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Pontederia crassipes Biosynthesised Silver Nanoparticles (AgNPs), Characterisation and Activity against Multidrug-Resistant Microbes

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Authors' contributions

This work was carried out in collaboration among all authors. Author TCS conceptualised the study, conducted the literature survey and wrote the paper. Author JC supervised the laboratory studies methodologies with animals. Authors JW and JC conducted the nano synthesis and characterisation. Author IM reviewed the study protocols and interpreted the results and findings. All authors read and approved the final manuscript.

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ABSTRACT

Introduction: *Pontederia crassipes* (water hyacinth) has been singled out as one of the major causes of enormous economic and ecological losses to tropical water systems and habitats. Almost impossible to eradicate, the ominous species is characterized by rapid multiplication, giving rapid and extensive spread that can choke entire rivers and water bodies. However, vast, diverse bioactive secondary metabolites are found in P crassipes which exhibit an expansive assortment of

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antibacterial properties used in traditional medicine. The rapid emergence of Multi Drug Resistant (MDR) pathogens to common drugs is a pandemic of global proportions. Global health institutions are calling for a coordinated, global action plan to develop new biomaterials to combat the rising threat by MDR strains and improve treatment outcomes and save lives. This study proposes consumption of the water hyacinth lily species through a novel, material biosynthesis approach not only to manage the aquatic infestation but to develop advanced multifunctional, anti-microbial Silver nanoparticles through bio-reduction and capping of Silver salts targeted at MDR microbes.

Methodology: After screening for active phytoconstituents of *P.crassipes*. The Biosynthesis of Novel AgNPs mediated by phenolic, antibacterial lyophilized hydro-ethanolic extracts from P crassipes as both bioreduction and capping agents was investigated. The resultant AgNPs characteristics, toxicological profiles and the antibacterial effects on 10 common microbes including MDR bacterial strains were evaluated.

Observations and Results: TEM revealed that cubic and spherical nanometric AgNPs structures were successfully synthesized with particle size range from 10-60nm. With an average zone of inhibition of 29mm and an MIC of 6.25 µg/ml .The nanoparticles were efficacious against selected MDR strains and common infectious bacterial strains tested. In-vivo acute oral toxicity evaluations confirmed the safety of *P crassipes*.

Conclusion: Biogenic *P.crassipes* mediated AgNPs are feasible, safe and efficacious and have immense potential for optimization into efficient broad spectrum MDR antibacterial agents.

Keywords: Pontederia crassipes; water hyacinth; antibacterial resistance; biosynthesis; silver nanoparticles; polyphenols.

1. INTRODUCTION

1.1 Pontederia crassipes

Pontederia Crassipes, a native plant species of the Amazon basin has been naturalized in tropical and subtropical regions, invading water bodies and water systems in more than 80 countries, including all territories in Southern Africa with catastrophic consequences [1]. The water hyacinth has been singled out as one of the major causes of enormous economic and ecological losses to tropical water systems and habitats2.This monocotyledonous free-floating, aquatic, notoriously invasive plant is listed as one of the 10 severest weed plants in the world by the International Union for Conservation of Nature (IUCN). Almost impossible to eradicate, the Species Specialist Group (ISSG), has also included this noxious species among the top 100 in the Global Invasive Species Database [2,3]. The University of Florida (UF/IFAS) Assessment, lists the species as prohibited and it is registered with the Florida Exotic Pest Plant Council (FLEPPC) as a Category I invasive species due to its ability to invade and displace native plant communities [3]. The ominous species is characterised by rapid multiplication, giving rapid and extensive spread that can choke entire rivers and water bodies [4]. The highly adaptive plant has remarkable tolerance to pH and wide variations in nutrient and temperature conditions. Incredibly, it has a capacity to reproduce both sexually and asexually using seeds and stolon's respectively [5]. Scientific reports indicate that it can treble its biomass in just 20 days under ordinary growth conditions. Another interesting report indicated that in just 8 months, 10 specimens of the water hyacinth were able to multiply to over 600,000 individual plants with titanic coverage's5. It is this coverage that has disastrous consequences on the food web, affecting water quality as well as providing habitats for various pathogens, parasites and vectors of endemic diseases including malaria and helminths in tropical countries [5]. The huge *Pontederia crassipes* mats choking water bodies' create problems including high demand on water oxygen and high nutrients consumption, which have deleterious effects on species diversity [4,6]. These worrying mats do not only obstruct light but challenge navigation through the affected water bodies as well. Various studies have reviewed or proposed technological devices and protocols including multi modal approaches to manage the relentless spread of this plague in tropical countries, most of which have limited practical use and offer very insignificant reprieve to the scourge [5]. Traditionally, local authorities and other agencies have regarded this menacing plant species as an intolerable pest to be eradicated from water bodies by all means necessary including, physical, chemical and biological methods with disappointing success rates [6].

Interestingly, various researchers allude to the vast diverse bioactive secondary metabolites found in *P crassipes* which exhibit an expansive assortment of pharmacological properties with favourable untapped use in biomedical applications [4-7]. Its ability to proliferate in polluted waters point to an ability to absorb and rearrange the morphology of heavy metal complexes acting as a sorbent [6]. Traditionally the plant infusions and extracts have been used for gastrointestinal conditions including dysentery and diarrhoea, helminthic parasitic infections, digestive disturbances and flatulence. The antioxidant, antimicrobial, antitumor, anti-inflammatory and wound healing attributes of this plant have extensively been reported [7].

Most of the reviews on the potential utilisation of the water hyacinth have mostly been limited to phytoremediation, bioenergy sources and stock feeds production. Its pharmacological applications and its ability to bio reduce and cap metallic oxides giving opportunities for conjugated nano-composites based on its pharmacological characteristics and phytoconstituents profiles for enhanced biomedical applications have been barely reviewed. This study proposes consumption of the water hyacinth plant species through a novel, material biosynthesis approach not only to manage the infestation but to develop advanced multifunctional, anti-microbial platforms by bio-reduction and capping of anti-microbial metallic complexes from silver by the various phenolic phytoconstituents of the species to develop new biomedical materials for pharmaceutical application.

