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AN ECO-FRIENDLY APPROACH FOR THE GREEN SYNTHESIS OF SILVER NANOPARTICLES USING PETUNIA LEAF EXTRACTS AND ASSESSING THEIR ANTIBACTERIAL, ANTIOXIDANT AND PHOTOCATALYTIC ACTIVITY

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ABSTRACT

Recent advances in nanotechnology have resulted in limitless applications of nanomaterials, an important role in medicine is one such application. Silver nanoparticles (AgNPs) have gained popularity, as it is ideal for biomedical applications due to their high antioxidant, antibacterial, biological functionality and non-toxicity. This study describes the antioxidant, photocatalytic and antibacterial activity of AgNPs synthesized using five different Petunia hybrida leaf extracts (black, white, pink, purple, pink-purple) for the first time. Initially, the colour change indicated the presence of produced AgNPs. UV-Vis spectroscopy and transmission electron microscopy (TEM) were used to analyse the formation, size, and shape of the produced AgNPs. The bandgap energy and TEM image of pink AgNPs indicated that the synthesized AgNPs were in the 50 nm range. The existence of phytocompounds was identified by qualitative phytochemical analysis. Carbohydrates, terpenoids and steroids were found in all Petunia samples. TFC, TPC, TAC, DPPH and IC50 tests were used to assess the antioxidant activity of leaf water extracts and AgNPs. The antioxidant assay findings demonstrated that AgNPs had stronger antioxidant activity than their respective leaf water extracts. The agar well diffusion technique revealed that the produced AgNPs had significant antibacterial activity against Escherichia coli compared to Staphylococcus aureus. Moreover, the photocatalytic activity of the produced pink-purple AgNPs at 100 ppm was investigated by removing malachite green dye from an aqueous solution under sunlight in the presence and absence of NaBH4 catalyst. In 40 minutes, 100 ppm pink-purple AgNPs demonstrated effective photocatalytic activity in the breakdown of malachite green. The findings suggested that Petunia is an eco-friendly source for AgNP biosynthesis, which can be employed as a novel antioxidant, antibacterial, and photocatalytic agent; hence, it may be used in a range of applications to contribute to a better life.

INTRODUCTION

Nanotechnology is a developing field of colloidal science that gained significant importance in the last decade. It is the study of materials at the nanoscale, with nanoparticles (NPs) being the fundamental structural unit, ranging from 1 to 100 nm in at least one dimension. Numerous forms of NPs have been synthesized and employed in a wide range of sectors including environmental remediation, food industries, drug delivery and medicine (Shaikh et al., 2021).

Silver nanoparticles (AgNPs) have been most beneficially employed among metallic NPs due to their superior

chemical, physical and biological properties, which are influenced, strongly by the shape, size, composition, structure and crystallinity compared to their bulk forms. (Lee and Jun, 2019).

There are various physical and chemical methods to synthesize AgNPs, however they are highly involved in the production of toxic compounds, low yield, high cost and complexity, hence the biological approach is a good alternative for the synthesis of AgNPs to overcome these limitations and also it addresses difficulties including stability, crystal development and particle aggregation (Fafal et al., 2017). It uses biological elements like plant extracts and microorganisms as eco-friendly alternatives. The use of plant resources for NP synthesis has several advantages, including the elimination of complex processes like maintenance of microbial cell cultures, compound purification procedures and lowers the risk of biohazard (Ravichandran et al., 2019). In NP synthesis, both top–down and bottom–up techniques are applied. The top-down technique incorporates a physical material that employs tools like lithography, etching and atomization procedures to allow structure shaping and size reduction. The bottom–up technique begins with a big structure and proceeds through chemical synthesis, self-assembly, and positional assembly, which are controlled by temperature, concentration, pH, covalent and ionic. The top-down technique has a less defined surface structure than the bottom-up approach. The biological approach follows the bottom-up approach (Indiarto et al., 2021).

The reduction, stabilization and capping of AgNPs are based on the biomolecules present in plant extracts. The composition and concentration of AgNPs vary according to different plants, especially medicinal plants, due to their rich source of antioxidants and phytochemicals. Terpenoids, carbohydrates, tannins, fats, enzymes, phenols and flavonoids are all capable of reducing silver to AgNPs. The complex biomolecules help to reduce metal ions and stabilize NPs into the desired size and shape. The plant-mediated synthesis of AgNPs requires only a plant extract and silver salt, followed by an extraction procedure that could be either water or ethanol like other solvents (Akintelu, Bo and Folorunso, 2020).

