Original Research Article

Eco-Friendly Biosynthesis of Silver Nanoparticles Using Elephantorrhiza elephantina: Characterization and Antimicrobial Activity against Mycobacterium tuberculosis Surrogate Models

ABSTRACT

The emergence of multidrug-resistant (MDR) and extensively-drug resistant (XDR) *Mycobacterium tuberculosis* (MTB) strains have scampered efforts to arrest the tuberculosis (TB) pandemic due to treatment failures based on current drugs. The need for novel therapeutics has never been more urgent. Research is leaning towards alternative composite antimicrobials such as silver nanoparticles (AgNPs). *Elephantorrhiza elephantina* (*E. elephantina*) is an indigenous Southern African rhizomatous plant which has been used for eons in African traditional medicine for the management of TB. The purpose of this study was to biosynthesize and characterize AgNps using *E. elephantina*, and to evaluate their antimicrobial effectiveness against surrogate MTB species. The study also sought to screen for the participating secondary metabolites in *E. elephantina* acting as bio reducing, capping and stabilising agents; and to determine the toxicity of the lyophilised root extract *in vivo*. Qualitative phytochemical screening techniques confirmed the presence of bioactive secondary metabolites with functional groups capable of mediating in the biosynthesis of AgNPs. UV-Vis spectrometry established the identity of the nanostructures as AgNPs. Transmission electron microscopy and dynamic light scattering confirmed the AgNPs to have an average size of 44 nm, with spherical and cubic morphologies.The biosynthesisedAgNPs were potent inhibitors of the model MTB species with an MIC of 19.53 µg/mL, and inhibition zones of 20mm and 18mm against *Mycolicibacterium aurum* and *Mycolicibacterium smegmatis* respectively at their MICs. Oral toxicity evaluations based on OECD guideline 425 determined the LD50 of the lyophilised*E. elephantina* root extract to be safe above 4000mg/kg body weight.The study concluded that biosynthesized AgNPs from *E. elephantina* root extract, using a one-pot green-synthesis technique, were both safe and effective, offering a promising alternative therapeutic strategy for combating multidrug-resistant TB.

Keywords: Elephantorrhiza elephantina, mycobacterium, biosynthesis, AgNPs, secondary metabolites, tuberculosis, antimycobacterial, nanoparticles.

1 Introduction

1.1 Elephantorrhiza elephantina

The species *Elephantorrhiza elephantina* (*E. elephantina*) (Burch.) Skeels, commonly known as eland's foot, is a wild tree that belongs to the Fabaceae family. Fabaceae is one of the largest plant families, found in many countries throughout the world. The Fabaceae family has at least 19,500 plant species, each with unique qualities that give them significant

therapeutic, industrial, food and ethno-veterinary uses in many cultures of the world. The most common uses in ethno-medicineinclude the treatment of respiratory infections such as tuberculosis (TB), sexually transmitted diseases, opportunistic infections in HIV/AIDS, mastitis, as well as analgesic and anti-inflammatory applications[1–3]. The different parts of the plant are all used for treatment, particularly the root, rhizome, stem bark and leaves. *E. elephantina* is indigenous only to Africa, along with eight other species in its genus. This genus thrives well in the African savannah. In African traditional medicine (TM), *E. elephantina* is highly valued for its remarkableusefulness in the treatment and management of multiple conditions in both man and animals [4-6].It is oftenadministered alone, or in combination with other plant species. The root and rhizome extracts are commonly prepared as decoctions for treating a wide array of illnesses in man. These include respiratory diseases, sexually transmitted diseases, impotencediarrhoea, cough, tuberculosis, pain relief, fever, among others[4,5]. It can also be applied topically for relief of skin ailments such as eczema and measles [6]. The presence of highly bioactive phyto-compounds in *E. elephantina* has been demonstrated by earlier scholars, and these support its widespread use in TM[7–9]. Flavanols and phenolic compounds have been linked to its high antimicrobial, including antimycobacterial activity. In our previous work with TM practitioners, the use of *E. elephantina*, and generally the use of herbal medicines,wasreportedly associated with high treatment success rates in patients with TB. The occurrence of drug resistance was rarely observed inpatients using TM, and the treatment regimens were often personalised for each individual patient[10]. This background gives some basis for utilising *E. elephantina* for improved formulations targeting difficult to treat microbes such as the *Mycobacterium* species.

1.2 Anti-bacterial resistance

One of the biggest health challenges of our time is the evolution of pathogenic microorganisms with new mechanisms of antimicrobial evasion [11]. Global trends have seen a rise in drug resistant pathogenic strains of clinical significance. These include multi-drug-resistant strains of *Mycobacterium tuberculosis* (MTB), methicillin resistant *Staphylococcus aureus* and vancomycin resistant *Enterococcus*. Most worrying is the emergence of extensively drug-resistant strains, which have limited susceptibility to currently available antibiotics. In the absence of successful interventions, mortality due to antimicrobial resistance is expected to surpass 10 million within the next two and half decades [12,13].

Microbes evade the host immune response and chemotherapy by employing multiple mechanisms such as the use of efflux pumps, use of transporter proteins, alterations in membrane permeability, degradation and bioconversion of drugs into less harmful metabolites, post translational modifications, development of persister populations, among others[14,15]. Resistance is partially attributed to the complex lipid-rich cell wall structure of *Mycobacterium*species, which is impenetrable to most antimicrobial agents. This increases the difficulty of treatment, lengthens treatment duration, and reduces the number of therapeutic options available to patients. In TB for instance, treatment success rates for drug resistant TB have historically been in the 54-65% range[16], only increasing in recent times following the inclusion ofbedalaquine®, pretomanid®, and linezolid® (BPaL) into treatment regimen for drug-resistant forms of TB[17]. These drugs are however associated with severe side effects and escalate the costs of treatment. Additionally, resistance to drugs in the BPaL regimen has already been recorded in clinical settings [18].Bioactive pharmaceuticals offer alternative modes of antimicrobial activity and work synergistically with conventional antimicrobial medicines; and hence have the capacity to counteract antimicrobial resistance [13].

