SUNTEXT REVIEWS

SunText Review of Material Science
ISSN: 2766-5100

Open Access
Research Article
Volume 5:1

Environmental Friendly Synthesis of Silver Nanoparticles Using 5 Varieties of Pentas Lanceolata Sp. Flower Extracts: Evaluating Their Antioxidant, Antimicrobial, Photocatalytic Properties and Potential Application to Test Melamine Adulteration in Milk

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Received date: 13 March 2024; **Accepted date:** 02 June 2023; **Published date:** 18 June 2024

Citation: Kirivasan K, Kandiah M, Perera O (2024). Environmental Friendly Synthesis of Silver Nanoparticles Using 5 Varieties of Pentas Lanceolata Sp. Flower Extracts: Evaluating Their Antioxidant, Antimicrobial, Photocatalytic Properties and Potential Application to Test Melamine Adulteration in Milk. SunText Rev Mat Sci 5(1): 125.

DOI: https://doi.org/10.51737/2766-5100.2024.025

Abstract

Nanotechnology is a growing field of study relating to nanoscale (1-100nm) structures and their diverse applications. Silver nanoparticles (AgNPs) have been used in various commercial products with increasing demand. Researchers are studying alternative approaches to synthesise AgNPs in a cost-effective and environmentally friendly way. Numerous studies have demonstrated the ability to utilise plant extracts to synthesise AgNPs. This research involved using Pentas lanceolata flower water extracts (WE) to synthesise AgNPs and evaluate their antioxidant potential, antibacterial activity against Escherichia coli and Staphylococcus aureus, photocatalytic degradation of methylene blue dye and the ability of the AgNPs to detect melamine adulterant in milk. Five varieties of P. lanceolata flowers (white, light purple, purple, pink, and red) were tested to synthesise AgNPs, except the purple variety, all other four varieties were able to synthesise AgNPs at room temperature. The formation of AgNPs was confirmed using ultraviolet-visible spectrometry peaks. Antioxidant assays were performed on both the WE and AgNPs namely total phenolic content (TPC), total flavonoid content (TFC), total antioxidant capacity (TAC) and 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay. The WE showed higher TFC compared to the synthesised AgNPs, however; TPC, TAC and DPPH activity was higher in AgNPs. The majority of the AgNPs showed similar antibacterial activity compared to WE. White AgNPs was selected for further analysis and showed 50-60nm spherical nanoparticles using scanning electron microscope. The white AgNPs were not able to degrade methylene blue dye under sunlight even following the addition of catalyst $NaBH_4$. Moreover, the white AgNPs was not able to detect melamine in milk. Further studies need to be performed on P. lancoelata AgNPs to identify possible applications.

Keywords: Water extracts (WE); Silver nanoparticles (AgNPs); Reactive oxygen species (ROS); Total antioxidant capacity (TAC); Staphylococcus aureus; Escherichia coli

Introduction

Nanoscience is the study of structures ranging in size between 1 and 100 nm, and the application of this knowledge to vast fields, such as physics, chemistry, medicine, and electronics, is known as nanotechnology. Although nanostructures have been used from way back as 4th century AD, by Romans to make dichronic glass, which change colour in different light conditions, the curiosity towards modern nanotechnology grew following a lecture named "There's plenty of Room at the Bottom" by Nobel Prize laureate Richard Feynman in 1959. Nanoparticles exhibit astonishing optical and physiochemical properties, allowing them to be utilised in numerous applications. Nanoparticle synthesis can be accomplished broadly by two approaches: top-down and bottom-up. In the top-down approach, bulk materials are broken down into nanostructures, whereas in the bottom-up approach, nanoparticles are formed from simple atoms. Different technologies can be used to analyse the synthesised nanoparticles, including Scanning Electron microscopy (SEM), Transmission Electron Microscopy (TEM), X-ray diffraction (XRD), and spectrophotometry. Compared with other metallic nanoparticles, silver nanoparticles (AgNPs) have gained much attention because of their unique electrical, optical, and biological properties. AgNPs have demonstrated high surface-enhanced Raman scattering, catalytic activity, antifungal, anti-inflammatory, antiviral, and antibacterial properties, making them useful in many commercial products, such as antiseptic agents, cosmetics, food packaging, bioengineering, and catalysis. AgNPs can be synthesised via three methods: physical (evaporation-condensation and laser ablation), chemical (reduction of silver ions to metallic silver using organic or inorganic reducing agents), and biological (also known as green synthesis, using biomass such as plant extracts, bacteria, and fungi). The biological synthesis of AgNPs is cheaper, less toxic, and more eco-friendly. Synthesising AgNPs using plant extracts has received the widest interest as plants contain high phytochemical content which reduces silver ions to metallic silver and stabilises AgNPs and have no biohazard issues compared to using microbes.6 Several plants have been studied for their potential use in synthesising AgNPs and their properties have been analysed. As the demand for AgNPs is continuously rising with the expected global AgNP production of approximately 800 tons by 2025, there is increasing interest in finding greener and cheaper methods to manufacture AgNPs. To the best of the authors' knowledge, this is the first study to utilise Pentas lanceolata to synthesise AgNPs. It is commonly known as the Egyptian Star Cluster and belongs to the family Rubiaceae. they are used to treat malaria in indigenous African medicine8. Pentas lanceolata aqueous flower extracts has shown to contain various antioxidants such as flavonoids, phenols and quinones, which would aid in synthesising AgNPs. Antioxidants are compounds that can neutralise harmful free radicals by donating or accepting electrons. Free radicals, such as reactive oxygen species (ROS), reactive nitrogen species (RNS), and reactive sulphur species (RSS), are highly unstable molecules with unpaired electrons, which are generated within the body either due to normal metabolism or external factors such as ultraviolet radiation or pollutants. They interfere with normal cellular processes and cause various diseases such as cancer. AgNPs have antioxidant properties which can reduce the action of free radicals. the emerging antibiotic resistance crisis, there is a growing need to identify alternative antimicrobials. Green-synthesised AgNPs have shown broad-spectrum antibacterial properties. The release of silver ions by AgNPs is thought to aid the destruction of microbes. It acts by increasing the permeability of the cell membrane and interfering with cellular respiration, DNA replication, and protein synthesis.Water pollution due to effluent dyes is a major concern as they are xenobiotic in nature, making them difficult to eliminate by conventional means. Dyes are colourants used in many industries, such as textiles and cosmetics, however, they are highly toxic, having a carcinogenic, mutagenic, and genotoxic potential. Among these dyes are organic dyes which consists of azo group (-N=N-), where nanocatalysis by AgNPs is being studied. The photocatalytic degradation of synthetic dyes using AgNPs has gained much attention over conventional methods as no hazardous products are produced and AgNPs have high adsorption coefficients, large surface area and high dispersion. Upon irradiation via sunlight or UV, electrons are excited from the valence band to the conduction band, forming electron-hole pairs on the AgNPs. Hydroxyl radicals are generated which oxidise dyes to form CO2 and water. Melamine is a toxic adulterant that is used to artificially increase the protein content of food products. In 2008, thousands of infants in China were diagnosed with kidney stones following the consumption of melamine-contaminated infant milk. Conventional melamine detection methods, such as high-performance liquid chromatography (HPLC), are both time-consuming and labour-intensive. Colorimetric assays utilising AgNPs have been studied as reliable and sensitive methods. The amino group of melamine interacts with AgNPs by forming hydrogen bonds, causing the nanoparticles to aggregate together, changing the visible colour of the AgNP solution to red due to the shift of the surface plasmon band to longer wavelengths. Synthesise AgNPs using water extracts of five different flower varieties (white, light purple, purple, pink, and red) of Pentas lanceolata, and characterise the AgNPs using SEM. The antioxidant activity of the water extracts and synthesised AgNPs was assessed using total flavonoid content (TFC), total phenolic content (TPC), total antioxidant capacity (TAC) and 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) assays. The antibacterial properties of the water extracts and synthesised AgNPs were determined against gram-negative Escherichia coli and gram-positive Staphylococcus aureus by the well diffusion method, and methylene blue was used to test the photocatalytic activity of the AgNPs. Additionally, the ability of the synthesised AgNPs to detect melamine was analysed by identifying the minimum detection limit. Achieving these aims, the synthesised AgNPs can be utilised in many commercial applications.