1.2 Anti-bacterial Resistance

Antimicrobial agents were medically popularised and first commissioned at the beginning of the Second World War in the 1940s as treatments for various serious bacterial infections. Penicillin was one of the most successful pioneering anti-bacterial agents which proved essential in combating bacterial infections among soldiers during the war [8]. Incidentally, the celebrations over of these new medical bacterial panacea was cut short when in the 1950s penicillin resistance emerged as a threat to treatment outcomes [9]. Since then, constantly emerging resistant bacteria strains continue to plague the health delivery systems eroding much of the progress achieved with antibiotics and rewinding the health benefits realised over the past 80 years in antimicrobial research and development. Bacterial antimicrobial resistance (AMR), a phenomenon that occurs when adaptations in bacteria mechanisms render the treatments less effective is a global crisis which has in many sectors been described as one of the leading public health threats of the modern world [10]. Frightening forecasts by the UK government commissioned “Review on Antimicrobial Resistance” forecast that AMR related fatalities could account for up 10 million yearly deaths before 2050 [11]. The Antibiotic resistance pandemic has been attributed by most researchers to a number of causes including: development of antibiotic resistance genes among populations, the careless and inappropriate prescription of drugs, abuse and sub-use as well as the duality use of most antibiotics in humans and agriculture [12]. With regards to antibiotic overuse, as early as 1940s, Sir Alexander Fleming warned the world regarding antibiotic overuse. The argument is that the overuse of antibiotics is the main driver behind the evolution of multidrug resistance [13]. Various epidemiological studies have also confirmed the direct correlation between antibiotic use and the rise of resistant bacteria strains. The inappropriate prescription of drugs especially antibiotics also exacerbate the rapid development of resistant bacteria. Studies conducted in the U.S concluded that diagnosis, choice and duration of the antibiotic treatment course is often incorrect in up to 50% of treatment clinical cases [14]. Globally, antimicrobials for human use are also used as drugs and supplements in livestock. The best example from the developing world is the Multi drug administration treatment for helminthisiasis with albendazole and mebendazole which are originally veterinary drugs since the 1970s. In the developed world, studies confirm that over 80% of the antibiotics used in healthcare in the U.S. are also used in animals, mostly as prophylaxis to prevent infection and as growth supplements [15,16]. These antimicrobial supplements inadvently find their way systemically through food consumption. These phenomena are compounded by the stalled research and development of new antimicrobial agents due to structural and regulatory barriers [17]. Oligopolistic mergers among multinational pharmaceutical companies have substantially restricted diversity of antimicrobial agents in healthcare and development of new molecules. For those still involved in drug discovery research and development of antimicrobials, obtaining regulatory approval is an insurmountable barrier [18-20]. The huge

challenges in obtaining regulatory approval reported include among other things, bureaucracy, dossier irregularities and lack of clarity on procedures as well as the complications and expense of running clinical trials [21].

1.3 Natural Antibacterial Plant Polyphenols

Plants have adapted to survive in almost all natural habitats of the earth. With regards to the various challenges posed by the different environments, plants have adapted to deal with, as well as to heal from herbivorous attacks and pests including various forms of microbial attacks [22]. Advances in forensic and analytical sciences have revealed that this capacity to survive is consequent of their ability to synthesise numerous arrays of metabolites. An individual plant species is believed to be able to synthesise up to 10 000 different metabolites [23]. These biologically active metabolites is the survival package sustaining plants amidst environmental threats and stresses.

Plants synthesise 2 main sub groups of metabolites. Primary metabolites are phytochemicals developed to sustain the plant growth, proliferation and subsistence. Primary metabolites include carbohydrates, proteins, vitamins and many other physiologically functional molecules. On the other hand, the secondary metabolites are the specialised functional phytochemicals developed by the plant for self-protection, preservation and survival in the environment. The secondary metabolites subgroup, which is of interest to this study are the ones that are referred to as phytochemical compounds [24]. Numerous studies have scientifically validated that phytochemicals have toxicological, pharmacological and physiological effects on the cells of mammals including humans and various microbiota. They can be loosely classified into 4 coherent groups : Phenolics which include flavonoids and tannins: volatile terpenes in all their configurations from mono to polyterpenes; non protein amino acids and other nitrogen containing compounds; Sulphur containing compounds including lectins [25].

The proved lethality potential of some of the secondary metabolites to pathogenic bacteria species including some microbial's that are resistant to traditional antibiotics have put them at the forefront in the battle against multidrug resistant bacterial species [26]. Different plants produce varying levels and different types of phytochemicals depending on the most prevalent threats in their domain of origin so as to guarantee their survival [27]. It is with this background that most plants with significant pharmacologically relevant phytochemicals have found cardinal uses in traditional medical practices as antimicrobial and anti-oxidant treatments.

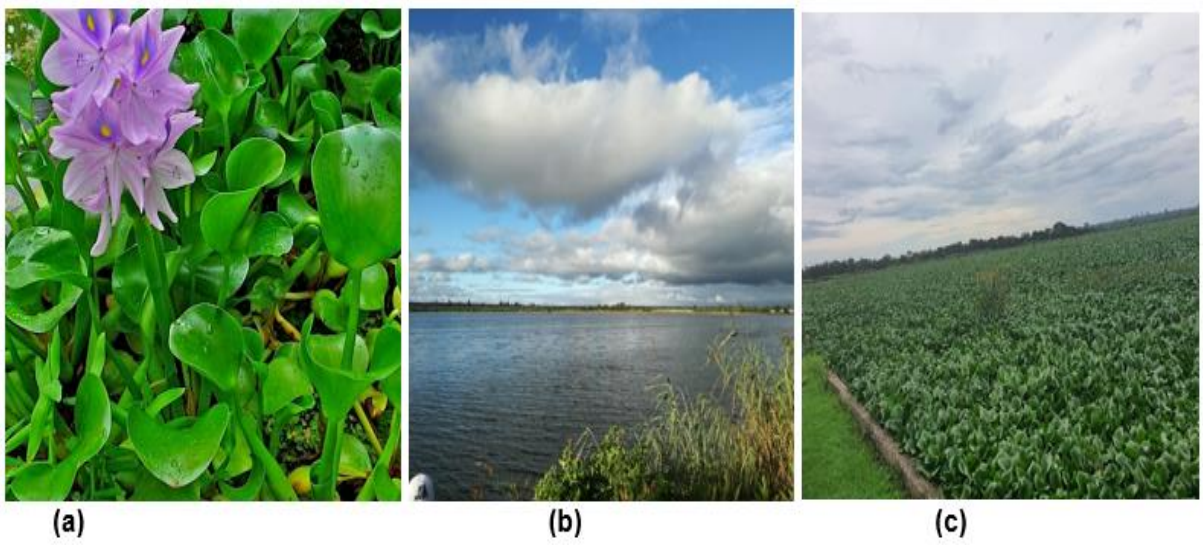

Fig. 1. (a) *P.crassipes* plant specimens floating on water (b) The Umguza Dam in Bulawayo Zimbabwe in 2016 before *P. crassipes* infestation (c) The Umguza dam in Bulawayo Zimbabwe after invasion by *P.crassipes* in 2022

1.4 Biosynthesised Metallic Nano-particles from Polyphenols

The increasing drug-resistance to most antibiotics has necessitated the development of new antimicrobial agents and alternative technology based treatments with improved treatment outcomes. Over the past few years advances in nanotechnology have ushered in new nano-scale biomaterial platforms with optimised antimicrobial actions towards most multidrug resistant pathogens. Metallic nanoparticles present advanced nanotechnological antimicrobial platforms, with high efficacies and advanced mechanisms of action [28]. The microcidal properties of Nano metric metallic oxides from various sources have been investigated and among the various synthesis techniques, biosynthesised metal nanoparticles present safer and easy one pot processed low dimensional structures for various microcidal applications. Biosynthesis as a novel alternative method has been inspired by the concerns regarding the synthesis of colloidal nanomaterials, such as those involving harmful solvents and toxicologically unsafe precursor chemicals which give rise to the production of unsafe toxic by-products [29]. This eco-friendly, synthesis technique employs biological living systems including, plants, yeasts, bacteria and viruses as bio-reducing and stability imparting capping agents. Biosynthesised metallic nanoparticles present a dynamic, safe and efficacious substitute to chemically-produced antimicrobial nano platforms [30].