1.3 *Mycobacterium* infections

The *Mycobacterium* genus can be classified into tuberculous and non-tuberculous mycobacteria (NTM). *Mycobacteriumbovis*, *Mycobacteriumafricanum*and *Mycobacteriummicroti* are some of the species that make up the *M. tuberculosis complex* (MTBC); and of this group, *M. tuberculosis* is the most pathogenic to man. All *Mycobacterium* species, except*Mycobacterium leprae* and MTBC, are referred to as NTM [19,20]. NTM such as *Mycobacteriumabscessus* are responsible for skin and soft tissue infection in man, and pathogens of the*Mycobacterium avium* complex cause pulmonary infection. The MTBC are the cause of TB, both active and latent TB infection. TB is an ancient public health threat which continues to be one of the leading causes of death in modern times. TB incidence is highest in low to middle income countries, including Africa, south of the Sahara. New trends of antimicrobial resistant TB have also been observed in previously non-endemic populations, where high mortality rates have been reported in recent times. These include states within the United States of America and some European countries where new strains of multi-drug-resistant TB (MDR-TB) led to outbreaks of TB in the last couple of years[17].

Natural antimycobacterial compounds derived from plant and marine sources offer some promise for effectively targeting tuberculous bacteria through novel modes of action. Fast-growing non-pathogenic*Mycobacterium* strains, such as *Mycolicibacteriumaurum* (*M. aurum*)and *Mycolicibacteriumsmegmatis*(*M. smegmatis*), are often employed as surrogatemodel strains to demonstrate anti-mycobacterial activities of new compounds in settings with limited laboratory infrastructure[21,22]. These strains were selected for our study as they have comparable antimicrobial susceptibility patterns with *M. tuberculosis*, and could be handled safely in our laboratory.

1.4 Natural antimycobacterial plant polyphenols

Various tuberculosis mitigating programs have been put in place since March 24, 1882, when Dr Robert Koch first alerted the world of the discovery of MTB[23]. However, the inadequacies of the mitigations are apparent to even the most casual observer. As highlighted above,global efforts have failed to eliminate TB due to the emergence of multi-drug resistant (MDR) and extensively-drug resistant (XDR) strains of MTB[17]. It is imperative therefore that drug discovery must focus on fashioning newer anti-TB drugs with novel drug targets. Whilst the ancient world knew about TB and was already facing its onslaughts, medicinal plants have always been the cornerstone for curing the disease[5,7,22,24,25]. Many researchers believe that novel alternative medicines can be developed from plant sources for the treatment of MDR and XDR-TB. A review by Sharma and Yadav [26]cites 72 anti-TB phytochemicals that have been isolated from various plants,and their respective mechanisms of anti-TB activity.

Plants produce secondary metabolites as a protective and adaptive mechanism to survive within their natural ecosystems. The presence of phyto-compounds such as flavonoids and phenolic compounds has been associated with high antibacterial activity *in vitro* against some clinically relevant pathogens, including some *Mycobacterium* species[27,28]. Phytocompounds employ multiple pathways such as eliciting an inflammatory response, inhibition of metabolic enzymes, and direct killing of bacilli. Polyphenols such as curcumin have been shown to increase the expression of genes related to autophagy and apoptosis[27]. Allicin, an alkaloid from garlic has demonstrated significant antimycobacterial activity against resistant and susceptible *M. tuberculosis* strains [29,30]. The phytochemical analysis of *E. elephantina* has revealed the presence of several bioactive secondary plant metabolites. Fatty acids, phenols and esters form the bulk of the phytochemicals; whilst flavonoids, anthraquinones, polysterols, and triterpenoids, are among the other phytoconstituents found in *E. elephantina*[7,9]. The bioactivity profiles of these natural

compounds can be improved through nano functionalisation. Silver nanoparticles have the ability to increase the bactericidal potential of medicinal plant extracts and their bioavailability[31–33]. Incorporating plant phytochemicals into nano formulations could potentially unravel alternative therapies with superior modes of antimicrobial activity. These have the potential to tackle the current antimicrobial resistance challenge.

1.5 Biosynthesised metallic nanoparticles from polyphenols

Nanotechnology is the manipulation of materials in the nanoscale range (1-100nm) in order to amplify material characteristics and achieve unique functionalities. It is an emerging discipline with converging applications. Some of its uses in the biomedical field include diagnosis and therapeutics, using for example liposomes and micelles; dendrimers, polymeric and metal nanoparticles[34,35]. These have found phenomenal success in translating medical research from the laboratory to the patient bedside.

Numerous studies have reported the multifunctionality of nanometric drug analogues and their improved bioavailability, chemical and therapeutic equivalence. Their targeted, controlled and sustained release profiles, imply lower drug dosages with less side effects[36]. These attributes have heightened interest in nanometric drug molecules and their envisaged contributions to drug efficacy and safety. Similarly, the promising potency of optimised natural bioactive materials has also influencedresearch in drug discovery from natural polypehenols. Functional groups from plant secondary metabolites have always been known to be able to rearrange metallic salts into their elemental form in nanometric dimensions[36]. This biosynthesis, also known as green synthesis, can be adapted to develop multifunctional biomedical platforms from metallic salts. Literature presents several successes in biosynthesising silver, gold, copper, zinc and iron nanoparticles with applications in the treatment of many diseases, including TB [34, 35, 37]. This simple, one pot biosynthesis technique relies on relevant functional groups from plant secondary metabolites, which act as bio reducing, capping and stabilizing agents.*E. elephantina* contains pharmacologically potent polyphenolic antimicrobial components. The amplified medicinal benefits from both the plant secondary metabolites and the nanometric metallic ions is worth exploring. Metallic nanoparticles created through biosynthesis are reported to offer an effective, safe, and dynamic alternative to antibacterial nanoplatforms synthesized chemically[36].Metallic nanocomposite formulations were seen to improve treatment outcomes in experimental animal models[39]. The applicability of metallic nano formulations in therapeutics is further enhanced by the capacity to regulate and refine the desired characteristics by optimizing the production parameters. The size and form of the metallic nanoparticles can also be pre-determined. This has a significant impact on chemical and bioequivalence, as well as phase aggregation[40]. In this study, we present the biosynthesis of AgNPs from the hydro-methanolic solvent extracted secondary metabolites of *E. elephantina*, as well as the efficacy evaluation of the nanoparticles against surrogate MTB species.