Methodology

Good laboratory practises were followed throughout the project to ensure the safety of lab personnel and environment, while generating high quality data.

Sample Collection

Five varieties of Pentas lanceolata flower samples were collected from Diyatha Uyana plant nursery, Battaramulla, Sri Lanka.

Sample processing

The flowers were separated from the plant and dried in a hot air oven at 70°C for 24 hours. To 2g of ground dried flower samples, 50mL of distilled water was added and incubated at 60°C for 15 minutes. The mixture was filtered using Whatman No.1 filter paper to obtain the water extracts (WE) and stored them in a 4°C refrigerator until further use.

Preliminary Phytochemical Screening

The water extracts were subjected for preliminary screening to test for the presence or absence of phytochemical constituents.

Xanthoproteic test

Few drops of conc. HNO3 were added to 0.5mL of WE.

Test for Carbohydrates

2 ml of the extract was added to a test tube. Then 2 ml of Molisch's reagent was added to the tube. Later, few drops of 100% H_2SO_4 were added along the test tube wall. If positive, formation of a purple or reddish colour ring can be observed.

Test for Tannins

To 0.5mL of WE, 1.5mL of distilled water and few drops of 10% FeCl3 were added.

Test for Saponins

2 ml of distilled water was mixed with 2 ml of the extract in a test tube. The tube was shaken vigorously. If positive, formation of a stable persistent froth can be observed.

Test for Phenols

Few drops of Iodine solution were added to 0.5mL WE.

Test for Glycosides

To 0.5mL of WE, 700µL of glacial acetic acid, 1 drop of 5% FeCl3 and conc. H2SO4 was added along the side of the test tube.

Test for Quinones

Few drops of conc. HCl were added to 0.5mL of WE.

Test for flavonoids

Few drops of 10% FeCl3 were added to 0.5mL of WE.

AgNPs synthesis and optimization

To 1mL of Pentas WE, 9mL of AgNO3 was mixed and incubated at various temperatures such as room temperature for 24 hours, 60°C and 90°C for 15, 30, 45 and 60 minutes to identify the optimum temperature for (AgNP) synthesis.

Characterization of Pentas AgNPs: Spectrophotometer. Synthesised AgNPs were checked for absorbance from 320-700 nm, using a UV-Visible spectrophotometer calibrated with distilled water as the blank.

Scanning Electron Microscopy (SEM)

2mL of the room-temperature synthesised white-AgNPs were centrifuged at 13000rpm for 5 minutes, following which the supernatant was discarded, and the pellet was dried using a hot-air oven. The dried samples were sent to Sri Lanka Institute of Nanotechnology (SLINTEC) for Scanning Electron Microscopy (SEM) using a Hitachi SU6600 SEM.

Antioxidant assays

Both the WEs and room-temperature-synthesised AgNPs were diluted 15-fold using distilled water and analysed their antioxidant activity in triplicates.

Total Flavonoid Content (TFC)

To 1.5mL of sample, 1.5mL of 2% AlCl3 was mixed and incubated at room temperature for 15 minutes. The absorbance was recorded at 415nm using distilled water as blank and concentration was expressed in equivalents of µg Quercetin (QE) equivalents per 100g (µg QE/100g).

Total Phenolic Content (TPC)

1mL of 10% Folin-Ciocalteu's reagent was vortexed with 200µL of the sample and incubated at room temperature for 4 minutes. After which, 800µL of 7.5% Na_2CO_3 was added and incubated again at room temperature for 60 minutes following which the absorbance was recorded at 765nm using distilled water as blank. The concentration was expressed in equivalents of mg Gallic acid equivalents (GAE) per 100g (mg GAE/100g).

Total Antioxidant Capacity (TAC)

1mL of the sample was mixed with 1mL of reagent consisting equal volume of 0.6M H_2SO_4, 28mM sodium phosphate and 4mM ammonium molybdate. The mixture was incubated at 95°C for 90 minutes and the absorbance was measured at 695nm using distilled water as blank. The concentration was expressed in equivalents of mg Ascorbic acid (AAE) per 100g.

2, 2-Diphenyl-1-picrylhydrazyl (DPPH)

1mM DPPH was prepared using methanol as solvent. 2mL of 1mM DPPH was mixed with 1mL of each sample. The mixture was incubated at room temperature for 20 minutes and the absorbance was measured at 517nm using methanol as a blank.
Percentage activity was measured using the following equation 1.