Fabrication of novel nano-composites through bioreduction of metallic salts by pharmacologically active polyphenols of known clinical value from medicinal plants is attracting increasing interest due to their potential to harness the medicinal properties of the natural polyphenolic extracts and conjugate them to the inherent amplified properties of the metallic oxides [31]. Biosynthesised metallic nano-composites promise enhanced therapeutic competencies in comparison with the simple natural polyphenols. In experimental models metallic nano-composites demonstrate remarkable augmented treatment outcomes due to the additive or synergistic dual activities of the constituents [30]. The ability to control and fine-tune resultant desired properties through varying fabrication conditions including pH, temperature, concentration and rate of reaction adds a valuable dimension to their practicality in therapeutics. The same fabrication conditions can also determine size and shape of the metallic nanoparticles which has a gigantic effect on phase aggregation as well as therapeutic, bio and chemical equivalence. In the present investigation we present the bio inspired fabrication of metallic nano platforms of known antimicrobial agent, ionic silver by the pharmacologically active polyphenolic antimicrobial constituents of *Pontederia crassipes.* We further investigate the morphology and toxicity profiles as well as the amplified antibacterial effects on multidrug resistant microbes.

2. MATERIALS AND METHODS

2.1 Materials, Equipment and Facilities

All chemicals, cell cultures, associated reagents, equipment and facilities for the *in-vivo* laboratory animal toxicity assays and the antibacterial inhibition determinations were obtained from the University of Zimbabwe College of health sciences laboratories, Harare, Zimbabwe. For the green synthesis and the nanoparticles characterisations, all chemicals and equipment were availed by the University of California, Los Angeles, Department of Chemistry and biochemistry. Los Angeles, CA 90095, USA.

2.1.1 Plant materials preparation

Whole *Pontederia Crassipes* plants were collected from Lake Chivero, 30 km South-West of Harare, Zimbabwe (17.9117° S, 30.7875° E). The plants were authenticated taxonomically by the national herbarium technical officers in Harare, Zimbabwe. The plants were washed under running water and the leaves were detached from the stems and tubers. The leaves were then solar dried naturally for 20 days in the shade. The dried leaves were ground into a powder using a coffee grinder. The powder was then stored in a tightly closed jar awaiting both hydro and ethanolic solvent extraction.

2.1.2 *Pontederia crassipes* leaf hydro-ethanolic extract preparation

In a 2000 ml round bottom flask, 300g of *Pontederia Crassipes* dried leaf powder was added to 900ml of a 30:70 hydro-ethanolic solution (70% ethanol) and left to cold extract for 3 days with daily 5 minute shakings twice a day. The obtained extract was filtered using filter paper (Whatman no. 1) into a suitable conical flask through a Buchner funnel. The resultant

supernatant was then evaporated under low pressure (Rotavapor® R-200, Buchi, Switzerland), and subsequently followed by lyophilization (Lyovapor l-200, Buchi, Switzerland) under 120Pa pressure and a temperature of –20°C. The lyophilized extract crystals were kept in a refrigerator at 4°C.

2.2 Phytochemical Screening

In a 200ml round bottomed flask, 10g of the lyophilized hydro-ethanolic extracts of *Pontederia Crassipes* were dissolved in 100g of distilled water and subjected to various phyto-screening techniques to confirm the presence or absence of relevant phytoconstituents of pharmacological interest to this study. The following qualitative tests were conducted on the extract liquor:

2.2.1 Tests for alkaloids

The presence of alkaloids was determined through the Mayer's test. To 5 ml of the lyophilized extract liquor in a test tube, two drops of Mayer's reagent were added. The presence of alkaloids was determined by the development of a white creamy precipitate at the bottom [32].

2.2.2 Tests for tannins and phenolics

The presence of tannins in the extract was determined by the ferric chloride test. To a test tube, 2-3 drops of ferric chloride was added to 5 ml of the prepared extract liquor. The test sample was observed for the presence of catechic tannins signalled by the development of a green-blue colour or a blue-black colour development which indicates the presence of Gallic tannins [33].

2.2.3 Test for flavonoids

The presence of flavonoids was determined by means of 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. 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 [34].

2.2.4 Test for terpenoids

To confirm the presence of terpenoids. To a test tube with 5ml of the extract liquor, 2 or 3 granules of tin metal in 2 ml thionyl chloride solution were added. The formation of a pink colour indicates the presence of terpenoids [35].

2.2.5 Tests for steroids

The presence of steroids in the hydro-ethanolic extract of *Pontederia Crassipes* was confirmed by adding 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_2SO4. The development of a reddish brown colour indicates the presence of sterols in the extract [36].

2.2.6 Test for saponins

To determine the presence of saponins in the test sample, the simplified foam test was used. In a 100ml measuring cylinder, 5ml of the extract liquor was added to 30ml distilled water, the mixture was shaken for 2 minutes and the development of at least 1 cm head of foam in the test tube confirms the presence of saponins [37].

2.2.7 Detection of proteins and amino acids

The detection of proteins and amino acids was done using Millon's Test. In a test tube with 5ml of the extract liquor, 2 ml of Millon's reagent was added and the test tube contents were heated in a water bath at 70°C for 5 minutes. The test tube was then cooled and 2 to 3 drops of sodium nitrite were added. The presence of proteins and amino acids is observed by the formation of a white thermo chromic precipitate which turns to a crimson red upon heating [38].

2.2.8 Detection of carbohydrates

The Molisch's test was used to determine the presence of carbohydrates in the lyophilised extract. In a test tube, to 5 ml of the extract liquor, 2 to 3 drops of alcoholic α-naphthol solution was added. The presence of carbohydrates is detected by the development of a violet ring at the junction [39].

2.2.9 Detection of glycosides

The modified Borntrager's assay was used to determine the presence of glycosides. 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°C for 10 minutes. After cooling, extraction was done with 10ml of benzene. The resultant benzene layer was decanted and treated with 5ml ammonia solution.

The mixture was observed for the development of a pink colour which signals the presence of anthranol glycosides [40].

2.2.10 Acute oral toxicity evaluation and treatment schedule

The acute oral toxicity profiling of *Pontederia Crassipes* was investigated through a modified OECD technical guideline 425 protocol [41]. In this study female laboratory albino rats were used. As per protocol requirements, the animals weighed approximately ±225g and were aged between 8 to 10 weeks old. The rats were introduced into the experimental housing conditions 7 days before the commencement of the experiments so that they can acclimatise to the environment. The rats were fed a standard commercial rodent diet and water adlibitum [41].

The modified investigation carried out consisted of single ordered dose progressions [41]. The test and control animals were dosed, in sequence, at 2 day 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. Based on our assumption that *Pontederia crassipes* was most likely non-toxic due to its prevalence in communal water sources and reported traditional medicinal uses without any reported adverse effects by the users, the starting dose investigated was 250mg/kg body weight and the limit dose was 2000mg/kg body weight, 12 female nulliparous female rats were used in this study. To facilitate precise identification of the individuals, the participating rats were marked prior to the investigations. Also prior to the first dose, the participating animals were fasted pellets for 18 hours but had access to water. The *Pontederia Crassipes* was orally gavaged in the following different concentration in a distilled water solution in 4 different doses of: 250, 500, 1000, and 2000 mg/kg body weight, A contracted veterinary specialist with no vested interests in the study observed the animals for mortality and in the absence of mortality they were investigated for any changes in behaviour and clinical signs and symptoms of toxicity every hour up to the first 12 hours on day 1 and thereafter, every day up to day 14 of the investigations.

