethanol to remove any unbound phytochemicals or impurities. The purified AgNPs were dried in a hot air oven at 100°C for 24 hours. Finally, to improve crystallinity and stability, the dried particles were calcined in a furnace at 500°C for 3 hours.

## Characterization of the biosynthesized AgNPs

The identity of the synthesized nanoparticles was verified using a UV-Vis spectrometer (Hitachi, UH5300) [19]. Transmission electron microscopy (TEM) was employed to analyze the size and shape of the biosynthesized AgNPs [19]. For TEM analysis, droplets of the AgNPs suspension were placed on a carbon-coated copper grid covered with a formvar film and allowed to air-dry. The dried samples were then loaded onto the specimen holder, and TEM measurements were performed at an accelerating voltage of 100 kV using a LEO912 AB OMEGA transmission electron microscope. Dynamic light scattering (DLS, Beckman Coulter LS 13 320 XR Particle Size Analyzer) was utilized to determine the size distribution and average sizes of the synthesized silver nanoparticles. TEM was also used to capture images of the synthesized nanoparticles and ascertain their size. Nanoparticle samples were diluted at ratios of 1:10 and 1:100 before being deposited onto the copper grids. Liquid chromatography-mass spectrometry (LC-MS) chromatograms were obtained for both the crude extract before biosynthesis and the supernatant following biosynthesis. This was done to confirm the involvement of metabolites from the crude extract in the biosynthesis process.

## Determination of antimicrobial activity

The potential antimicrobial effects of both *Ficus ingens* silver nanoparticles (AgNPs) and the free *Ficus ingens* extract were investigated. *Mycobacterium* *smegmatis* served as a key model organism for evaluating anti-tubercular potential. Alongside this, the antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* was also determined to provide a broader understanding of the compounds' antimicrobial properties against Gram-negative and Gram-positive bacteria, respectively.

## Preparation of plant extracts

The preparation of plant extracts for the assessment of antimicrobial activity followed a procedure adapted from the methodology described by Deniz and co-workers [42]. Briefly, a precise quantity of 0.1g of the lyophilized *Ficus ingens* extract was accurately weighed and dissolved in 10 ml of distilled water, resulting in a stock concentration of 10mg/ml. To ensure complete dissolution, this solution underwent brief vortex followed by sonication in a temperature-controlled water bath at room temperature. This standardized stock solution was subsequently diluted in the Nutrient broth to achieve a working solution with a concentration of 5mg/ml. To maintain aseptic conditions crucial for microbiological assays, the prepared extracts were filter-sterilized using sterile nylon membrane syringe filters with a pore size of 0.22µm. A two-fold serial dilution series was then freshly prepared to generate a range of test concentrations, spanning from 5mg/ml to 0.009766mg/ml, for immediate use in the bioassays.

## Determination of minimum inhibitory concentration (MIC)

The microbroth dilution test was used for MIC determination as described by Banfi et al. (2003) [43] with slight modifications. White costar 96-well microtitre plates were used for the assay. Two-fold dilutions of the extracts and AgNPs were prepared by diluting with Nutrient broth with supplements in the test wells to obtain 100µL of each required dilution between 0.009766mg/mL and 5mg/ml. One hundred µL of the synthesized *Ficus ingens* AgNPs stock solution was also added, and similarly diluted. The bacterial suspensions (100µL) were added to each well containing extract or NPs. This was done in triplicate for each of the concentrations plated. Control wells containing no extract, as well as Nutrient broth and rifampicin (positive control) were also included. The plates were sealed and incubated at 37°C for 48hours. The MIC was qualitatively determined as the lowest concentration at which there was inhibition of bacterial growth

## Zone of inhibition test

Overnight cultures of M. *smegmatis*, *Staphylococci* *aureus* and *E. Coli* in Nutrient broth were adjusted to a 0.5 McFarland turbidity standard (BaCl2 and H2SO4), which is equivalent to 1,5 x 108CFU/mL. Bacteria were cultured onto Mueller–Hinton agar plates using a sterile glass spreader. Sterile filter disks of 6 mm diameter were impregnated with extract solutions and *Ficus ingens* AgNPs at their MICs. Replicates were included for each test. Rifampicin (2µg/mL) was used as a positive control. Nutrient broth was used as a negative control. The plates were incubated at 37°C for 48 hours. At the end of the incubation period, the antibacterial activity was evaluated by measuring the size of the inhibition zone.