$\%Activity =$(Abs of DPPH-Abs of Sample/ Abs of DPPH) * 100
Equation 1: Percentage activity

Antibacterial efficacy of AgNPs versus water extracts

Antibacterial potential was evaluated against Escherichia coli and Staphylococcus aureus using the well diffusion technique on Mueller-Hinton agar. A colony from *E. coli* and *S. aureus* nutrient agar plate was diluted with saline solution and streaked onto 20ml Mueller-Hinton agar petri plates. Three wells were prepared on each plate for the negative control (saline), duplicates of sample (S1 and S2). Gentamycin discs was used as the positive control. The agar plates were left for incubation for 24 hours incubation at 37°C in an incubator. Following that, the zone of inhibitions (ZOI) was measured using a ruler. The procedure was performed under ascetic conditions.

Photocatalytic degradation of Methylene Blue

Room temperature synthesised white-AgNPs was selected for assessing the photocatalytic activity. 1mL of 4000ppm and 276ppm AgNPs samples were mixed with 100mL of 1mM methylene blue dye solution separately in a beaker. The mixture was kept under sunlight, and absorbance was measured every 30 minutes from 320-800 nm using water as a blank. The entire procedure was repeated with the addition of 1mL of 0.2M $NaBH_4$ to the dye mixture.

Detection of melamine adulterant in milk

Room temperature synthesised white-AgNPs was selected to evaluate its application in melamine adulterant detection in milk. Initially, distilled water was used as the solvent to prepare five serial concentrations (10mM, 8mM, 6mM, 4mM, and 2mM) of melamine. 1000µL of white-AgNPs sample was mixed with 600µL of 5 different concentrations of melamine and recorded the absorbance spectrum from 320–800 nm. Visual colour changes were recorded. Prior to testing with milk sample, milk was pre-treated according to (Ma *et al*., 2011a). 4g of milk was mixed with 30mL of 61mM Trichloro acetic acid and 10mL of acetonitrile, vortexed for 30 minutes and centrifuged at 4000rpm for 45 minutes. The supernatant was filtered using a syringe filter (0.2µm) and adjusted the pH to 6.8 using $NaHCO_3$ and TCA. 1000µL of AgNPs was mixed with 600µL of pre-treated milk and recorded the absorbance spectrum from 320–800 nm. Same procedure was repeated for 10mM melamine spiked milk.

Statistical analysis

The generated data were evaluated by one-way ANOVA using Microsoft Excel (Microsoft 365). Pearson's correlation test was applied to determine the correlation between TFC, TPC and TAC using SPSS version 28.

Results

Phytochemical profile

Protein was present in all five varieties of Pentas flowers, while other phytochemicals such as glycosides were present only in few varieties (Table 1).

Silver nanoparticle synthesis and optimization

As shown in (Figure 1). The colour of the solution changed to dark brown due to the formation of AgNPs. AgNPs peaks were observed for four varieties, white, light purple, pink and red at 420-460nm. The purple variety didn't form AgNPs during incubation at room temperature for 24 hours (Figure 2). AgNPs peaks were observed for four varieties, white, light purple, pink and red at 420-460nm. The purple variety didn't form AgNPs during incubation at room temperature for 24 hours (Figure 2).Room temperature synthesised AgNPs were selected for further analysis

Scanning Electron Microscopy (SEM) analysis

SEM images of White AgNPs showed 50-60nm AgNPs, morphology being spherical with aggregation (Figure 3).

Antioxidant assays

Total Flavonoid Content (TFC)

We shows significantly higher TFC compared to AgNPs (Figure 4). And the one way ANOVA shows there is a significance difference between WE and AgNP with the $P\text{-value}<0.05$ (Table 3).

Total Phenolic Content (TPC)

AgNP shows higher TPC compared to WE (Figure 5). And the one way ANOVA shows there is a significance difference between WE and AgNP with the $P\text{-value}<0.05$ (Table 4).

Total Antioxidant Capacity (TAC)

AgNP shows higher TAC compared to WE (Figure 6). And the one way ANOVA shows there is a significance difference between WE and AgNP with the $P\text{-value}<0.05$ (Table 5).

DPPH assay

Synthesised AgNPs showed significantly higher DPPH free-radical scavenging activity compared to WE (Figure 7). The one way ANOVA shows there is a significance difference between WE and AgNP with the $P\text{-value}<0.05$ (Table 6).

Antibacterial activity

Escherichia coli

White-AgNP shows higher ZOI compared to WE (Figure 9) and the one way ANOVA shows there is no significance difference between WE and AgNP with the P-value<0.05 (Table 6).

Staphylococcus aureus

White-AgNP shows higher ZOI compared to WE (Figure 11) and the one way ANOVA shows there is no significance difference between WE and AgNP with the P-value<0.05 (Table 7). The one way ANOVA shows there is no significance difference between E. coli and S. aureus of WE and AgNP with the P-value<0.05 (Table 8).

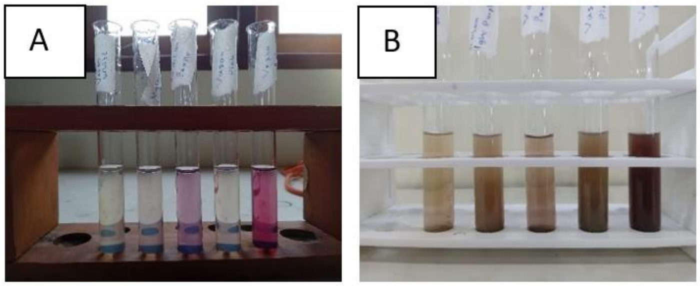


Figure 1: *Visual colour change before (A) and after (B) AgNPs synthesis. Sample order from left to right: White, Light Purple, Purple, Pink, and Red*