2 Methodology

Prior to the investigations, animal use and research ethics approvals were obtained from the Joint Parirenyatwa Research Ethics Committee (JREC/04/2021) which is the local research Institutional Review board for the University of Zimbabwe. Animals were handled according to ethical animal use and those exhibiting any form of adverse reactions were to be euthanized upon completion of the experiments.

2.1 Materials, equipment and facilities

All reagents, chemicals, consumables and equipment used for plant extraction, phytochemical characterization and antimicrobial assays were obtained from the Harare

Institute of Technology's Pharmaceutical Technology Laboratory. The *in vivo* toxicity studies using laboratory animals were conducted at the University of Zimbabwe's Faculty of Medicine and Health Sciences, animal laboratory, and all associated reagents were provided by the laboratory. All chemicals, equipment and facilities for the green synthesis of silver nanoparticles and their characterization were availed by the Department of Chemistry and Biochemistry, at the University of California, Los Angeles.

2.1.1 Test microorganisms

M. aurum and M. smegmatis were the selected surrogate model strains for MTB. The *M. aurum* and *M. smegmatis* cultures were generously provided by Prof Mukanganyama at the Biochemistry Unit, University of Zimbabwe. The purity and identities of the cultures were confirmed through Gram staining and biochemical identification tests.

2.1.2 Plant collection and preparation

E. elephantina roots were harvested in South-Eastern Zimbabwe in Buhera District. Buhera lies along latitude -19° 29' 9.59" S and longitude 31° 49' 23.99".This is within agro-ecological regions III-V, with low to moderate rainfall, high summer temperatures, and mild winters. The roots were collected in the dry season, when plants were flowering, just before the onset of the summer rains in November 2022. Sustainable harvesting practices were followed throughout sample collection. The identity of the plant was taxonomically authenticated by the National Herbarium in Harare, Zimbabwe, and a voucher specimen was deposited. The plant material was individually washed with distilled water to remove dirt and debris. Samples were then air dried at room temperature for 3 weeks, to obtain a constant dry weight. The dried roots were pulverised into a fine powder (sieve size 0.5-0.9mm) prior to extraction.

2.2 Hydro-methanolic extract preparation and characterisation

Dried *E. elephantina* (260g) root powder was weighed on an analytical balance (AXIS®) and transferred to a sterile 2L amber bottle. The plant powder was macerated at room temperature for 5 days with shaking, in 1.5L of solvents with various polarities. The polar solvents had the highest extraction yields and, thus 80% methanol was selected for further tests. The extract solution was first filtered through cotton wool, then under vacuum in a Büchner funnel using Whatman No.1 filter paper (125mm, Cat No. 1001125, Whatman International Ltd, Maidstone, England). The extracts were thereafter evaporated under low pressure (Rotavapor® R-300, Buchi, Switzerland), followed by lyophilization (Lyovapor l-200, Buchi, Switzerland) under 140Pa pressure and ultra-low temperature of -50°C. The dried extracts were stored in airtight, sterile amber bottles at 4°C until required.

2.2.1 Qualitative Phytochemical screening

Qualitative tests were conducted for some selected phytochemicals with known antimycobacterial activity, using standard screening tests as reported by Chifambaand Zengeni[41]in related metabolomics studies.

Table 1: Metabolomics screening tests done on the lyophilised extract

Metabolomics	Screening test done	Results interpretation
Test for alkaloids	Mayer's test. To 5 ml of the lyophilized extract liquor in a test tube, two drops of Mayer's reagent were added[40].	The presence of alkaloids was determined by the development of a white creamy precipitate.

Tests for tannins and phenolics	Ferric chloride test. To a test tube, 2-3 drops of ferric chloride was added to 5 ml of the prepared extract liquor[41].	The presence of catechic tannins signalled by the development of a green-blue colour, a blue-black colour which indicates the presence of Gallic tannins.
Test for flavonoids	The alkaline reagent test. To 5ml of the lyophilized liquor in a test tube, 2 to 3 drops of a 50 % NaOH lye were added [42].	The development of a deep yellow colour which gradually pales to a colourless hue after the addition of 3 to 4 drops of dilute HCL, confirms the presence of flavonoids.
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 H_2SO_4[43].	The development of a reddish-brown colour indicates the presence of sterols in the extract.
Tests for steroids	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 H_2SO_4[43].	The development of a reddish-brown colour indicates the presence of sterols in the extract.
Test for saponins	The simplified foam test. In a 100ml measuring cylinder, 5ml of the extract liquor was added to 30ml distilled water, the mixture was shaken for 2 minutes[44].	The development of at least 1 cm head of form in the test tube confirms the presence of saponins.
Test for glycosides	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 80^oC for 10 minutes. After cooling, extraction was done with 10ml of benzene. The resultant benzene layer was decanted and treated with 5ml ammonia solution[41,45].	The mixture was observed for the development of a pink colour which signals the presence of anthranol glycosides.

2.2.2 Quantification of total flavonoid and tannins

The total flavonoid content of the lyophilised *E. elephantina* was estimated spectrophotometrically at 510 nm. In the test, 1mg of extract was dissolved in 2mL of distilled water as per earlier methods [39]. To this solution, 0.5mL of 1M sodium nitrite was added together with 2mL of a 1M, NaOH solution, distilled water was then added to make the volume to 10mL. The solution was shaken and allowed to stand at room temperature for 15 min and the absorbance was subsequently measured. The total flavonoid content was estimated as mg of quercetin equivalent (mg QE/g extract) on a dry weight basis using the standard curve. The Prussian Blue Assay was used to quantify tannins, using optical density (OD) values at 700nm [44].