Petunia hybrida is a member of the Solanaceae family, which originated in South America. They are easily identified in gardens, due to their diverse flower colours and patterns. This popular garden plant, however, is a vital model species for plant biologists, and the availability of its parents' genomes makes it much more relevant. It is used to treat chest pains, digestive disorders, heart problems, lung ailments and insomnia (Vandenbussche et al., 2016). Free radicals are atoms or groups of atoms with unpaired electrons in their valence shell and are formed when oxygen interacts with specific compounds (Figure 1). They are created as a result of environmental pollutants such as chemicals, toxins, radiation, and physical stress, leading to a decrease in antioxidants in the immune system as well as the activation of protein mutations. A significant amount of free radicals can cause continuous oxidative damage, which can lead to cancer, cell damage, ageing, cardiovascular, hepatic, neurological and renal system diseases (Madamanchi, Vendrov and Runge, 2005).

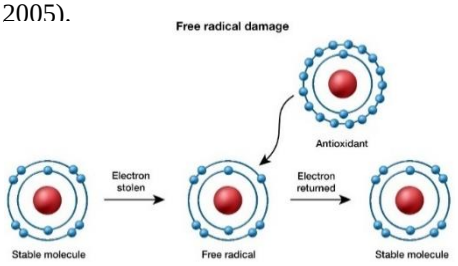

Figure 1: Free radical damage (Alumier, 2019)

To eliminate the free radicals generated from the body, an antioxidant defence system is present. They act as protective agents in the body, reducing the damage caused by oxidative stress generated by reactive oxygen species (ROS) and delaying the onset of many chronic disorders. As a result, many types of research are being conducted on antioxidant-rich substances to determine their potential efficacy as dietary supplements and adjuvants for use in the treatment of free radical-related disorders. Secondary metabolites are produced by plants to scavenge excessive quantities of ROS and free radicals to repair the damage. Petunia has been shown to have significant antioxidant activity due to its presence of flavonoids and phenolic acids (Pizzino et al., 2017).

AgNPs are the most thoroughly researched antibacterial nanoagents due to thier broad-spectrum antimicrobial properties and efficiency against microorganisms. Many physicochemical features including size, surface, shape and stability are considered to be crucial in its antibacterial action. Antibacterial action is mediated by the attachment of AgNPs to the cell releasing silver ions, affecting DNA and other cellular organelles (Figure 2) (Tang and Zheng, 2018).

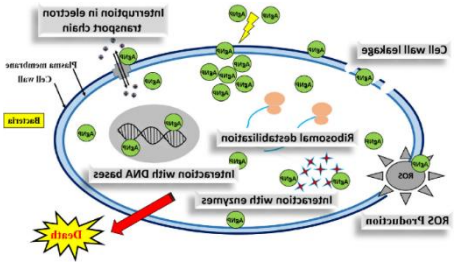

Figure 2: Mechanisms of antibacterial activity (Patil and Kim, 2016)

Many harmful organic chemicals, particularly azo dyes and industrial effluents, have been released directly into the ecosystem due to the industrial revolution. As these contaminants possess toxic, mutagenic and carcinogenic properties, several procedures including UV radiation, ozonization, flocculation and ion exchange have been used to eliminate them. However, the use of AgNPs as photocatalysts is a major advantage for the degradation of Malachite green, Methyl orange like dyes as it utilizes both UV and visible light due to their bandgap energy being small and surface plasmon resonance (Figure 3). In addition, it is cost-effective, abundant, simple and rapid. Hence, AgNPs are perfect for dye degradation among other metallic nanoparticles (Bhakya et al., 2015).

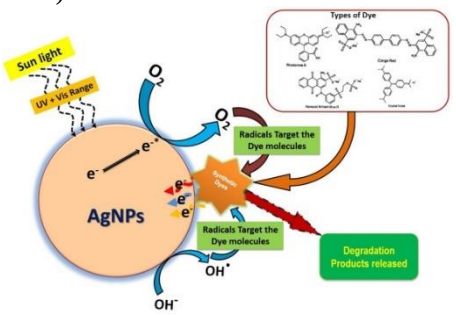

Figure 3: Mechanisms of antibacterial activity (Marimuthu et al., 2020)

The present study aims to synthesize AgNPs, evaluate the antioxidant properties, photocatalytic activity and antibacterial activity of aqueous leaf extracts of Petunia (Black, white, pink, purple, pink-purple). To achieve this, phytochemical screening will be performed initially. Then the antioxidant activity will be tested by Total Flavonoid Content (TFC), Total Phenolic Content (TPC) and Total Antioxidant Capacity (TAC). The free radical scavenging ability will be tested by DPPH assay, photocatalytic property of AgNPs will be evaluated using Malachite green and finally, the antibacterial activity will be done according to the well diffusion

method using E. coli and S. aureus. This study is expected to provide valuable insight on Petunia as a natural antioxidant source and promote their use as a functional food and their medicinal values in the future.