2.3 Biosynthesis of Silver Nanoparticles

The green synthesis route was followed for the biogenic fabrication of the augmented *Pontederia Crassipes* AgNPs through bioreduction of $AgNO_3$ by the extract liquor's polyphenolic phytoconstituents. For the synthesis, 100ml of a 1 mM solution of silver nitrate was prepared using distilled water. For the bioreduction of Ag+ ions, 20 mL *Pontederia Crassipes* 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 15 minute intervals and overnight for optimal synthesis of AgNPs. The synthesis of silver nanoparticles 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.

2.3.1 Transmission Electron Microscopy (TEM) analysis of AgNPs

The biosynthesised AgNPs were analysed by TEM for their size and shape. 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 then 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 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.

2.4 Antibacterial Activity Test

2.4.1 Test microorganisms

The bacterial species; *S.triphimurium, P.Aeruginosa, B. subtilis, M. luteus, streptococcus and pseudomona*, multi drug resistant strains ® of gram positive *Streptococcus pyogenes* and meticillin sensitive *Staphylococcus Aureus* as well as gram negative *Escherichia coli* and *Klebsiella pneumoniae* were obtained from the University of Zimbabwe Department of medical microbiology in Harare, Zimbabwe. The antibacterial, evaluation laboratory studies were performed according to CLSI guidelines.

2.4.2 Determination of the zone of inhibition

A modified, simplified Kirby-Bauer test [42] was configured and used in this study to determine and compare the susceptibility or resistance of the chosen pathogenic bacteria to the *Pontederia Crassipes* AgNPs, Colloidal AgNPs as well as crude *Pontederia Crassipes* through zone of inhibition observations and MIC determination through serial dilution techniques.

2.4.3 Zone of inhibition MIC test requirements

- Mueller-Hinton agar plates
- Sterile swabs and forceps
- Pure bacterial cultures
- Samples of *Pontederia Crassipes* mediated AgNPs, Ionic silver, *Pontederia Crassipes* extract liquor

2.4.4 Zone of inhibition measurement and MIC determination test protocol

Bacterial inoculum suspensions were spread uniformly on solidified Muller−Hinton Agar (MHA) using sterile swab [43]. The bacterial strains used in the first study to confirm the broad spectrum antibacterial effects of *Pontederia crassipes* were *S.triphimurium, P. Aeruginosa, Bacillus subtilis, Micrococcus luteus, streptococcus and pseudomona.* The second evaluation used multi drug resistant strains ® of gram positive. *Streptococcus pyogenes* and meticillin-sensitive *Staphylococcus aureus* as well as gram negative *Escherichia coli* and *Klebsiella pneumoniae* alongside their susceptible reference stains denoted by R in the tests.

In the procedure: To the Mueller-Hinton agar plates, swabs of the pure bacterial cultures were evenly spread over and 2 -3 drops of the test samples were placed in the media plate using sterile forceps. The petri plates were incubated for 24 hours at 36^{o}C with controlled humidity. After the incubation period and diffusion of the test samples, the clear area (zone of inhibition) around the point of introduction of the samples was observed and measured. The size of the zone of inhibition is directly proportional to the antibacterial activity.

For the determination of MICs the same experimental set up was used. Serial dilutions of the test materials with distilled water was done and the minimum concentrations of the test materials needed to inhibit the ability of the microorganism's ability to produce any visible growth in the agar plates was noted. In this simplified modified method, the lowest concentration of the antimicrobial agents (in µg/ml) which prevented the appearance of visible growth of the microorganisms within a 24 hour period were determined as the MIC.

3. RESULTS AND DISCUSSION

The preliminary phytochemical analysis of the leaf extract revealed the presence of several primary metabolites including carbohydrates, proteins and amino acids (Table 1). This correlates well with other studies which have identified the heteropolysaccharides L-galactose, D-xylose and others in *P.crassipes* [22-24]. Studies have also confirmed the presence of hemicellulose, triacylglycerol's and glycolipids. Other investigations have identified various amino acids including leucine and glutamine. This heavy presence of primary metabolites is perhaps the reason behind the lily's ecological resilience, high capacity for propagation and multiplication in aquatic systems [24], however for the purposes of this study, it is the presence of various pharmacologically interesting secondary metabolites that captures our attention. The crude extract showed strong presence of phenolic compounds in both the hydro-ethanolic extract as well as the hydro-extract [46]. Quantitative studies by other researchers isolated simple phenols represented mostly by 2 (and 4)-methylresorcinol, catechol and resorcinol. Polyphenolic acids detected in the plant leaves by comparative studies include, petioles, p-hydroxybenzoic (parabens), and

gentisic, and p-coumaric, Gallic and salicylic acids4 [25-45]. All these listed phenolic acids which are known to have considerable pharmacological activity are also believed to be the ones responsible for the bioreduction and capping of $AgNO_3$ in the biosynthesis process. Our analysis also revealed the strong presence of flavonoids and their glycosides. This collaborates well with other studies that have identified flavonoids including tricin, luteolin, kaempferol and quercetin [25, 26]. Flavonoids are widely known in the medical world for their medicinal benefits, including antibacterial, antiviral, antifungal, anticancer, antioxidant and anti-inflammatory attributes [46-48]. The alkaloids, saponins, terpenoids and Phytosterols present in the crude extract all point towards a plant with high medicinal application potential and also relates well with its high adaptability to different harsh environments including water with high toxin levels as well as resilience to parasites [49]. The presence of these metabolites substantiate the traditional use of *Pontederia crassipes* as an antibacterial agent in traditional medicine and also suggest its potential use in biosynthesis of nano-platforms from metallic salts.

3.1 Acute Oral Toxicity Evaluation

The test results and observations from the toxicity profiling substantiate that the hydro-ethanolic leaf extract of *Pontederia crassipes* is safe for internal use up to the standardised toxicity cut off dose of 2000mg/kg body weight. The behavioural 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 journalised by an experienced veterinary specialist (Table 2). 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.

Table 1. Phytochemicals present in *Pontederia crassipes* hydro-ethanolic and distilled extracts

Test	Presence in hydro-ethanolic extract	Presence in distilled water extract
Alkaloids	++	+
Phytosterols	+	-
Flavonoids	+++	+
Saponins	-	-
Proteins and Amino Acids	+	+
Fixed oils and fats	-	-
Phenolic compounds	+++	+
Tannins	++	+
Carbohydrates	++	+
Glycosides	+	+
Terpenoids	+	+

(-) Indicates absence, (+) Indicates presence, (+++) indicates strong presence.