2.3 Acute oral toxicity testing of lyophilised *E. elephantina*

The OECD technical guideline 425 protocol was used with some modifications[45]. A total of 24 female nulliparous Wistar albino rats aged between 8 and 10 weeks, and weighing approximately ±235g, were used. The rats were conditioned in the animal house to

acclimatise for 10 days prior to testing and were fed commercial rodent feed and water *ad libitum* during the period prior to the experiments. The rats which were specifically bred for laboratory studies, were selected after inspection by a veterinary officer to assess good health. The test animals were put into 2 groups of 12 animals each. The first being the test group and the second being the control. Our modified investigation protocol consisted of serial single ordered dose progressions. The test and control animals were individually dosed, in sequence, at 48-hour intervals. The first animal received a dose below a randomly selected estimated LD50. After dosing the first animal, the next animal was given an increased dose which was double the first one based on the determined toxicity susceptibility observations on the condition of the previous animal dosed over the 2-day observation interval. The starting dose chosen for this study was 250mg/kg body weight and the limit dose was 4000mg/kg body weight. The 12 rats in each study group were individually marked prior to the investigations to guarantee precise identification. Prior to administering the first dose, the participating animals were fasted pellets for 18 hours with unlimited access to water. The *E. elephantina*, was administered through oral gavage in the following different concentration in a distilled water solution in 5 different doses of: 250, 500, 1000, 2000 and 4000 mg/kg body weight. The veterinary specialist observed the animals for mortality and in the absence of mortality they were investigated for any changes in behaviour and agreed clinical signs and symptoms of toxicity every hour up to the first 12 hours on day 1 and thereafter, every day up to day 21 of the investigations. The rats were weighed on days 1(first day), 7, 14 and 21 (last day) of the studies.

2.4 Biosynthesis of Silver Nanoparticles

Biogenic fabrication of the augmented *E. elephantina*, AgNPs was carried out through bio-reduction of $AgNO_3$ by functional groups in the lyophilised extract liquor's phytoconstituents. Prior to the synthesis, 100mL of a 1 mM solution of $AgNO_3$ was prepared using distilled water. For the bio reduction of Ag^+ ions, 20 mL *E. elephantina* lyophilised extract liquor was added drop wise into the 100 mL of 1 mM aqueous solution of $AgNO_3$ and heated to between 60–80°C for 1 hour in an Erlenmeyer flask. The reaction variables including reactants concentration (silver nitrate, and lyophilised extract), reaction time, medium pH, reaction temperature, were monitored and optimized for the one pot synthesis of AgNPs. The reaction was monitored at pH 1, 3, 7, and 10, and optimization was achieved by adjustments with 0.1 N HCl and 0.1 N NaOH accordingly. The resultant nanoparticles were observed from 0 to 2 hours at 30-minute intervals and overnight for optimal synthesis of AgNPs. The biosynthesis was monitored at temperatures of 25, 50, and 100°C. The concentration of $AgNO_3$ was optimized at different concentrations from 0.50 to 3.0 mM. The concentration ratio of the lyophilised plant extract to the $AgNO_3$ was also varied and the synthesis and reaction parameters were observed at various lyophilised extract to the metallic salt solution ratios. The supernatant with the AgNPs solution was centrifuged for 30 minutes at 8,000 rpm (Sigma 3K30). The resulting precipitate was washed with ethanol and the resultant after decanting the supernatant was lyophilised (lyophilizer: Christ Alpha 1-4 LD) to reveal crystalline AgNPs. The nanoparticles were calcined at 400^oC for 2 hours.

2.4.1 Characterisation of the biosynthesised AgNPs

The nanoparticles' identity was confirmed using UV-Vis spectrometer (Hitachi, UH5300). The biosynthesised AgNPs were analysed by TEM for their size and shape.In the analysis procedure, drops of the AgNPs suspension were placed on a carbon-coated copper grid covered with a formvar film, which were then allowed to dry by evaporation in air. After drying, the samples were subsequently loaded onto the specimen holder. The TEM measurements were conducted at a voltage of 100 kV (A LEO912 AB OMEGA transmission electron microscope). Dynamic light scattering (DLS, (Beckman Coulter LS 13 320 XR Particle Size Analyzer) was used to determine the size distribution or average sizes of

synthesized silver nanoparticles. The TEM was used to obtain images of the synthesized nanoparticles and determine their size. Nanoparticles samples were diluted 1:10 and 1:100 before being dropped onto the copper grids. LCMS chromatograms were obtained for the crude extract before the biosynthesis as well as for the supernatant after the biosynthesis so as to confirm participation of metabolites from the crude extract in the biosynthesis.

2.5 Determination of antimycobacterial activity

The antibacterial activities of *E. elephantina*AgNPs, as well as free *E. elephantina*, were determined using *M. aurum* and *M. smegmatis* as model strains for anti-tuberculosis activity.

2.5.1 Preparation of plant extracts

Plant extracts for antimycobacterial activity were prepared as described by Deniz and colleagues [46] with a few modifications. Briefly, 0.1g of the lyophilised extract was weighed and dissolved in 10mL of 10% DMSO to obtain a 10mg/mL stock solution of the extract. The solution was vortexed briefly, then sonicated in a water bath at room temperature to aid dissolution. The standardised stock solution was diluted in Middlebrook 7H9 broth with supplements (OADC, Tween 80 (0.05%) and glycerol (0.2%) to obtain a 5mg/mL working solution. The extracts were filter sterilized under aseptic conditions using nylon membrane syringe filters (0.22µm). A 2-fold serial dilution series was then carried out to obtain various concentrations of the extracts from 0.009766mg/mL to 5mg/mL. These were freshly prepared for use in bioassays. The final DMSO concentration was <2% for all the extracts.

2.5.2 Determination of Minimum inhibitory concentration (MIC)

The microbroth dilution test was used for MIC determination as described by Banfi *et al.*[47] with slight modifications. White costar 96-well microtitre plates were used for the assay to enable visualization of colour at the end point. Two-fold dilutions of the extracts and AgNPswere prepared by diluting with Middlebrook 7H9 broth with supplements in the test wells to obtain 100µL of each required dilutionbetween 0.009766mg/mL and 5mg/mL.One hundred µL of the synthesized *E. elephantina*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 DMSO (2%) andrifampicin (positive control)were also included. The plates were sealed and incubated at 37°C. After 24 hours of incubation, 0.02% resazurin (30 µl) was added to each well, and the plates incubated for a further 24 hours at 37°C. The MIC was qualitatively determined as the lowest concentration at which there was inhibition of bacterial growth (blue colour) in contrast to a colour change of blue to pink in wells where bacterial growth was not inhibited.