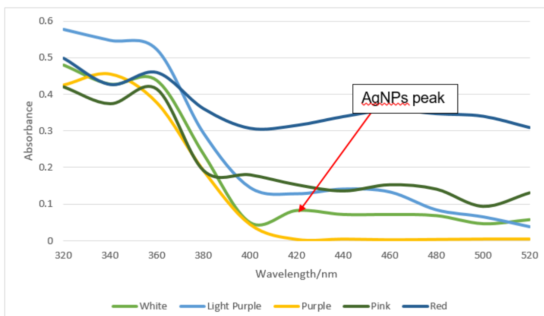
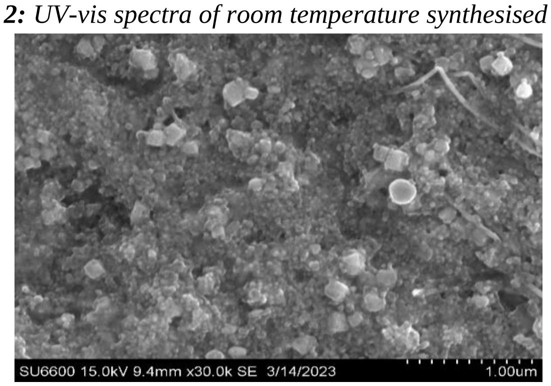
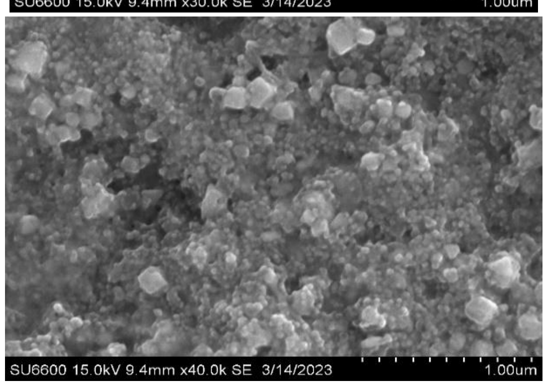


Figure 2: *UV-vis spectra of room temperature synthesised AgNPs.*





Citation: Kirivasan K, Kandiah M, Perera O (2024). Environmental Friendly Synthesis of Silver Nanoparticles Using 5 Varieties of Pentas Lanceolata Sp. Flower Extracts: Evaluating Their Antioxidant, Antimicrobial, Photocatalytic Properties and Potential Application to Test Melamine Adulteration in Milk. SunText Rev Mat Sci 5(1): 125.

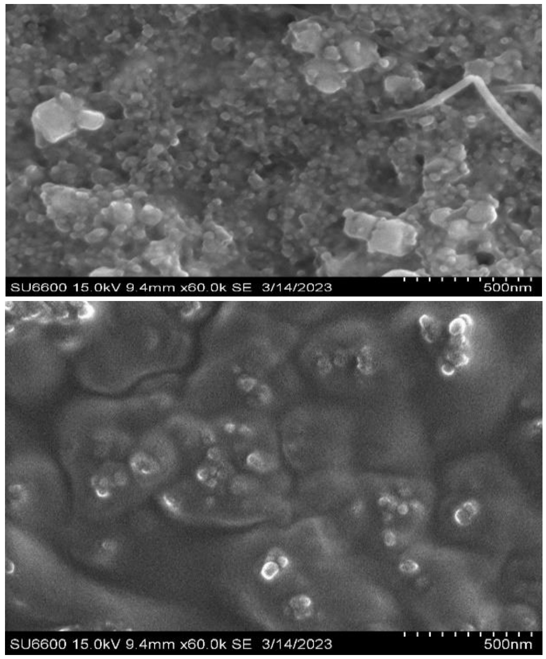


Figure 3: *SEM images of white AgNPs in different magnifications. A) 15.0 kV 9.4mm x 30.0k. 1µm, B) 15.0 kV 9.4mm x 40.0k, 1µm C) 15.0 kV 9.4mm x 60.0k, 500nm and D) 15.0 kV 9.4mm x 60.0k, 500nm.*