MATERIALS AND METHODOLOGY

The five species of Petunia leaves, black, white, pink, purple and pink-purple were collected from Diyatha Uyana plant nursery Battaramulla and Nuwara Eliya, Sri Lanka.

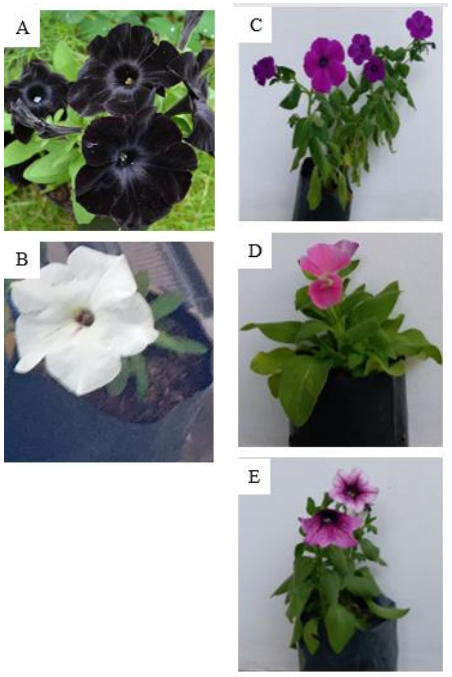

Figure 4: Varieties of Petunia used (A) Black Petunia (B) White Petunia (C) Purple Petunia (D) Pink Petunia and (E) Pink-purple Petunia

Sample preparation

The samples were cut into small pieces and left in the oven for 3 days at 40°C. Then 2 g of each sample was mixed with 50 mL of distilled water, incubated at 80°C for 15 minutes and filtered using Whatman No.1 filter paper into falcon tubes. The filtrations were stored at 4°C for further use.

Phytochemical analysis of the samples

The following phytochemical tests were conducted on water extracts.

Table 2: Methodology of the phytochemical analysis

Phytochemical	Methodology
Carbohydrates	2 mL of extract was added to 1 mL of Molischs reagent and a few drops of conc. sulphuric acid (Auwal *et al.*, 2014).
Tannins	1 mL of extract was added to 2 mL of 5% ferric chloride (Gonfa, Teketle and Kiros, 2020).
Saponins	2 mL of extract and 2 mL of distilled water was added to a graduated cylinder and was shaken for 15 minutes lengthwise

	(Gonfa, Teketle and Kiros, 2020).
Terpenoids	0.5 mL of extract was treated with 2 mL of chloroform and conc. sulphuric acid (Das *et al*., 2014).
Anthraquinones	A few drops of 10% ammonia solution was added to 1 mL of extract (BaoDuy, Trang and Trang, 2015).
Steroids	1 mL of extract and chloroform was added and a few drops of conc. sulphuric acid (Gonfa, Teketle and Kiros, 2020).
Proteins	To 2 mL of extract few drops of 0.2% ninhydrin reagent was added and heated for 5 minutes (Deshmukh and Theng, 2018).

Green synthesis of AgNPs

1 mL of leaf extract from each sample was mixed with 9 mL of 1mM AgNO3 solution made with 0.102 g and 600 mL distilled water. The samples were incubated at 90oC and 60oC for 15 minutes, 30 minutes, 45 minutes and 60 minutes. In addition, the solutions were also incubated at room temperature (RT) for 72 hours. Absorbance was measured from 320 nm to 520 nm.

1 mL of water extracts and synthesised AgNPs were diluted with 14 mL of water and the following assays were performed using diluted water extracts and AgNPs in triplicates.

Determination of Total Flavonoid Content (TFC)

According to the AlCl3 colorimetric technique, 2.4 mL of sample was added to 0.05 mL of 1 M potassium acetate. This was incubated at RT for 30 minutes and absorbance was measured at 415 nm against a distilled water blank. The findings were given in µg Quercetin equivalents per 100 g (µg QE/100 g) (Zia et al., 2017).