2.5.3 Zone of inhibition test

Overnight cultures of *M. smegmatis* and *M. aurum* in Middlebrook 7H9 broth with supplementswere adjusted to a 0.5 McFarland turbidity standardat an OD of 600 nm, which is equivalent to 1,5 x 10^8CFU/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*E. elephantina*AgNPsat their MICs. Replicates were included for each test. Rifampicin (2µg/mL) was used as a positive control. Supplemented Middlebrook 7H9 and 2% DMSO were used as negative controls. 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.

3 Results and discussion

3.1 Plant collection and preparation

The rhizome of *E. elephantina* was collectedfrom multiple flowering shrubs to make a pooled sample. The location chosen was away from major roads, settlements, farming and mining activities. This was done to minimise the effect of environmental pollutants as inherent variations in the phytochemical composition of plants and the presence of contaminants make it rather difficult to achieve consistent surface morphology and monodispersion in the synthesis of NPs [48]. The presence of impurities also affect the pharmacological properties of the plant.The plants were air dried at room temperature in order to preserve any heat liable phyto-compounds.

3.2 Phytochemical profiling of *E. elephantorrhiza*

From the qualitative tests (Table 1), flavonoids, tannins and phenolic compounds were the most abundant phytochemicals found in the extract of *E. elephantina*.Similar trends were observed in previous studies[9]. The successful use of E. elephantina in TM has been linked to its diverse phytochemical composition [7–9].

Table 2Phytochemical screening of hydro-ethanolic, hydro-methanolic and distilled water extracts of E. elephantina

Phytoconstituent	Presence in hydro-ethanolic extract (70%$^{v}/_{v}$)	Presence in hydro-methanolic extract (80%)	Presence in distilled water extract
Alkaloids	+	+	++
Phytosterols	-	-	-
Flavonoids	+++	+++	+++
Saponins	+	+	+
Phenolic compounds	+++	+++	+
Tannins	+++	+++	++
Glycosides	+	+	+
Terpenoids	+	+	+

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

The results of UV-visible spectroscopy procedure allowed quantification of flavonoids(53.5mg GAE)and tannins (90.0mg QE). These values are however lower than cited in other studies, possibly due to variations in geographical location and extraction procedures [8]. The high tannin content contributes to the reddish colour of the *E. elephantina* rhizome. We can infer that it is the presence of these phytochemicals that was responsible for the ionic interactions with metallic ions in the biosynthesis of the biogenic *E. elephantina*AgNPs.Some of the phenolic acids which have shown significant antimycobacterial activity include kaempferol, quercetin 3-*O*-β-D-glucosde, catechin and gallic acid[27,29,30,49].

3.3 Oral toxicity profiling of lyophilised *E. elephantina* in distilled water

The experimental rats were routinely observed, and their behaviour monitored during the experiments for changes in body weight and other observable indicators of poor health effects. Preliminary acute toxicity evaluation of root extract of *E. elephantina* on Wistar rats showed no major irreversible physiological and behavioural changes in the test animals. There were no mortalities recorded over the test period (Table 3). The observed staggered

defecation rate and slight drowsiness at the highest dose resolved within 48hours after conclusion of the experiments. No animals were therefore euthanised after the test period, but were utilised for other experiments after a washout period. Even though there was no mortality of test animals, the results somewhat correlate with traditional beliefs that overdose of the root extracts has been associated with constipation and disorientation. In oral tradition there are reports of fatalities from overdosing of the rhizome extracts which however have not been corroborated by scientific studies. Our results correlate well with findings by Maphosa *et al*. and other studies, following the same or related acute oral toxicity protocols [52]. Studies by Hutchings *et al.*[51] reported spontaneous cutaneous necrosis and pulmonary oedema following subcutaneous injection of aqueous extracts of *E. elephantina*. Hutchings also observed other symptoms of poisoning which we did not observe. This is most likely due to the differences in dosage forms and route of administration[53]. Correlation analysis indicates that exposure route of drugs is an important factor in the relationship between dosage and toxicity or lethality. Various compounds with low intestinal absorption have been demonstrated to exhibit much less toxicity when administered orally than via the injection route[53]. Despite the fact that drugs are metabolized in the body regardless of the administration route, it is scientifically noted that first-pass metabolism only occurs via the oral route and not the injection route. The decrease in toxicity of most compounds taken orally compared with injection route stems from this phenomenon. However, since the traditional administration of the plant is oral, we believe that our results somewhat substantiateits traditional usage. Even though traditional medicines are sometimes administered subcutaneously in much of Southern Africa through blade incision called '*nyora'* or '*inhlanga*' in Zimbabwe, there are no reports of *E. elephantina* being administered through that route. In relation to the above, one weakness from our study was the lack of sub-acute studies to observe whether there is any dose related internal organ damage, blood, and hormonal changes of toxicological concern.

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Table 3: Observations from toxicity profiling of E. elephantina

Observed parameter	Dose of *E. elephantina* in mg/kg body weight				
	250mg	**500mg**	**1000mg**	**2000mg**	**4000mg**
Food intake	Normal	Normal	Normal	Normal	Reduced
Water intake	Normal	Normal	Normal	Reduced	Reduced
Death	Alive	Alive	Alive	Alive	Alive
Breathing	Normal	Normal	Normal	Normal	Staggered
Defecation	Normal	Normal	Normal	Staggered	Staggered
Urination	Normal	Normal	Normal	Normal	Increased
Skin colour	Normal	Normal	Normal	Normal	Normal
Drowsiness	Not observed	Not observed	Not observed	Slight	Slight
Erection of Fur	Not observed	Not observed	Not observed	Not observed	Not observed

3.3.1 Bodyweight observations.

The weights of all the rats in the observation groups including the control were recorded weekly during the test period. Animals were weighed on the initial day and on the 7th, 14th and the 21st day thereafter. In all the recorded weights, animals in the treatment groups did not exhibit statistically relevant deviations in body weight in comparison with the control group. Significant irregular growth patterns and weight fluctuations in growing animals is

characteristic of poor health and physical stress conditions. Toxicity effects can result in internal failures of the homeostatic processes. This causes disturbances in metabolic processes, indicated by uncharacteristic weight gain or loss. Apart from nutritive failures, sick animals struggle to feed either due to incapacitation or lack of appetite. In the current study, the animals were all fasted overnight prior to dosing. Only water was provided during fasting. The animals were then weighed and checked for any adverse health indications. The initial weights for all the rats selected for the study were within specifications for normal healthy nulliparous Wistar rats of their age (Figure 1). There wasa progressive increase in weight over a 21-day period of *ad libitum* feeding, in both test and control animals (Figure 2). Our studies are in agreement with other studies by Maphosa and colleagues [50], who also recorded dose dependent changes in body weight. However, there was a significant aberration in expected growth patterns in the 2000 and 4000mg/kg body weight groups. The observed changes are not surprising given the observation by the veterinary specialist of reduced food and water intake, respiratory difficulties and constipation at higher doses(Table 3).The noted changes resolved within 48 hours following administrations. In the absence of sub-acute toxicity evaluations of organs and body fluids, the exact source ofthe weight fluctuations cannot be ascertained.