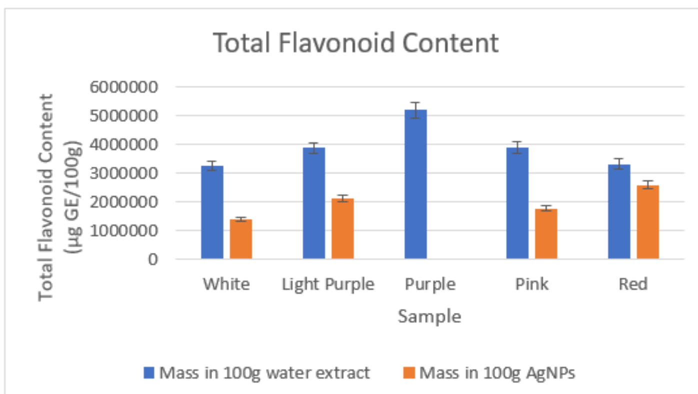


Figure 4: *TFC of WE and AgNPs.*

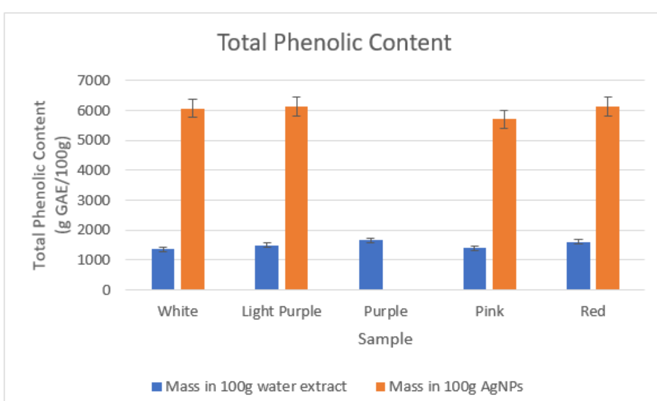


Figure 5: *TPC of WE and AgNPs.*

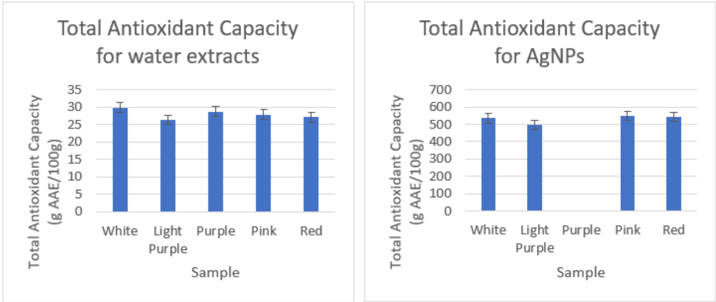


Figure 6: *TAC of WE and AgNPs.*

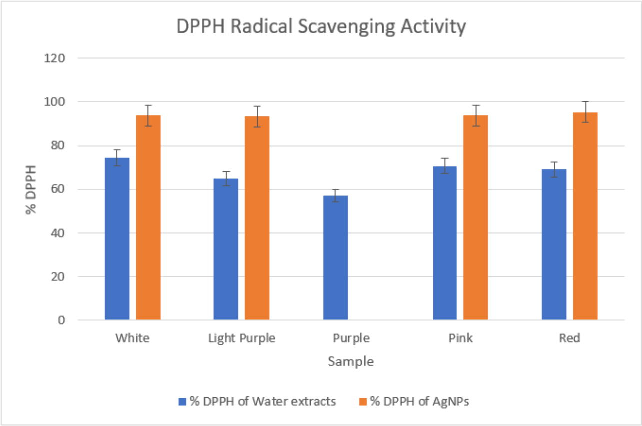


Figure 7: *DPPH activity of WE and AgNPs.*

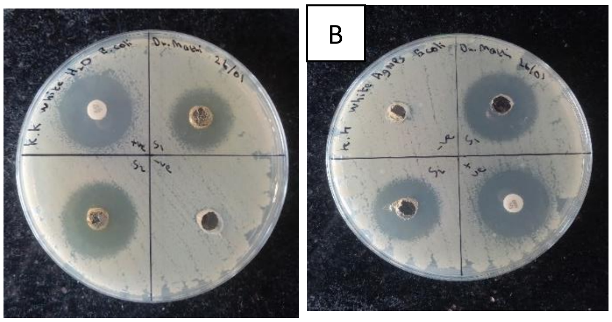


Figure 8: *Zone of Inhibition of E. coli for white WE (A) and AgNPs (B).*

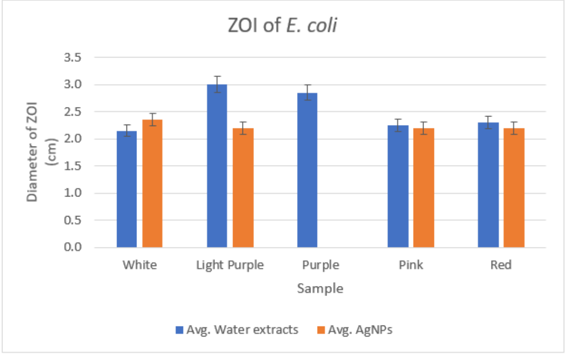


Figure 9: *ZOI of E. coli for the WE and AgNPs.*

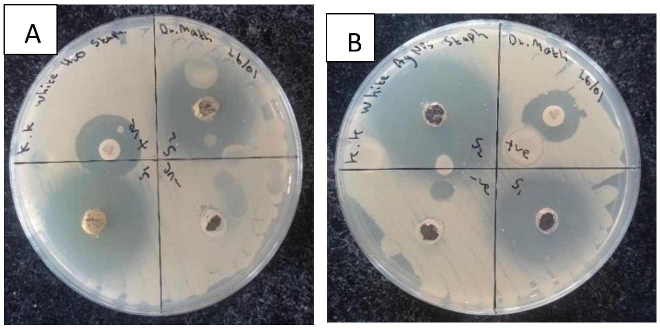


Figure 10: *Zone of Inhibition of E. coli for white WE (A) and AgNPs (B).*

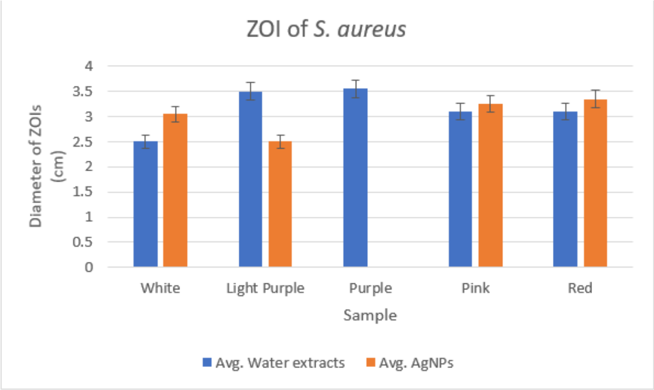


Figure 11: *ZOI of S. aureus for the WE and AgNPs.*

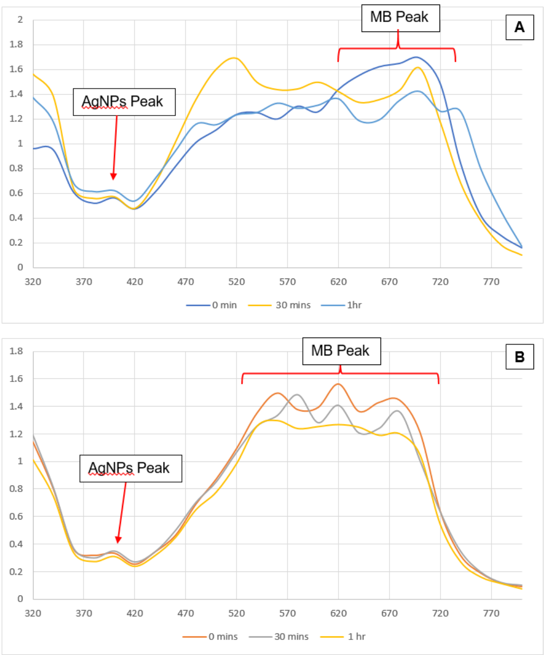


Figure 12: *MB dye degradation under sunlight with 267ppm (A) and 4000ppm (B) of white AgNPs*