Table 2. Observations for behaviour and appearance of rats during studies

Observed parameter	Dose of *Pontederia crassipes* in mg/kg body weight			
	250mg	500mg	1000mg	Control
Food intake	Normal	Normal	Normal	Normal
Water intake	Normal	Normal	Normal	Normal
Death	Alive	Alive	Alive	Alive
Breathing	Normal	Normal	Normal	Normal
Diarrhoea	Not observed	Not observed	Not observed	Not observed
Urination	Normal	Normal	Normal	Normal
Skin colour	Normal	Normal	Normal	Normal
Drowsiness	Not observed	Not observed	present	Not observed
Erection of Fur	Not observed	Not observed	Not observed	Not observed

3.1.1 Behavioural pattern and LD50

In the absence of viable and technically appraised non animal alternatives, the use of animals in research still continues as the cornerstone of safety studies. The rise in animal rights activism, has raised concerns regarding moral issues, cruel methodology and translational science issues for decades now. It is therefore imperative that animal studies should be reviewed by Local Animal Ethics Committees so as to safeguard research animals and guarantee that they are treated in respect of the 3Rs (Replace, Reduce, Refine) principles [50]. Despite these concerns no alternative method has been able to approximate the complete effects of metabolites and their fate in living systems. Scientific safety studies seek to understand organism's metabolic responses to toxins and drugs in order to gain a fuller knowledge of the metabolism of drugs inside the organisms and non-animal alternatives fall short of such capacities [51]. It is on these basis that we conducted animal studies after seeking approval from a local ethics board and the studies were conducted under strict adherence to the 3Rs as well as international protocols and guidelines.

The OECD technical guideline 425 with minor modifications was used in this study. As per the guideline, only healthy adult animals were used. The females chosen for the study were nulliparous and non-pregnant. The rats were specifically bred for such studies and the ages which were already known were between 8-12 weeks as required by the guidelines. 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 up and down test study of the *Pontederia crassipes* extracts was carried out using rat models at doses of 250, 500, 1000 and 2000mg/kg body weight. The rats were continuously 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 noted changes observed in all rats for all categories. The study concluded that *Pontederia crassipes* was toxicologically safe at 2000mg/kg body weight and therefore LD50 is concluded to be beyond 2000mg/kg body weight. With reference to the Hodge and sterner classification for toxicity, the hydro-ethanolic leaf extract of *Pontederia crassipes* is classified as nontoxic.

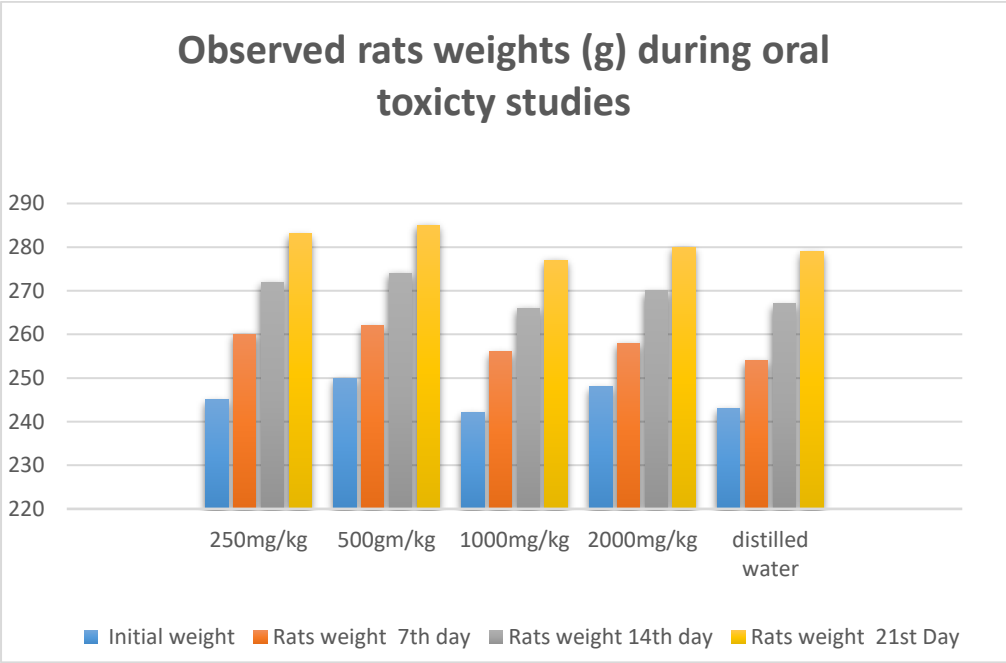

Fig. 2. Observations for rat's weights over the experimental period

3.1.2 Body weight observations

As part of the observations for toxicity signs, the body weights of the animals under test were monitored during the study period. The weights for all animals in the five groups were taken weekly for the test period starting on the initial day and every 7th day thereafter until day 21. In all the recorded weights, all 4 treated groups did not exhibit statistically relevant or significant aberrations in body weight in comparison with the control group.

Underlying health conditions and physical stress is usually accompanied by irregular weight changes in animals [51]. Uncharacteristic and unexplained gains or losses in weights are indicative of internal changes in metabolism and failure of the homeostatic processes. Loss or appetite and failure to feed is directly related to adverse health conditions latent or otherwise [51, 52]. The initial weights for all the rats selected for the study were within specifications for 8 to 10 week old laboratory rats. Interpretations of the observations confirmed the expected gradual weight increase over a progressive three week period of normal feeding expected from normal healthy animals. The body weight changes among the groups and the control were not statistically significant. The normal progressive body weight increases observed and the continued normal feeding appetite is a confirmation of the absence of any toxicity effects on the rats from *Pontederia crassipes* and correlates well with the absence of any deaths among the animals under the tests. This gives confidence that *Pontederia crassipes* leaf extracts do not interfere with the normal metabolism and health of the animals.

3.2 Biosynthesis and Characterisation of *Pontederia crassipes* Mediated AgNPs

For our purposes, the green synthesis of AgNPs from the leaf extract of *Pontederia crassipes* provided a safer, economical 1 pot, sustainable and eco-friendly method. In this investigation, the polyphenols in the leaf extract were the reducing and capping agents for the synthesis of AgNPs. The change in colour which was observed from dark brown to reddish brown indicated the formation of AgNPs. The colour change, which is due to the excitation of surface plasmon vibrations, was noted to have spectroscopic confirmation of AgNPs formation [53]. The UV–Visible spectrum confirmed peeks between 400-500nm which is the expected UV–Visible absorption maximum range for AgNPs. The poly dispersed range of the observed peaks might confirm the varied polyphenols in the *P.crassipes* extract. TEM analysis revealed that the obtained AgNPs were primarily a mixture of cubic and spherically configured structures (Fig 3). Their size ranged from 10 to 60nm and DLS confirmed that average size of the nanoparticles was 35.5nm.

LCMS chromatograms (Fig 4) of the crude extract and the supernatant after the synthesis reveal the disappearance of many peaks at elution times 3.34, 5.41, 5.43, 6.10, 6.10, 6.31 and 6.82. These peaks indicate the major phytoconstituents consumed in the synthesis process. No new major peaks emerged in the LCMS supernatant which confirms the complete removal of the developed AgNPs. Further work is imperative to identify the exact participating polyphenols involved in the bioreduction and capping noted by these chromatograms.