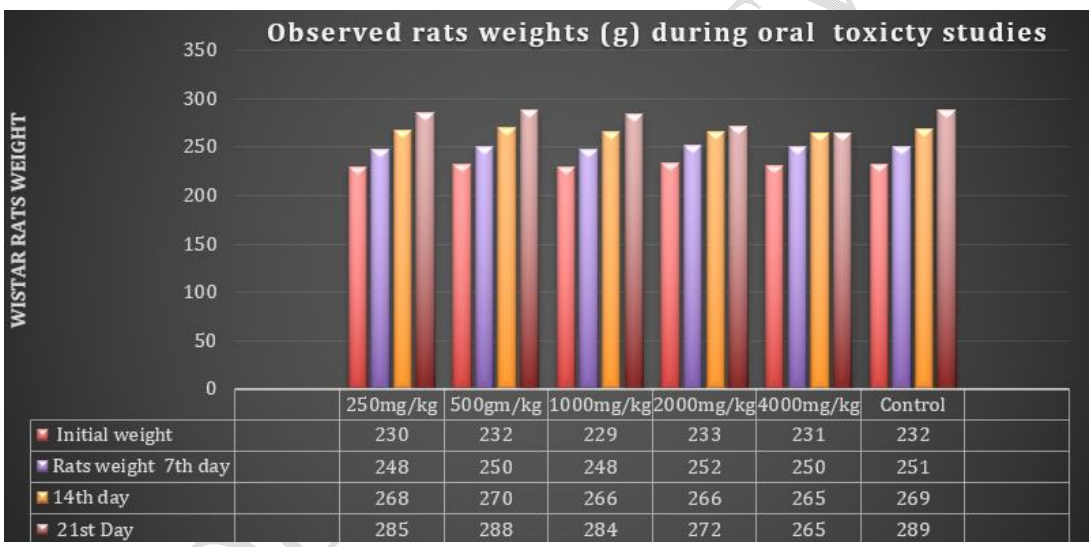

Figure 1: Wistar rat weights over the investigation period

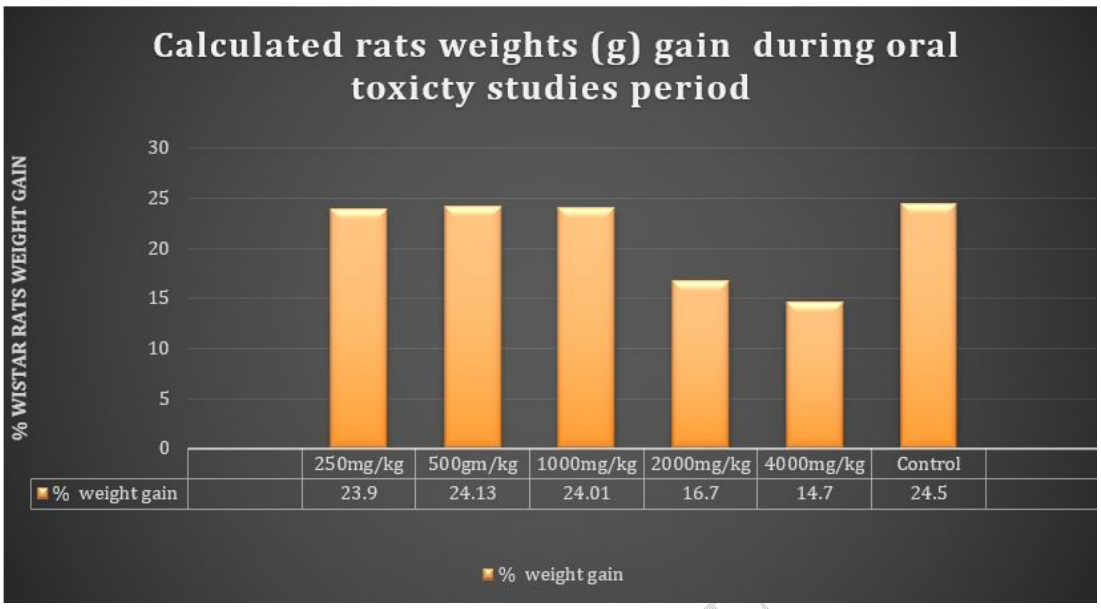

Figure 2: Percentage Wistar rat weight gains over the investigation period

As recorded above, there were no mortalities, nor withdrawals due to adverse health symptoms of participating animals. Despite the absence of mortality, it can be inferred that lyophilised extracts of *E. elephantina*could potentially cause toxicity at elevated concentrations. Larger doses should be administered with caution in medicinal preparations. The study concluded that despite the potential for toxicity of *E elephantina,* the lyophilised extracts were not lethal at any dose below 4000mg/kg body weight. According to the Hodge and Sterner classification for toxicity, an LD_{50} of >4000mg/kg body weight was obtained for the *E elephantina* lyophilised extracts[54].