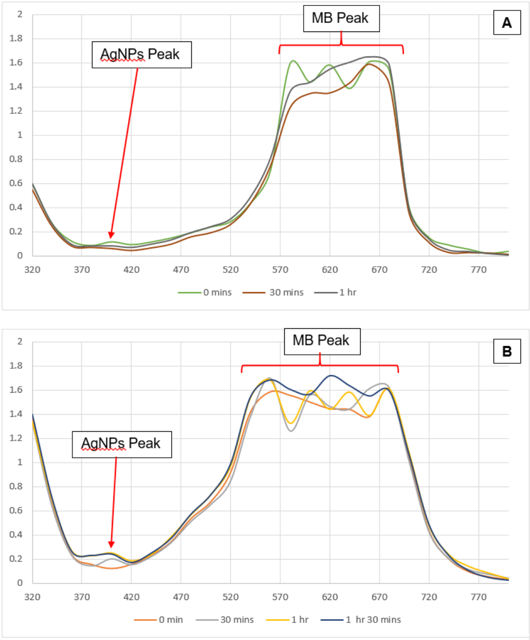
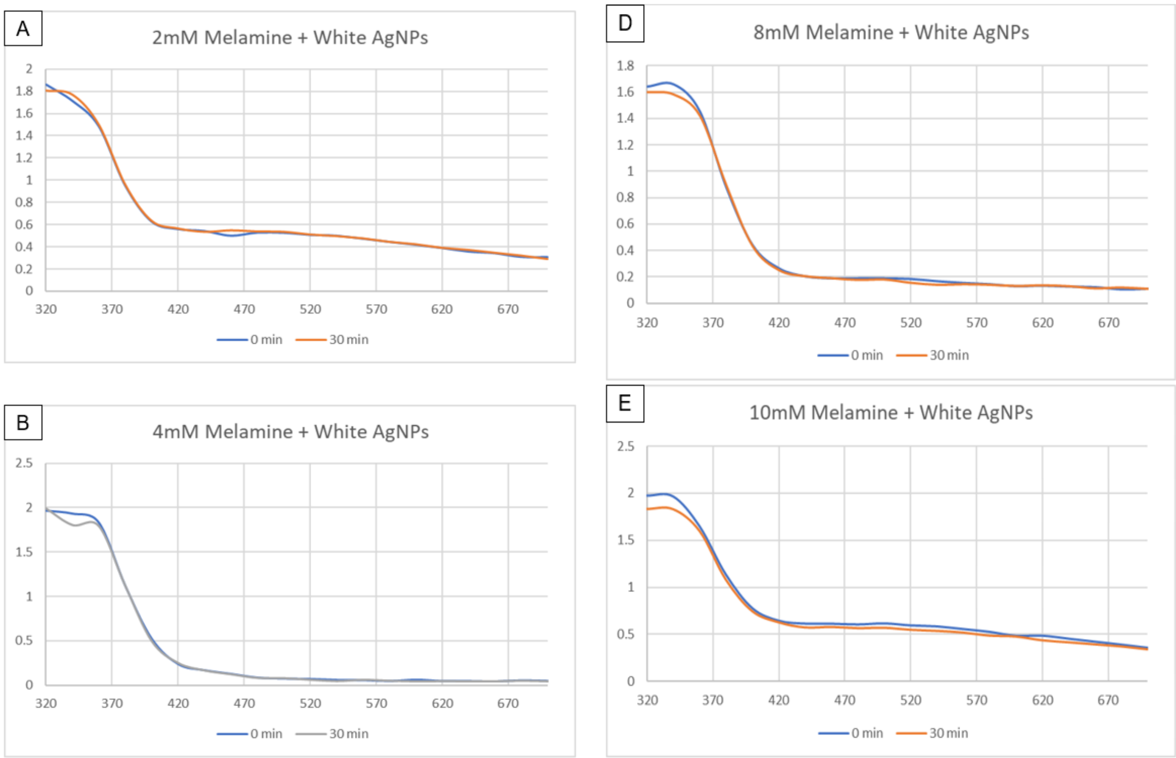


Figure 13: *MB dye degradation under sunlight in presence of catalyst-NaBH4 with 267ppm (A) and 4000ppm (B) of white AgNPs*



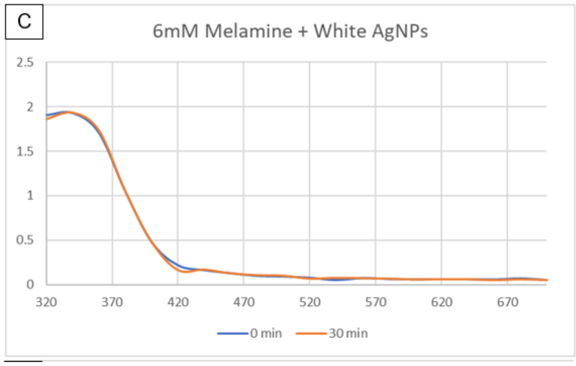


Figure 14: *Absorption spectra for different concentrations of melamine in distilled water with white AgNPs A) 2mM melamine B) 4mM melamine C) 6mM melamine D) 8mM melamine E) 10mM melamine*

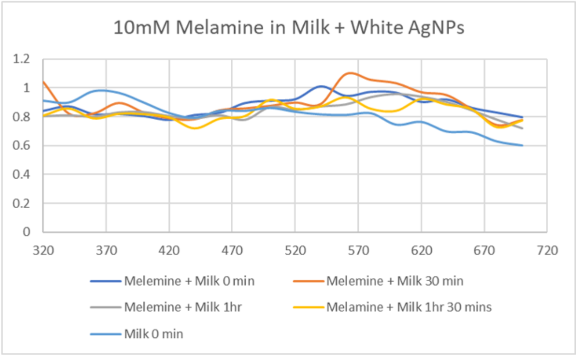


Figure 15: *Absorption spectra for 10mM melamine in milk with white AgNPs.*

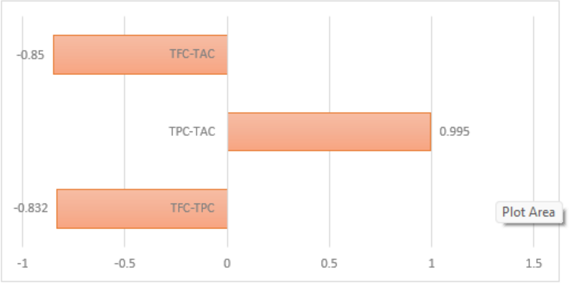


Figure 16: *Pearson correlation graph for the antioxidant assays.*

Photocatalytic activity

Dye degradation under sunlight

No degradation of MB dye was observed till 1 hour.

Dye degradation under sunlight in presence of NaBH4

Even following the addition of catalyst NaBH4, MB was not degraded by white AgNPs (Figure 17 and 18) even at 1.30 hours.

Melamine detection

Melamine detection in distilled water

No visible colour change or absorption peak deviations above 500nm wavelength were observed following the addition of white AgNPs to different concentrations of melamine prepared using distilled water (Figure 14).

Melamine detection in milk

No visible colour change or absorption peak deviations above 500nm wavelength were observed following the addition of white AgNPs to melamine spiked milk (Figure 15).