# RESULTS AND DISCUSSION

## Plant Collection and Preparation

The bark of *Ficus ingens* was collected from several mature trees to create a representative pooled sample. The selected collection site in Buhera was intentionally located away from major roads, settlements, farming, and mining activities. This precaution was taken to minimize the potential influence of environmental pollutants on the plant's chemical composition. It is known that inherent variations in a plant's phytochemical makeup, as well as the presence of contaminants, can affect its properties [44]. To preserve any heat-sensitive compounds present in the *Ficus ingens* bark, the collected samples were air-dried at room temperature.

## Phytochemical Profiling of Ficus ingens

Qualitative analyses (Table 1) reveal that flavonoids, tannins, and phenolic compounds were the most prevalent phytochemicals in the *Ficus ingens* extract, a finding consistent with earlier research [6]. The traditional medicinal use of *Ficus ingens* has been attributed to its rich and varied phytochemical makeup [6]. UV-visible spectroscopy enables the quantification of flavonoids (53.5 mg GAE) and tannins (90.0 mg QE). However, these levels were lower than those reported in other studies, potentially due to differences in geographical origin and extraction methods. We can deduce that these phytochemicals facilitated the ionic interactions with metallic ions during the biosynthesis of the biogenic *Ficus ingens* silver nanoparticles (AgNPs). Notably, several phenolic acids, including kaempferol and gallic acid, have demonstrated significant antimycobacterial activity [45].

Table 2. Results for phytochemical screening of hydro-ethanolic and distilled water extracts of *Ficus ingens*

|  |  |  |
| --- | --- | --- |
| Metabolite | Presence in hydroethanolic extract (70%v/v) | Presence in distilled water extract |
| Alkaloids | + + | + + |
| Tannins | + + + | + + |
| Phlobotanins | + + + | + + + |
| Flavonoids | + + | + |
| Saponins | + | + |
| Terpenoids | + | + + |
| Phenols | + + + | + |
| Glycoside | + + | + + |
| Anthocyanins | + | + + |

*Absent (-), Present (+), Present in moderate amounts (++), Present in abundance (+++)*

## Oral toxicity profiling of lyophilized *Ficus ingens* in distilled water

The test results and observations from the toxicity profiling validate that the hydro-ethanolic bark extract of *Ficus ingens*, is safe for internal use up to 4000mg/kg body weight (Table 3). These results were expected, as the plant is generally considered to be safe from the accumulated experiences and testimonies of traditional medicinal practitioners over a long history of usage in Southern Africa [8, 9]. Our literature search could not find any report of any adverse effects arising from consumption of bark part of the *Ficus ingens* plant. The behavioral factors under assessment which include signs of restlessness among the study animals, painful response to touch, urine characteristics and urination frequency, skin texture, morphology and colour, fur condition and erection, as well as food and water intake were periodically journalized by an experienced veterinary specialist (Table 3). No adverse observations were noted with regards to symptoms and signs of toxicity for all the parameters under review and no deaths were recorded for the entire duration of the testing period. All changes observed in these acute oral toxicity studies were within normal physiological ranges.

Table 3. Observations from toxicity profiling of *Ficus ingens*

|  |
| --- |
| Observed parameter Dose of *Ficus ingens* in mg/kg body weight |
|  | 250mg | 800mg | 2560mg | 4000mg |
| Food intake | Normal | Normal | Normal | Normal |
| Water intake | Normal | Normal | Normal | Normal |
| Death | Alive | Alive | Alive | Alive |
| Breathing | Normal | Normal | Normal | Normal |
| Defecation | Normal | Normal | Normal | Normal |
| Urination | Normal | Normal | Normal | Normal |
| Skin colour | Normal | Normal | Normal | Normal |
| Drowsiness | Not observed | Not observed | Not observed | Not observed |
| Erection of Fur | Not observed | Not observed | Not observed | Not observed |

## Bodyweight observations

The up and down test as outlined by OECD technical guideline 425 with minor modifications was used in this study. As per the guideline, only healthy adult nulliparous and non-pregnant females were chosen for the study [28]. The rats were aged between 8-12 weeks as required by the technical guideline 425. The animals were all fasted before dosing overnight with only water provided for them. Before commencement of dosing, the animals were weighed and checked for any adverse health indications. The acute oral toxicity profile study of the *Ficus ingens* extract was carried out using Wistar laboratory bred rat models at doses of 250 mg, 800 mg, 2560 mg and 4000 mg/kg body weight. The experimental rats were routinely observed, and their behavior monitored during the experiments for changes in body weight and other observable indicators of poor health effects. As reported, above there were no deaths and no withdrawals from the study due to adverse health symptoms of participating animals. There were no notable changes observed in all rats for any of the categories. The study concluded that *Ficus ingens* was toxicologically safe at 4000 mg/kg body weight, and therefore LD50 is concluded to be beyond 4000mg/kg body weight. Concerning the Hodge and Sterner classification for toxicity, the hydro-ethanolic bark extract of *Ficus ingens* is classified as nontoxic.