3.4 Biosynthesis of *E. elephantina*AgNPs

A characteristic colourchange from dark brown to reddish brownwas the first confirmation that the biosynthesis of nanometricAgNPs was successful. A similar colour change was observed by Mohammad *et al.* [55] and Semu*et al.*[56] in the green synthesis of AgNPs from related plant sources. Constant monitoring of the reaction for the reduction of Ag^{+} by taking the OD from 200–700 nm in a double beam UV–Vis spectrophotometer showed spectra around between 410 and 460nm which is characteristic of AgNPs. The surface plasmon resonance (SPR) confirmed concentrated absorbance spectra of AgNPs at 430, and 452nm respectively. Shameli *et al.*[57]reported that the characteristics of the fabricated nanostructures including the shape and size influence SP bands. Various reports on biosynthesis of AgNPs theorise that spherical metallic nanostructures only give a unitary SPR band, while anisotropic particles produce multiple bands depending upon the shape and size of the resultant nanostructures[58]. Our investigation observed multiple peaks, which relate well with the TEM images (Figure 4) that show varying nanostructure morphologies. It can be logically theorised that the secondary plant metabolites' functional groups served as bio reducing, capping and stabilising agents.Our studies also determined that the reaction medium pH directly affected the particle size and the stability of the nanoparticles. Nanoparticles at pH 1, 3 and 10 had smaller nanoparticles, thanthose at pH 7. This observation is most likely consequent from the possible aggregation of the nanoparticles. This is because changes in the zeta potential of the system affect the isoelectric point of the fabricated nanoparticles. Phenomenon including aggregation, coalescence and flocculation are therefore related to the zeta potential which in turn is

related to the pH. For smaller desired nanoparticles, the optimum pH was therefore below 3, and at 10.An increase in temperature was observed to also increase the size of the nanoparticles. The reaction and fabrication notably increased with temperature,and corresponding larger nanoparticles formed at 50 and 100 °C.

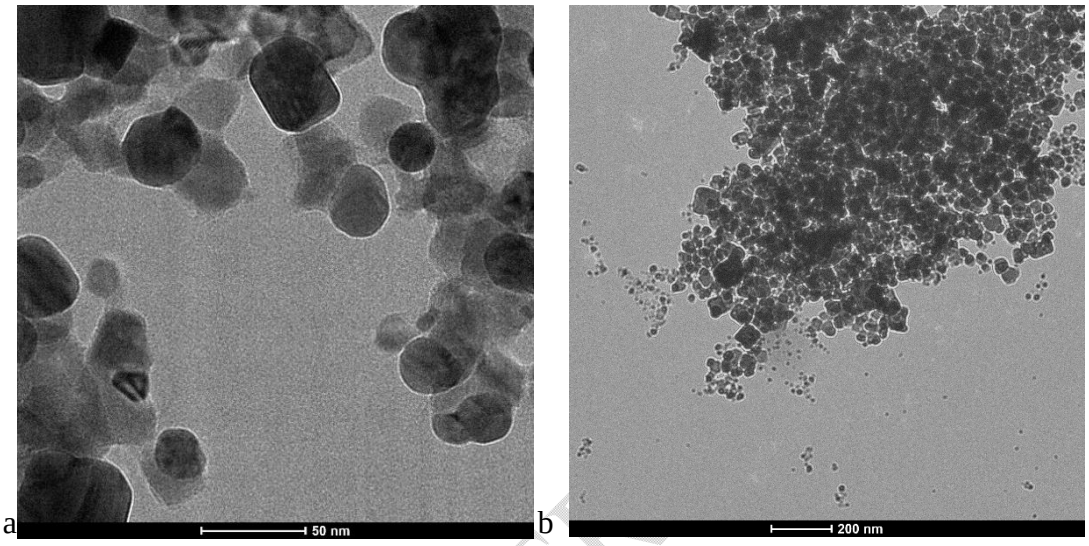

Figure 3: TEM images of E.elephantina mediated AgNPs nanoparticles showing the spherical and cubic mixture of nanostructures obtained

TEM confirmed that the morphology of the fabricated AgNPs included both spherical and cubic nanostructures as well as other minor constituent shapes (Figure 4). DLS confirmed an average NP size of 44 nm. On closer observation, the TEM images also showed agglomerated and scattered nanometric structures with varying morphologies, which most probably indicate the participation of competing functional groups from the lyophilised extract.

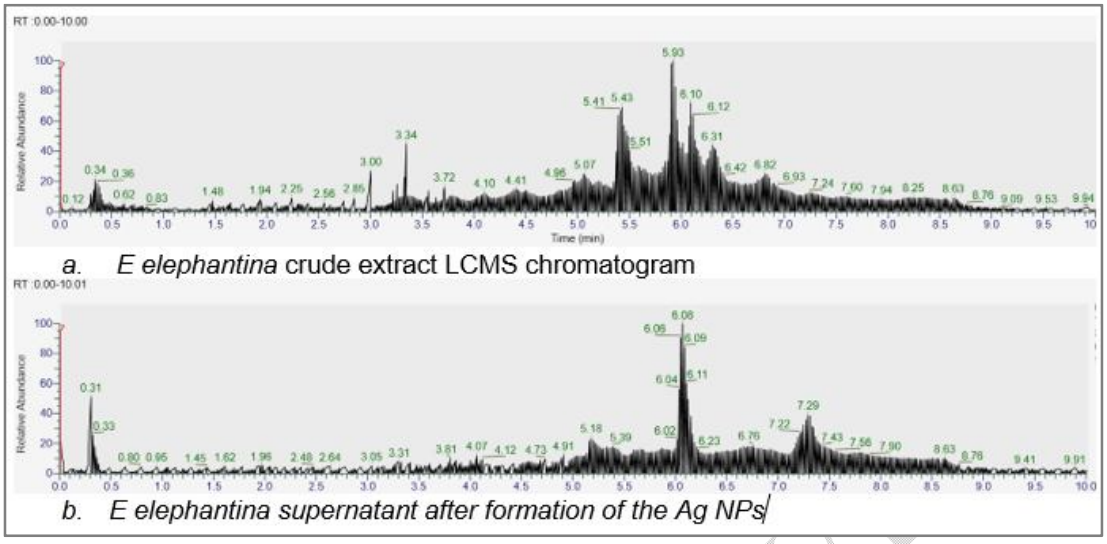

Figure 4: E elephantina crude extract and supernatant LCMS chromatograms showing disappearance of peaks of participating secondary metabolites

Though the UV-Vis, TEM an DLS can confirm the identity, and measure the configuration of the fabricated structures, one complication from biosynthesis using plants with numerous secondary metabolites like *E. elephantina* is the identification of the actual phytoconstituents that participated in the biosynthesis. This allows for the prediction of the biomedical activity of resultant platforms. Comparison of LCMS images of the crude extract before the biosynthesis, and of the supernatant after the reaction, give us a glimpse into the nature of the participating bio compounds (Figure 4). The disappearance of various peaks from the supernatant demonstrates the consumption of specific metabolites by the reaction. Better understanding of the identity of these compounds will aid in determining the exact amount of reducing agent needed, since excessive amounts results in larger unwanted aggregated nanoparticles. The one pot synthesis can be further optimized by varying the phytochemicals participating in the reaction.