Determination of Total Phenolic Content (TPC)

To the 100 µL portion of the sample, 3.1 mL of distilled water was added. 10-fold dilution was carried out to the Folin-Ciocalteu reagent and 0.2 mL of it was added to the sample and kept for 6 minutes. Thereafter, 0.6 mL of 20% sodium bicarbonate was added and incubated for 1 hour at RT followed by measuring absorbance at 765 nm against a distilled water blank. The findings were given in g Gallic acid equivalents per 100 g (g GAE/100 g) (Sulastri et al., 2018).

Determination of Total Antioxidant Capacity (TAC)

0.2 mL aliquot of sample was mixed with 2 mL of the reagent solution which was made with 3.3 mL sulphuric acid, 0.23 g sodium phosphate and 0.0392 g of ammonium molybdate. Each of these were topped up to 50 mL and mixed. The tubes were incubated at 95oC for 90 minutes. The absorbance was measured at 695 nm against a blank containing reagent solution without the sample. The findings were given as g ascorbic acid equivalents per 100 g (g AAE/100 g) (Abhayawardena and Kandiah, 2020).

Determination of 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) Scavenging Activity

1 mL of sample was added to 2 mL of 0.004% DPPH. Incubated for 30 minutes at RT. Absorbance was measured at 517 nm against a methanol blank. Using the equation below (Figure 5) percentage DPPH scavenging activity was calculated (Kandiah and Chandrasekaran, 2021).

Inhibition (%) = A_control - A_sample/A_control × 100

Figure 5: Percentage DPPH scavenging activity equation

Determination of Median Inhibition Concentration (IC50)

2 mL of 0.004% DPPH solution was added to 1 mL of a series of four concentrations (100%, 80%, 60% and 20%) prepared using distilled water and samples. Methanol was used as the blank to measure absorbance at 517 nm. The percentage DPPH scavenging activity was calculated according to figure 5 (Kandiah and Chandrasekaran, 2021).

Photocatalytic activity of AgNPs under sunlight

2.9 mL of 2 mM Malachite green was diluted with 200 mL of distilled water. Into this 1 mL of 100 ppm, Pink-purple Petunia AgNP sample was added. The solution was placed under sunlight and the absorbance was measured at 30minute intervals for 2 hours against a distilled water blank. The same procedure was carried out using 0.04 µL of NaBH4 at 10minute intervals for 40 minutes (Lakshmi, Dhanya and Sheeba, 2016).

Determination of the Antibacterial activity

Escherichia coli and Staphylococcus aureus were used to swab the Muller-Hinton agar plates. Wells were made for duplicates of each sample (S1 and S2) and negative control (Figure 6). 1 mL of samples were added to the wells. 1 mL of saline was used as the negative control (-), positive control (+) was gentamycin. The plates were incubated for 24 hours at 37oC and the diameter of the zone of inhibition (ZOI) was measured using a ruler (Kandiah and Chandrasekaran, 2021).

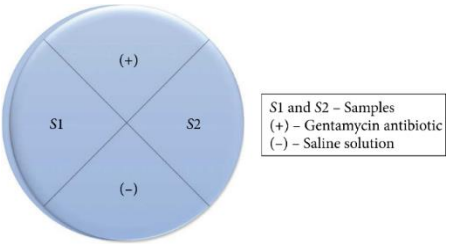

Figure 6: Labelling of the petri plates (Kandiah and Chandrasekaran, 2021)

Transmission electron microscopy (TEM)

10 mL of PK AgNPs were centrifuged for 5 minutes at 5000 rpm and repeated 6 times. It was then dried completely in a hot air oven. TEM analysis was carried out at the Sri Lankan Institute of Nanotechnology (SLINTEC), Homagama using JEOL JEM-2100.

Statistical analysis

The data from the above assays were statistically analysed using Microsoft-Excel and one-way ANOVA. Statistical difference is defined as $p < 0.05$ and F crit < F value respectively.

RESULTS AND ANALYSIS

Phytochemicals

Water extracts have been used to analyse the phytochemical contents.

Table 3: Phytochemical analysis

Phytochemical	White	Purple	Pink	Black	Pink-purple
Carbohydrates	✓	✓	✓	✓	✓
Tannins	×	×	✓	×	×
Saponins	×	✓	×	×	×
Terpenoids	✓	✓	✓	✓	✓
Anthraquinones	×	×	×	×	×
Steroids	✓	✓	✓	✓	✓
Proteins	×	×	×	×	×

Carbohydrates, terpenoids and steroids are present in all samples. Tannins were only present in Pink (PK). While saponins were only found in Purple (P). Petunia samples did not indicate the presence of anthraquinones and proteins.