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 Fig. 2. Weight changes over the investigation period

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Fig. 3. Percentage rat weight gains over the investigation period

## Biosynthesis of Ficus ingens AgNPs

The biosynthesis of silver nanoparticles (AgNPs) using *Ficus ingens* bark extract, following a green synthesis approach, readily demonstrated the successful formation of nanometric AgNPs through a discernible colour change. Upon the addition of silver nitrate solution to the *Ficus ingens* bark extract, the initial light-yellow hue of the extract gradually transformed into a distinct brown coloration. This visual transition, mirroring observations in the green synthesis of AgNPs from other Ficus species such as *Ficus microcarpa, Ficus religiosa*, *Ficus cordata*, and *Ficus benghalensis*, served as a preliminary indication of AgNPs formation [33]. The increasing intensity of the brown colour for the reaction suggested the progressive reduction of silver ions into their nanoparticle form.

Further spectroscopic analysis using a double-beam UV-Vis spectrophotometer corroborated the formation of *Ficus ingens* AgNPs. Constant monitoring of the reaction mixture within the 200-700 nm range revealed a characteristic surface plasmon resonance (SPR) absorption band centered at 445 nm. This prominent peak falls within the typical range of 400-500 nm reported for silver nanoparticles and aligns with SPR values observed for AgNPs synthesized using other Ficus species, such as 451 nm for *Ficus microcarpa*, 424 nm for *Ficus religiosa*, 460 nm for *Ficus cordata*, and 430-435 nm for *Ficus benghalensis* [33]. The strong absorbance at 445 nm definitively confirmed the presence of concentrated *Ficus ingens*-derived AgNPs in the solution.

 

 Fig 4: TEM image of the AgNPs biosynthesised mediated by *Ficus Ingens*

Transmission electron microscopy (TEM) provided valuable insights into the morphology and size characteristics of the synthesized *Ficus ingens* AgNPs (Figure 4). TEM analysis revealed a predominantly spherical morphology for the nanoparticles, although a minor population of cubic structures was also observed. This finding is consistent with reports from other Ficus-mediated syntheses, which often yield spherical nanoparticles alongside other less prevalent shapes [33]. The TEM images further indicated a range of nanoparticle sizes, with both well-dispersed and agglomerated structures visible. Dynamic light scattering (DLS) measurements determined an average nanoparticle size of 38 nm, which is within the size range reported for AgNPs synthesized using other Ficus species like Ficus benghalensis (10-50 nm) and Ficus cordata (average 23 nm). The observation of both agglomerated and scattered nanometric structures with varying morphologies likely reflects the involvement of multiple competing functional groups present in the complex phytochemical mixture of the lyophilized *Ficus ingens* bark extract.

The biosynthesis of AgNPs using *Ficus ingens* bark extract is theorized to be mediated by the diverse array of secondary plant metabolites present in the bark. These phytochemicals, including polyphenols, flavonoids, and terpenoids, are known for their potent reducing and stabilizing capabilities. They likely facilitated the reduction of silver ions (Ag+) to elemental silver nanoparticles (Ag0) and simultaneously capped the nanoparticles, preventing their uncontrolled aggregation. Comparative analysis of LCMS images of the crude *Ficus ingens* bark extract before the biosynthesis and the supernatant after the reaction reveal the disappearance or significant reduction in the concentration of specific phytochemical compounds. These depleted compounds can be inferred as the key biomolecules actively participating in the reduction and stabilization processes. Identifying these specific phytoconstituents is crucial for optimizing the synthesis process, allowing for a more precise control over the size and morphology of the resulting AgNPs by adjusting the concentration of the participating reducing agents.

While this study focused on the successful biosynthesis and initial characterization of *Ficus ingens* AgNPs, a detailed toxicity evaluation was not within the scope. The toxicity of AgNPs is a multifaceted issue influenced by factors such as size, shape, surface coating, and dosage, alongside various endpoint measurements and exposure routes. Therefore, a comprehensive assessment of the *Ficus ingens* AgNP toxicity would necessitate a separate, independent investigation employing standardized characterization techniques to allow for meaningful comparisons with other AgNPs.