Discussion

Eco-friendly synthesis of AgNPs makes use of phytochemicals such as carbohydrates, proteins, enzymes and other complex compounds to aid in synthesising AgNPs. Chemical synthesis of AgNPs is the frequently used method for commercially manufacturing of AgNPs, however, they are expensive and produce toxic by-products.24 Therefore, and there is an increasing need for finding alternative green sources for AgNP synthesis. In this study, Pentas lanceolata flower water extracts (WE) were used to synthesise AgNPs and studied their antioxidant characteristics and potential applications. To the best of our knowledge, there has not been a report on AgNPs synthesised using Pentas lanceolata. Water was used as the solvent for phytochemical extraction as it is non-hazardous and has applications in the biomedical and pharmaceutical industries.25 Phytochemical profiling of the WE were performed to identify the compounds which aid in reducing silver ions to form AgNPs. Overall, proteins, flavonoids, phenols, tannins, quinones, carbohydrates and glycosides were present in the WE (Table 1).To identify the optimum temperature for AgNP synthesis using Pentas flower WE, synthesis was performed at different temperatures for different incubation durations, while keeping all other parameters constant. The colour of the solution turned reddish-brown as reported by various researchers due to the reduction process of silver ions to form AgNPs. Noble metal nanoparticles have free electrons yielding a surface plasmon resonance (SPR) absorption band, by mutual vibration of the electrons in resonance to light wave. The SPR peaks can be observed between 400-500nm using UV-vis spectra to confirm the presence of AgNPs in the sample. The peak wavelength and intensity of the SPR depend on the synthesised AgNPs size, shape, nanoparticle aggregation, and environment such as substrate, solvent and adsorbates. Room temperature synthesised AgNPs were chosen for further analysis as they showed the most intense colour change and it is a greener approach as it requires less energy for AgNPs synthesis.SEM analysis was performed to identify the size and morphology of the AgNPs. The synthesised white AgNPs showed a size of 50-60nm, which lies in the nanomaterial size range, and morphology was spherical with aggregation (Figure 3). Bandgap energy measurement is essential in the study of nanomaterials. The nanomaterials can be identified as semiconductors or insulators (Table 9) by their bandgap energies <3eV and >4eV respectively.The bandgap is inversely proportional to the nanoparticle size. It was determined by Planck's equation. Oxidative stress is caused when the equilibrium between the cellular antioxidant mechanisms and oxidants present is disrupted. The presence of oxidants results in oxidative modifications of biological systems causing cellular damage and accelerating cellular death. They are linked to several diseases such as Alzheimer's disease and cancer. Although there are synthetic antioxidants such as Butylated hydroxytoluene and Butylated hydroxyanisole, studies have reported that synthetic antioxidant consumption cause toxic effects, requires high costs and exhibits less efficacy compared to natural antioxidants. Therefore, curiosity towards natural antioxidant sources is rising. In this study, antioxidant assays (TFC, TPC, TAC and DPPH) were performed to quantify the antioxidant activity of WE and synthesized AgNPs. TFC was quantified using the aluminium chloride colorimetric technique. The principle of this method is that aluminium chloride forms acid stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols. In addition, it also forms acid labile complexes with the ortho-dihydroxyl groups in the A- or B-ring of flavonoids.The WE exhibited significantly higher TFC compared to the synthesised AgNPs in the order Purple > Pink = Light Purple > Red = White (Figure 4) at P-value<0.05, F-value (18.23816) > F-critical (5.591448) (Table 3). Similar finding was observed by Ngamlai et al. (2022) where they have quantified and concluded the TFC of Hedyotis scandens methanolic leaf extract (Family Rubiaceae) was higher than the TPC, similar to the P.lanceolata sample. TPC was identified using the Folin-Ciocalteu reagent. The Folin-Ciocalteu reagent is formed from a mixture of phosphotungstic acid and phosphomolybdic acid which after oxidation of the phenols, is reduced to a mixture of blue oxides of tungsten and molybdenum. The blue colouration produced has a maximum absorption in the region of 750 nm and is proportional to the total quantity of phenolic compounds originally present.The AgNPs showed significantly higher TPC readings compared to the WE, in the order, White = Light purple = Red > Pink (Figure 5) at P-value<0.05, F-value (1626.242) > F-critical (5.591448) (Table 4). Ngamlai et al. (2022) reported the TPC of Hedyotis scandens methanolic leaf extract (Family Rubiaceae) to be 27.17±0.27 GAE mg/g, in comparison, P.lanceolata flowers have higher TPC. TAC of the samples was identified using the phosphomolybdate assay, wherein the presence of antioxidants, Mo (VI) is reduced to Mo (V) and forms a green-coloured phosphomolybdenum V complex, which shows a maximum absorbance at 695 nm.34 The synthesised AgNPs showed high TAC compared to WE, in the order, Pink > Red = White > Light Purple (Figure 6) at P-value<0.05, F-value (879.7935) > F-critical (5.591448) (Table 5). Ngamlai et al. (2022) based on their study on Hedyotis scandens concluded that flavonoids and polyphenols present in the plant have contributed to its TAC. DPPH assay was performed to evaluate the ability of the sample to transfer electrons and neutralise the reactive DPPH free radical. When DPPH accepts an electron or a hydrogen radical, it transforms from a stable synthetic free radical to a stable molecule. Antioxidants convert the DPPH radical to the non-radical form in the DPPH. As a result, there is less absorption, and the DPPH solution turns from purple to yellow.34 AgNPs showed equal and higher free-radical scavenging activity compared to the WE (Figure 7), reported AgNPs have high DPPH activity synthesised using Rubia cordifolia L. (family Rubiaceae) aqueous leaf extract AgNPs show higher antioxidant properties due to the presence of phenolic compounds, terpenoids, and flavonoids in plants, allowing nanoparticles to act as singlet oxygen quenchers, hydrogen donors, and reducing agents. Pearson correlation factor (Figure 16) was performed on all antioxidant assays, and the statistical correlations of the TFC-TAC and TFC-TPC assays showed a strong negative (-0.85 and -0.832 respectively) linear statistical correlation. However, TPC-TAC showed a strong positive correlation (0.995). This implies the presence of phenolic compounds on the surface of AgNPs and their role in eliciting antioxidant activity. AgNPs have shown excellent antimicrobial activity towards both gram-negative and gram-positive, including multidrug-resistant strains. Development of new antibiotics is a complicated process and therefore, AgNPs could be used to treat microbial diseases without promoting the appearance of new resistances.