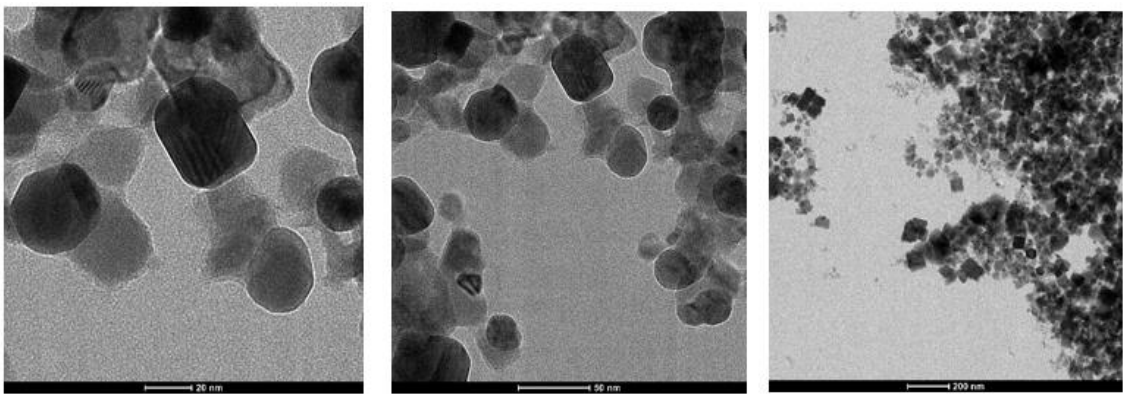

Fig. 3. TEM images of *Pontederia crassipes* AgNPs nanoparticles in different size range images

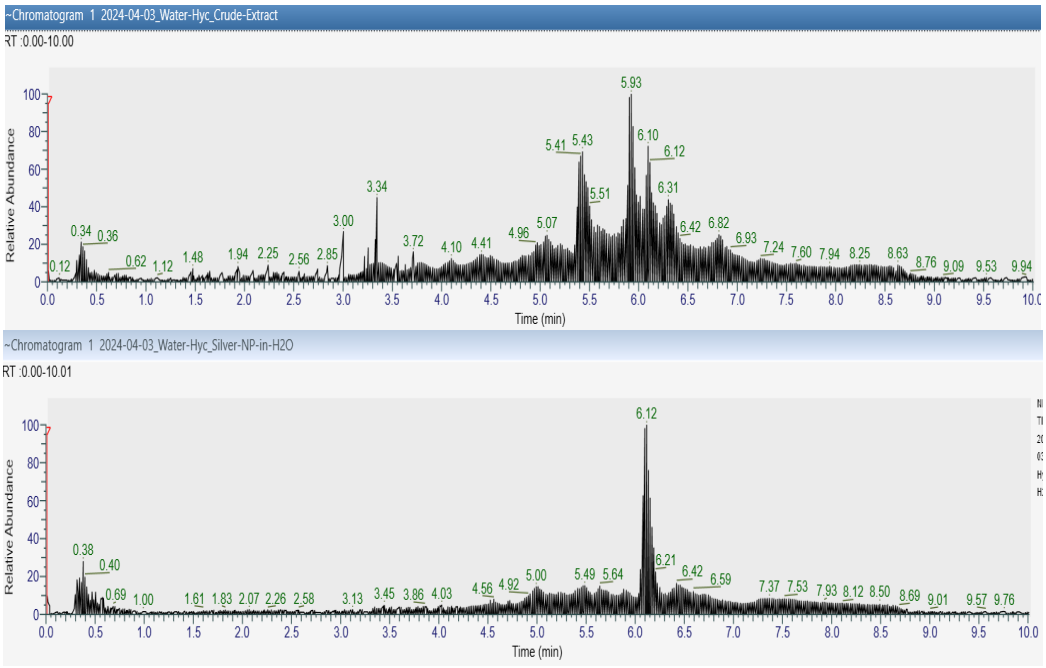

Fig. 4. LCMS images of *Pontederia crassipes* AgNPs chromatograms for the crude extract above and the supernatant below after nanoparticle fabrication

Table 3. Antibacterial zones of inhibition for *P. crassipes, P crassipes* AgNPs as well as penicillin on common microbes

Test Sample	µg/ml	Inhibition zone in diameter (mm)					
		S. triphimurium	***P. Aeruginosa***	***M. Luteus***	***Streptococcus***	***B subtilis***	***Pseudomonas***
P Crassipes	25	17±0.9	18±0.3	16±0.6	17±0.5	16±0.8	17±0.7
P C (AgNPs)	25	27±0.7	28±0.8	28±0.4	29±0.7	29±0.7	30±0.7
Penicillin	-	18±0.7	18±0.7	18±0.7	18±0.7	18±0.7	18±0.7

**All values are mean ± standard deviation of 3 determinations, S.triphimurium =Salmonella enterica serovar Typhimurium, P. Aeruginosa =Pseudomonas aeruginosa, B subtilis =Bacillus subtilis, M. luteus = Micrococcus luteus*

Table 4. Antibacterial activity zones of inhibition for *P.crassipes, P crassipes* AgNPs as well as penicillin on multidrug resistant strains

Test Sample	µg/ml	Inhibition zone in diameter (mm)					
		Gram positive bacteria			Gram negative Bacteria		
		MRSA®	***MSSAR***	***S.Pyogen®***	***E.coli®***	***E.coliR***	***K.pne®***
P Crassipes	25	18.03±0.7	19.24±0.3	17±0.5	21.18±0.9	22.30±0.5	23.22±0.7
P Crassipes (AgNPs)	25	27.24±0.5	28.45±0.8	28.45±0.4	29.35±0.5	29.68±0.1	30.56.±0.6
Penicillin	-	-	18.56±0.2	-	-	18.24±0.8	-
Colloidal silver	-	27.24±0.0	28.45±0.8	28.45±0.4	29.35±0.6	29.20±0.7	31.52.±0.6

**All values are mean ± standard deviation of 3 determinations, ® =the multidrug resistant strain, R =Reference strain (susceptible), S. pyogenes= Streptococcus pyogenes, MSSAR =meticillin-sensitive Staphylococcus aureus, MRSA =meticillin resistant Staphylococcus aureus E.coli =Escherichia coli, K.pne =Klebsiella pneumoniae*

Table 5. Minimum inhibition concentrations for *P. crassipes, P crassipes* AgNPs as well as penicillin on multidrug resistant strains

Bacterial strain	Minimum inhibitory concentrations µg/ml		
	P. Crassipes extract	***P. Crassipes* with AgNPs**	**Colloidal AgNPs**
MRSA®	28	6.25	6.25
S.Pyogen®	25	6.25	4
E Coli®	28	6.25	4
K. pneumoniae®	30	6.25	6.25

3.3 Anti-Bacterial Assays

In the antibacterial tests against susceptible common microbes, both the lyophilised *Pontederia crassipes* as well as *Pontederia crassipes* mediated AgNPs exhibited considerable antibacterial effects. The lyophilised plant extract was slightly lower in activity when comparable to the commercial drug used as standard. However the AgNPs exhibited antibacterial effect much higher than the commercial drug. (Table 3). The commercial drug was ineffective against multidrug resistant strains (Table 4). *Pontederia Crassipes* AgNPs were effective against multidrug resistant strains and their effect on these and their susceptible reference strains was not significantly different. The developed nanomaterials had comparable MICs to the commercially sourced colloidal AgNPs used as the standard (Table 5).