## Antimycobacterial activity of extracts

The investigation into *Ficus ingens* focused on evaluating the in vitro antimicrobial potential of its extract and synthesized silver nanoparticles (AgNPs) against *Mycobacterium* *smegmatis*, *Escherichia coli*, and *Staphylococcus aureus* (Table 4). The free extract of *Ficus ingens* demonstrated significant antimycobacterial activity against M. *smegmatis*, exhibiting a Minimum Inhibitory Concentration (MIC) of 95.2 µg/ml and an inhibition zone of 15 mm, a level consistent with findings from other *Ficus* species. Similarly, the extract also showed antibacterial activity against *E. coli* (MIC 175 µg/ml, 12 mm inhibition zone) and *S. aureus* (MIC 140 µg/ml, 14 mm inhibition zone), aligning with existing literature on other Ficus species (Table 5).

Silver nanoparticles synthesized using the *Ficus ingens* extract through a green synthesis method showed a marked enhancement in antimycobacterial activity against M. *smegmatis*, with an MIC of 18.5 µg/ml and an increased inhibition zone of 23 mm, suggesting a synergistic effect between the plant-derived compounds and the silver nanoparticles. The *Ficus ingens*-derived AgNPs also displayed significantly enhanced antibacterial activity compared to the crude extract against both *E. coli* (MIC 32 µg/ml, 20 mm inhibition) and *S. aureus* (MIC 25 µg/ml, 22 mm inhibition) [5]. This potentiation of activity is likely due to the multiple mechanisms of action exhibited by silver nanoparticles, including cell membrane disruption and interference with intracellular processes, potentially working in concert with the inherent antimicrobial properties of the various phytochemicals like tannins, saponins, phenols, and flavonoids present in *Ficus ingens* that likely contribute to the observed activities and may play a role in the enhanced activity of the AgNPs. The observed enhancement underscores the potential of green-synthesized silver nanoparticles from *Ficus ingens* as a more effective antimicrobial agent compared to the crude extract, offering a promising avenue for developing novel therapeutic strategies against bacterial and mycobacterial infections.

Table 4. Minimum inhibitory concentrations for *Ficus ingens* and *Ficus ingens* AgNPs against *M. smegmatis*, *E. Coli* and *S. aureus*

|  |  |
| --- | --- |
| Test culture | Minimum inhibitory concentrations (µg/ml) |
|  | Rifampicin | *Ficus ingens* extract  | *Ficus ingens* AgNPs  |
| *M. smegmatis* | 2.00 | 95.2 | 18.5 |
| *E. coli* | 4.00 | 175 | 32 |
| *S. aureus*  | 0.25 | 140 | 25 |

Table 5. Antibacterial activity of *Ficus ingens* and *Ficus ingens* AgNPs at their respective MICs against M. *smegmatis*, *E. Coli* and *S. aureus*

|  |  |
| --- | --- |
| Test culture | Inhibition zone in diameter (mm) |
|  | Rifampicin | *Ficus ingens* extract  | *Ficus ingens* AgNPs  |
| *M. smegmatis* | 35 | 15 | 23 |
| *E. coli* | 10 | 12 | 20 |
| *S. aureus*  | 20 | 14 | 22 |

# CONCLUSION

The green synthesis of silver nanoparticles using *Ficus ingens* bark extract was successfully achieved, demonstrating a simple and eco-friendly approach. The resulting AgNPs exhibited significant antimicrobial activity against *M*. *smegmatis*, *E. coli,* and *S. aureus*, with enhanced potency compared to the crude plant extract. This suggests that *Ficus ingens*-derived AgNPs hold promise as a potential source for developing novel and effective antimicrobial agents.

Further research is needed to identify the specific bioactive compounds responsible for this activity, elucidate the precise mechanisms of action, conduct thorough toxicity assessments, evaluate in vivo efficacy, and investigate formulation and stability for potential therapeutic development.

# DISCLAIMER (ARTIFICIAL INTELLIGENCE)

The authors explicitly state that no generative AI technologies, including Large Language Models (such as ChatGPT and COPILOT) and text-to-image generators, were employed in the writing or editing of this manuscript.

# ETHICAL APPROVAL

The University of Zimbabwe’s Joint Parirenyatwa Research Ethics Committee reviewed and approved all experiments conducted in this study (JREC/01/2025).

**COMPETING INTERESTS DISCLAIMER:**

Authors have declared that they have no known competing financial interests OR non-financial interests OR personal relationships that could have appeared to influence the work reported in this paper.

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