With regards to toxicity of the biosynthesised AgNPs, our studies were primarily concerned with the feasibility of biosynthesised AgNPs from *E. elephantina* and their potential antimycobacterial activity.A detailed toxicity evaluation of the AgNPs was excluded because studieshave shown that toxicity of AgNPs is governed by multiple factors including size, shape, coating, and dose. Additionally, there are numerous endpoint measurements of toxicity, which are also affected by the exposure route, translocation, accumulation, and duration of exposure. Given the lack of adequate and standardised characterization techniques that can compare results from different studies, a meaningful evaluation can only be done in an independent investigation outside the scope of this paper.

3.5 Antimycobacterial activity of extracts

Table 4: Minimum inhibitory concentrations for E. elephantina and E. elephantinaAgNPs against M. aurum and M. smegmatis

Test Culture	Minimum inhibitory concentrations (µg/mL)		
	Rifampicin	*E. elephantina* extract	*E. elephantina* AgNPs
M. aurum	2,00	78,13	19,53
M. smegmatis	2,00	78,13	19,53

Table 5: Antibacterial activity of E. elephantina and E. elephantinaAgNPs at their respective MICs against M. aurum and M. smegmatis

Test Culture	Inhibition zone in diameter (mm)		
	Rifampicin	***E. elephantina extract***	***E. elephantina* + AgNPs**
M. aurum	24	18	20
M.smegmatis	35	16	18

*All values are mean ± standard deviation of 3 replicates.

M. aurum and *M. smegmatis* are fast growing, and non -pathogenic microbes which are closely related to MTB. Theyshare similar metabolic pathways to MTB thus can be adopted as models for screening antimycobacterial compounds under lower biosafety laboratory conditions [21,22].The free extracts of *E. elephantina* had significant activity against both *M. aurum* and *M. smegmatis*, with an MICof 78.13 µg/mL, and inhibition zones of 18mm and 16mm respectively. There was a broad range of MIC values in literature.Madisha[59] found an MIC of 39µg/mL against *M. smegmatis* and much higher valuesof 2.5mg/mL were observed in studies byMukanganyama and colleagues against *M. aurum*using ethanolic extracts of *E. elephantina* [60]. The observed differences could be attributed to the choice of extraction solvents, and possibly differences in the phytochemical compositions of the plants. Plant phytochemistry is influenced by multiple factors such as seasonal variation and geographical location and these contribute to differences in pharmacological activities of plant extracts[40].

AgNPs were selected for this study because the metal is highly regarded as superior in terms of its antimicrobial activity [61], and relatively safe to mammalian cells compared to other metallic ions used in the fabrication of NPs [60]. The AgNPs are thought to exhibit multiple mechanisms of action including cellular damage and interference with metabolic pathways,membrane leakageand cell wall rapture[61, 63]. AgNPs have also been shown to have immune modulatory activities in the pathogenesis of MTB [64]. Phenolic compounds, flavonoids, and tannins were present in significant quantities in the extract (Table 2), and these are known to possessantimycobacterial activity [49]. Phytochemicals aid nano fabrication through their reducing and stabilizing properties.Combining NPs with plant phytochemicals improves the activity of the NPs, and counteracts the occurrence of drug resistance[31,65]. In this study, the antibacterial activity of the *E. elephantina* extracts increased when the extract was combined with AgNPs (Table 5), possibly due to synergism between the two.The MIC values obtained were in agreement withprior studies using 50nm

AgNPs, where MICs of between 1 -32 µg/mL were obtained against laboratory and clinical strains of *M. tuberculosis* and *Mycobacteriumbovis*[33].It is important to note that these NPs were created using the chemical reduction method, whereas our study utilised the green synthesis method, which is deemed a safer and more eco-friendly alternative.

Antimicrobial activity is also influenced by size, charge, and surface properties of the NPs. The average size of the NPs of 44nm, was sufficient to increase antibacterial activity compared to the free *E. elephantina*, but remained lower than the rifampicin control. Variations in antibacterial activity with size of NPs have been observed, where possibly due to the increased surface areas of smaller NPs, they have generally been associated with higher potencies than larger NPs[66]. The effect of the heterogeneous morphologies of the synthesized NPs (Figure 3) was not investigated, though previous studies have shown a relationship between physico-chemical properties of NPs and antibacterial activity [32].

4 Conclusion

The stabilising and reducing properties of phytochemicals allowed for successful fabrication of *E. elephantina*AgNPs with an average size of 44nm, and with demonstrable antimicrobial activity against *M. smegmatis* and *M. aurum in vitro*.This activity was enhanced by conjugation with plant phytochemicals as indicated by a 4 fold-reduction in MIC in *E. elephantina*AgNPs compared to the free *E. elephantina*. AgNPs possess unique properties which allow them to achieve antibacterial action through multiple mechanisms of action, and hence can find usefulness in eliminating drug resistant microbes including multiple drug resistant MTB. However, to avoid toxicity, caution should be applied when *E. elephantina*AgNPs are used in large doses above 4000mg/kg. It is also important to note that the effectiveness of plant conjugated NPs is potentially affected by varying phytochemical composition typical in plants.

Further work needs to be carried out to determine if similar antimicrobial activity patterns are observed with laboratory and clinical strains of susceptible and drug resistant MTB. Exploring the effects of the *E. elephantina*AgNPs in combination with conventional drugs is also a worthwhile area of research, as this could possibly potentiate action of conventional antimicrobials, leading to reduced doses and hence minimise adverse effects of current anti-TB drugs. With the wider adoption of nano technologies, future studies will also need to investigate the long-term effects of nanoparticles on human and environmental health.

5 Ethical Approval

All experiments in this study were examined and approved by the University of Zimbabwe's Joint Parirenyatwa Research Ethics Committee (JREC/04/2021).

6 Disclaimer (Artificial intelligence)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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