Table 1: *Phytochemical analysis of WE.*

	Flower variety				
Phytochemical	White	Light Purple	Purple	Pink	Red
Protein	✓	✓	✓	✓	✓
Flavonoid	✓	✓	✓	✓	×
Phenol	×	✓	×	✓	×
Tannin	✓	✓	✓	✓	×
Quinone	✓	×	×	×	×
Carbohydrate	×	✓	✓	✓	✓
Reducing sugars	×	✓	✓	✓	✓
Glycoside	✓	×	×	✓	×

Table 2: *AgNPs synthesis optimisation results.*

Temperature	Incubation duration	Flower Variety				
		White	Light Purple	Purple	Pink	Red
Room temperature	24 hrs	✓	✓	×	✓	✓
60°C	15 mins	×	×	×	×	×
	30 mins	✓	✓	✓	✓	✓
	45 mins	×	×	×	✓	✓
	60 mins	×	✓	✓	✓	✓
90°C	15 mins	×	✓	✓	✓	×
	30 mins	×	×	×	×	×
	45 mins	×	×	×	×	×
	60 mins	×	✓	✓	✓	✓

Though the exact mechanism of action is not known, numerous explanations are put forward. The first is that AgNPs act at the membrane level of microorganisms and damage their integrity increasing permeability. Second, AgNPs interact with sulphur and phosphate groups present in DNA and proteins, altering their structure and subsequently their function. And third mechanism is the release of silver ions from the nanoparticles interrupting the metabolic pathways of the microorganism. Pentas was previously studied to show antimicrobial properties.37 However, in this study, there was no significant improvement in the antibacterial activity of AgNPs compared to the WE for both E. coli (Table 6) and S. aureus (Table 7) at P<0.05, F-value < F-critical. Chandran, 2016 showed similar results using AgNPs synthesised from root extracts of Morinda pubescens (Rubiaceae family) and showed higher antibacterial activity with increase in AgNPs concentration. Methylene blue (MB) is an important cationic dye employed by textile industries because of its high solubility in water, however, they cause water pollution when released to water bodies.

Table 3: *One-way ANOVA table for TFC.*

Anova: Single Factor						
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
Water extracts	5	19567708.33	3913542	6.07E+11		
AgNPs	4	7927083.333	1981771	2.52E+11		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	8.29275E+12	1	8.29E+12	18.23816	0.003698	5.591448
Within Groups	3.18285E+12	7	4.55E+11			
Total	1.14756E+13	8				

Table 4: *One-way ANOVA table for TPC.*

Anova: Single Factor						
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
Water extracts	5	7492.142857	1498.429	17401.94		
AgNPs	4	24012.85714	6003.214	41500.85		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	45095765.18	1	45095765	1626.242	1.5E-09	5.591448
Within Groups	194110.3061	7	27730.04			
Total	45289875.49	8				

Table 5: *One-way ANOVA table for TAC.*

Anova: Single Factor						
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
Water extracts	5	139.8295455	27.96591	1.984762		
AgNPs	4	25.71525	6.428813	0.087394		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1030.770066	1	1030.77	879.7935	1.28E-08	5.591448
Within Groups	8.201232009	7	1.171605			
Total	1038.971298	8				

Recently, metallic nanoparticles are widely studied for photocatalytic degradation of MB removal from wastewater due to their high adsorption capacity, and less diffusion resistance with large surface area. The mechanism involves the excitation of AgNP electrons from the valence band to the conduction band upon irradiation forming electron-hole pairs on the surface. The excited electrons convert oxygen molecules and hydroxyl ions into oxygen radicals and hydroxyl radicals, respectively. These radicals oxidize MB dye and generate holes in the nanoparticles.

Table 6: *One-way ANOVA table for DPPH.*

Anova: Single Factor						
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
Water extracts	5	336.0783	67.21567	43.39637		
AgNPs	4	376.1212	94.03031	0.76054		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1597.834	1	1597.834	63.59824	9.3E-05	5.591448
Within Groups	175.8671	7	25.12387			
Total	1773.701	8				

Table 7: *One-way ANOVA for E. coli*

Anova: Single Factor						
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
Water extracts	5	12.55	2.51	0.14925		
AgNPS	4	8.95	2.2375	0.005625		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.165013889	1	0.165013889	1.881648906	0.2124938	5.59144785
Within Groups	0.613875	7	0.087696429			
Total	0.778888889	8				

Table 8: *One-way ANOVA for S. aureus.*

Anova: Single Factor						
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
Water extracts	5	15.75	3.15	0.1775		
AgNPs	4	12.15	3.0375	0.143958		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.028125	1	0.028125	0.172414	0.690407	5.591448
Within Groups	1.141875	7	0.163125			
Total	1.17	8				

The formed holes gain the electrons from dye molecules that adsorbed on the surface of nanoparticles, causing further MB degradation. Since AgNPs have a large surface area with high pores, they exhibit high photodegradation under sunlight. In this study, synthesised white AgNPs were evaluated for their photocatalytic degradation of MB dye with NaBH4 as a catalyst. No reduction in the absorption peak of MB dye was observed following the addition of the AgNPs (Figure 12) even in presence of the catalyst (Figure 13), therefore the synthesised AgNPs were not capable of degrading the dye. Melamine is a common toxic adulterant used in milk to artificially increase its protein content. Several studies have shown the potential for AgNPs to be used as a colorimetric sensor for melamine detection.In this study, synthesised white AgNPs were used to assess their potential to detect melamine. Initially, the AgNPs were added to varying concentrations of melamine prepared using distilled water and following that, were tested with spiked milk samples. No absorbance change was observed with 2-10mM concentrations of melamine in distilled water (Figure 13).

Table 9: *One-way ANOVA for E. coli and S. aureus combined.*

Anova: Single Factor						
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
Water extracts	5	12.55	2.51	0.14925		
AgNPs	4	8.95	2.2375	0.005625		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.165013889	1	0.165013889	1.881648906	0.2124938	5.59144785
Within Groups	0.613875	7	0.087696429			
Total	0.778888889	8				

Table 9: *Conductivity of synthesised AgNPs.*

AgNPs sample	Band energy/eV	Type of nanomaterial
White	2.95	Semiconductor
Light Purple	2.82	Semiconductor
Pink	2.7	Semiconductor
Red	2.7	Semiconductor

Before testing with raw milk, the spiked milk sample was pre-treated to remove the proteins and lipids.41 There was no change in absorbances for the spiked milk sample above 500nm (Figure 14). No visible colour change was observed in the milk sample as reported in previous studies.

Conclusion

Finally, in this study Four out of the five P.lanceolata WE synthesised AgNPs in room temperature incubation for 24 hours and SEM analysis showed the presence of spherical 50nm sized AgNPs. All synthesised AgNPs were semiconductors. The TFC assay showed higher antioxidant activity for WE compared to AgNPs whereas TPC. TAC and DPPH showed higher antioxidant activity for AgNPs compared to WE. There was no significant difference in the antibacterial activity of the AgNPs and WE. The AgNPs were not able to degrade the dye and further optimisations are needed to be used for melamine detection. Therefore, Pentas AgNPs has the potential to be used to cure diseases caused by oxidative stress and can act as a potent antioxidant.

Acknowledgements

The authors thank BMS for funding.

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