The antibacterial activity of the lyophilised hydro-ethanolic extract obtained at 25 µg/ml is much better than those reported in many studies. The *Pontederia crassipes* AgNPs also possessed comparable zone of inhibitions to colloidal AgNPs. There was not much difference in antibacterial activities of AgNPs between the bacterial strains, despite the structural differences in bacterial morphologies between gram positive and gram negative types. The present results show slightly higher antibacterial efficacies most likely due to the complimentary antibiotic effect from the *Pontederia crassipes* abundant secondary metabolites. The synergistic effect from the association of metallic nanoparticles with pharmacologically active polyphenols in the extract against Multidrug resistant bacteria points to new perspectives and safer options in the treatment of drug resistant infectious diseases [54-57]. This will also go a long way in alleviating the weaknesses associated with colloidal AgNPs as antibacterial agents including toxicity, high cost, low solubility, and side effects. These results therefore disclose the immense opportunity in curtailing anti-bacterial resistance by associating natural antibiotics and antibacterial metallic oxides through safe biosynthesised optimised nano-conjugates which can even be further functionalised to enhance activity due to the known versatility of multifunctional green synthesised nano platforms [58,59].

4. CONCLUSIONS

The current investigation informs, confirms, and validates the potential for optimising antibacterial affectivity of polyphenol rich plant extracts through biosynthesis of amplified metallic salts nanoparticles as arsenal for multidrug resistant strains of bacterial strains. The *Pontederia crassipes* mediated AgNPs synthesised in this simple, affordable, low risk (nontoxic structures) process exhibited appreciable antimicrobial responses here, that are scientifically valid enough to draw reliable affirmations and conclusions that, biogenic *Pontederia crassipes* AgNPs are feasible, safe and efficacious and presage a suitable vehicle in the journey towards the development of new and more efficient Multi drug resistant antibacterial agents.

CONSENT

It is not applicable.

ETHICAL APPROVAL

Prior to the investigations, animal use and research ethics approvals were obtained from the Joint Parirenyatwa Research Ethics Committee (JREC) which is the local research Institutional Review board for the University of Zimbabwe.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Harun I, Pushiri H, Amirul-Aiman AJ, Zulkeflee Z. Invasive water hyacinth: Ecology, impacts and prospects for the rural economy. Plants. 2021;10:1613. Available:https://doi.org/10.3390/plants10081613
2. Ben Bakrim W, Ezzariai A, Karouach F,Sobeh M, Kibret M, Hafidi M, Kouisni L, Yasri A. Eichhorniacrassipes (Mart.) solms: A comprehensive review of its chemical composition, traditional use, and value-added products. Front. Pharmacol. 2022;13:842511. DOI: 10.3389/fphar.2022.842511
3. Available:https://www.iucngisd.org/gisd/100_worst.php
4. Kunatsa T, Madiye L, Chikuku T, Musademba D. Feasibility study of biogasproduction from water hyacinth a case of lake chivero – harare, Zimbabwe. Int. J. Eng. Technol. 2013;3(2): 119– 128.
5. Kumar D, Karthik M, Rajakumar R. GC-MS analysis of bioactive compounds from ethanolic leaves extract of eichhornia crassipes (Mart) solms. And Their Pharmacological Activities. Pharma Innov. J. 2018a;7:459–462.
6. Kumar D, Karthik M, Rajakumar R. In-silico antibacterial activity of active phytocompounds from the ethanolic leaves extract of eichhornia crassipes (Mart) solms. Against Selected Targe; 2018b.
7. Guerena D, Neufeldt H, Berazneva J, Duby S. Water hyacinth control in Lake Victoria: Transforming an ecological catastrophe into economic, social and environmental benefits. Sust. Prod. Consumpt. 2015;3:59−69.
8. Nadgir CA, Biswas DA. Antibiotic Resistance and Its Impact on Disease Management. Cureus. 2023;15 (4):e38251. DOI: 10.7759/cureus.38251 PMID: 37261148; PMCID: PMC10226836
9. Davies J, Davies D: Origins and evolution of antibiotic resistance. Microbiol Mol Biol Rev. 2010;74:417-33. DOI: 10.1128/MMBR.00016-10
10. Zaman SB, Hussain MA, Nye R, Mehta V, Mamun KT, Hossain N: A review on antibiotic resistance: Alarm bells are ringing. Cureus. 2017;28:e1403. DOI: 10.7759/cureus.1403
11. Available:https://amrreview.org/#:~:text=%E2%80%9CThe%20Review%20on%20Antimicrobial%20Resistance,actions%20to%20tackle%20it%20internationally
12. Larsson DG, Flach CF. Antibiotic resistance in the environment. Nat Rev Microbiol. 2022;20:257-69. DOI: 10.1038/s41579-021-00649-x
13. Aslam B, Khurshid M, Arshad MI, et al. Antibiotic resistance: One health one world outlook. Front Cell; 2021
14. Available:https://www.pbs.org/newshour/health/the-real-story-behind-the-worlds-first-antibiotic
15. Bengtsson-Palme J, Kristiansson E, Larsson DG: Environmental factors influencing the development and spread of antibiotic resistance. FEMS Microbiol Rev. 2018;42:fux053. DOI: 10.1093/femsre/fux053
16. Kunhikannan S, Thomas CJ, Franks AE, Mahadevaiah S, Kumar S, Petrovski S. Environmental hotspots for antibiotic resistance genes. Microbiologyopen. 2021;10:e1197. DOI: 10.1002/mbo3.1197
17. Savoldi A, Carrara E, Graham DY, Conti M, Tacconelli E. Prevalence of antibiotic resistance in helicobacter pylori: A systematic review and meta-analysis in World Health Organization regions. Gastroenterology. 2018;155:1372-1382. e17. DOI: 10.1053/j.gastro.2018.07.007 10.
18. Doan T, Worden L, Hinterwirth A, et al. Macrolide and nonmacrolide resistance with mass azithromycin distribution. N Engl J Med. 2020;383:1941-50. DOI: 10.1056/NEJMoa2002606
19. Zhuang M, Achmon Y, Cao Y, et al. Distribution of antibiotic resistance genes in the environment. Environ Pollut. 2021;285:117402. DOI: 10.1016/j.envpol.2021.117402
20. Siddiqui AH, Koirala J. Methicillin-Resistant Staphylococcus aureus. In: StatPearls [Internet]. Treasure Island (FL): Stat Pearls Publishing; 2024.

Available:https://www.ncbi.nlm.nih.gov/books/NBK482221
21. Elward AM, McAndrews JM, Young VL. Methicillin-sensitive and methicillin-resistant Staphylococcus aureus: Preventing surgical site infections following plastic surgery. Aesthet Surg J. 2009;29(3):232-44.
22. Lakhundi S, Zhang K. Methicillin-Resistant Staphylococcus aureus: Molecular Characterization, Evolution, and Epidemiology. Clin Microbiol Rev. 2018; 31(4).
23. Shahkarami F, Rashki A, Rashki Ghalehnoo Z. Microbial Susceptibility and Plasmid Profiles of Methicillin-Resistant Staphylococcus aureus and Methicillin-Susceptible S. aureus. Jundishapur J Microbiol. 2014;7(7):e16984.
24. Sabbagh P, Riahi SM, Gamble HR, Rostami A. The global and regional prevalence, burden, and risk factors for methicillin-resistant Staphylococcus aureus colonization in HIV-infected people: A systematic review and meta-analysis. Am J Infect Control. 2019;47(3):323-333.
25. Piergiorgio Pietta, Markus Minoggio, Lorenzo Bramati,Plant Polyphenols: Structure, occurrence and Bioactivity,Editor(s): Atta-ur Rahman, bStudies in Natural Products Chemistry, b Elsevier. 2003;28:257-312.
ISSN 1572-5995,ISBN 780444514158
26. Quideau W, et al. Plant Polyphenols: Chemical Properties, BiologicalActivities, and Synthesis**StØphane Q 011 Wiley-VCH Verlag GmbH and Co. KGaA, WeinheimAngew. Chem. Int. Ed. 2011;50: 586 – 62.
27. Haslam E. Phytochemistry. 2007;68:2713 – 2721.[9] a) Robins RJ, Gibbons S, Marston A. Phytochemistry. 2007;68:2699 – 2704 b) Harborne JB. Phytochemistry. 1988;27:5-7; c) Harborne JB, Phytochemistry. 1988;28:7-8.[10]
28. Swain T, Bate-Smith EC. In comparative Biochemistry, (Eds.: Mason HS, Florkin AM), Academic Press, New York. 1962;3:755 – 809.
29. Alowaiesh BF, Alhaithloul HAS, Saad AM, Hassanin AA. Green Biogenic of Silver Nanoparticles Using Polyphenolic Extract of Olive Leaf Wastes with Focus on Their Anticancer and Antimicrobial Activities. Plants (Basel). 2023;12(6):1410.
DOI: 10.3390/plants12061410
PMID: 36987100; PMCID: PMC10057938.
30. Iravani S. Green synthesis of metal nanoparticles using plants. Green Chem. 2011;13:2638–2650.
31. Pandit C, Roy A, Ghotekar S, Khusro A, Islam MN, Emran TB, Lam SE, Khandaker MU, Bradley DA. Biological agents for synthesis of nanoparticles and their applications. J. King Saud Univ.-Sci. 2022;34:101869.
32. Alagesan V, Venugopal S. Green synthesis of selenium nanoparticle using leaves extract of withania somnifera and its biological applications and photocatalytic activities. Bionanoscience. 2019;9:105–116.
33. Manikandan DB, Sridhar A, Sekar RK, Perumalsamy B, Veeran S, Arumugam M, Karuppaiah P, Ramasamy T. Green fabrication, characterization of silver nanoparticles using aqueous leaf extract of Ocimum americanum (Hoary Basil) and investigation of its *In vitro* antibacterial, antioxidant, anticancer and photocatalytic reduction. J. Environ. Chem. Eng. 2021;9:104845.
34. Available:https://www.oxfordreference.com/display/10.1093/oi/authority.20110803100142343
35. Available:https://www.colorado.edu/lab/lecture-demo-manual/o638-identification-phenols-ferric-chloride-test
36. Available:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7035446/#:~:text=Tests%20for%20flavonoids,(b)%20Shinod's%20test.
37. Junaid R Shaikh ,Matsyagandha Kishanrao Patil Qualitative tests for preliminary phytochemical screening: An overview: ~ 603 ~International Journal of Chemical Studies. 2020;8(2):603-608.
38. Available:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3757278/
39. Available:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2816600/
40. Available:https://microbenotes.com/millons-test/
41. Available:https://www.vedantu.com/chemistry/molisch-test
42. Available:https://gpatindia.com/chemical-tests-for-glycosides-general-and-specific/
43. Available:https://www.oecd.org/env/test-no-425-acute-oral-toxicity-up-and-down-procedure-9789264071049-en.htm
44. Available:https://asm.org/getattachment/2594ce26-bd44-47f6-8287-0657aa9185ad/Kirby-Bauer-Disk-Diffusion-Susceptibility-Test-Protocol-pdf.pdf

45. Available:https://www.sciencedirect.com/topics/immunology-and-microbiology/mueller-hinton-agar
46. Pottail Lalitha et al. Secondary Metabolites of Eichhornia crassipes (Waterhyacinth): A Review (1949 to 2011): Natural Product Communications. 2012;7(9):1249 – 1256.
47. Bakr TMA, El-Shemi NM, Mesallam AS. Isolation and chemical evaluation of protein from water hyacinth. Plant Foods for Human Nutrition (Formerly Qualitas Plantarum). 1984;34:67-73.
48. Virabalin R, Kositsup B, Punnapayak H. Leaf protein concentrate from water hyacinth. Journal of Aquatic Plant Management. 1993;31:207-209.
49. Greca MD, Lanzetta R, Mangoni L, Monaco P, Previtera L. A bioactive benzoindenone from Eichhornia crassipes Solms. Bioorganic and Medicinal Chemistry Letters. 1991;1:599-600.
50. Greca MD, Lanzetta R, Molinaro A, Monaco P, Previtera L. Phenalene metabolites from Eichhornia crassipes. Bioorganic and Medicinal Chemistry Letters. 1992;2:311-314.
51. Center TD, Hill MP, Cordo H, Julien MH. USDA Forest Service Publication, 41. Weed Science Society of America (WSSA). (1984) Composite list of weeds. Weed Science, 32, 1. Agunbiade FO, Bamidele I, Olu O, Adebowale; 2002.
52. Hubrecht RC, Carter E. The 3Rs and Humane Experimental Technique: Implementing Change. Animals (Basel). 2019;30;9(10):754. DOI: 10.3390/ani9100754 PMID: 31575048; PMCID: PMC6826930.
53. Husain Asif[1]; Meenakshi, Dhanalekshmi U.[2,3]; Ahmad, Aftab[4,5]; Shrivastava, Neelima[1]; Khan, Shah A.[2,3]. A review on alternative methods to experimental animals in biological testing: Recent advancement and current strategies. Journal of Pharmacy and Bioallied Sciences. 2023;15(4):165-171. DOI: 10.4103/jpbs.jpbs_380_23
54. Talbot SR, Biernot S, Bleich A, et al. Defining body-weight reduction as a humane endpoint: a critical appraisal. Laboratory Animals. 2020;54(1): 99-110. DOI: 10.1177/0023677219883319
55. Available:https://sites.uw.edu/oawrss/iacuc/policies/permissible-weight-loss-in-research-animals/
56. Margueritat J, Gonzalo J, Afonso CN, Mlayah A, Murray DB, Saviot L. Surface plasmons and vibrations of self-assembled silver nanocolumns. Nano Lett. 2006;6(9): 2037-42. DOI: 10.1021/nl061237h. PMID: 16968022.
57. A probabilistic model of competitive multi-ecological interactions for analyzing the Impact of Noise within Sight of Aquatic Hyacinth; 2023.
58. Dynamics of Water Hyacinth In A Fishery System With Delay Effect; 2016.
59. Spatial Temporal Variations of Water Hyacinth in a Delayed Fisheries System;2016.

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