Biosynthesis of AgNPs

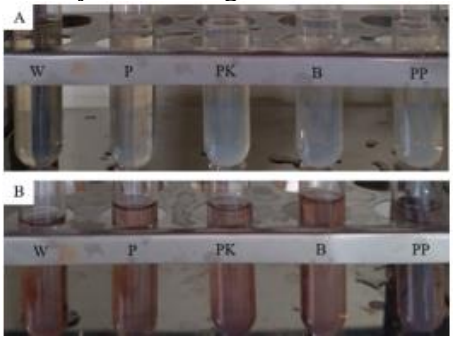

Figure 7: Biosynthesis of AgNPs using Petunia leaf extracts. The AgNO3 solution changed from pale yellow (A) to deep brown (B).

A change in colour after incubation confirms the presence of AgNPs.

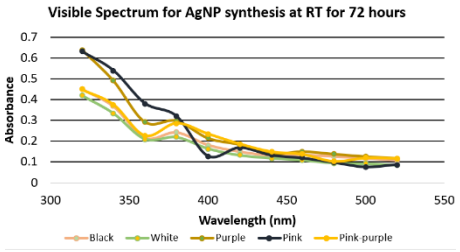

Figure 8: Graph demonstration spectrometric analysis by varying the wavelength of AgNPs synthesized using five varieties of Petunia water extracts.

PK and PP show a peak between 400-480 nm, which confirms the AgNPs synthesis.

Table 4: Spectrophotometry analysis data of AgNP synthesis optimization

Temperature (°C)	Time (mins)	Samples				
		Black *Petunia* (B)	White *Petunia* (W)	Purple *Petunia* (P)	Pink *Petunia* (PK)	Pink-purple *Petunia* (PP)
RT	4320	×	×	×	✓	✓
60	15	×	×	×	×	×
	30	✓	×	×	×	×
	45	×	×	×	×	×
	60	×	×	×	✓	×
90	15	×	×	×	✓	×
	30	×	×	×	×	×

✓ Presence of NPs

× Absence of NPs

According to the above table, PK produced AgNP absorbance peaks at RT, 60°C for 60 min and 90°C for 15 min. The PP produced AgNP absorbance peak only at RT whereas B produced a peak only at 60°C for 30 min. The rest of the sample did not produce any peak at any temperature or time interval.

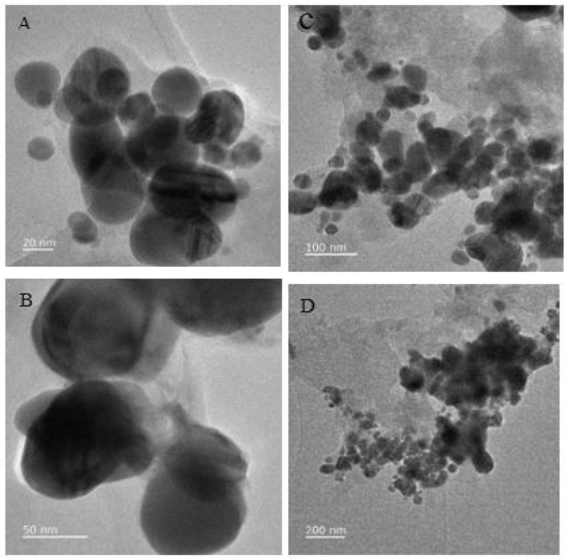

Figure 9: TEM images of PK AgNPs at 20 nm (A), 50 nm (B), 100 nm (C) and 200 nm (D).

The TEM images indicated that the synthesized AgNPs were spherical and 50 nm in range.

Determination of Total Flavonoid Content (TFC)

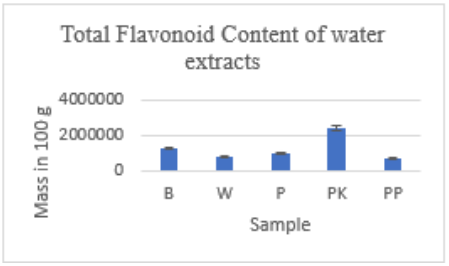

Figure 10: TFC of the five varieties of Petunia leaf water extract.

TFC was found to be highest in PK and lowest in PP out of all the water extracts.

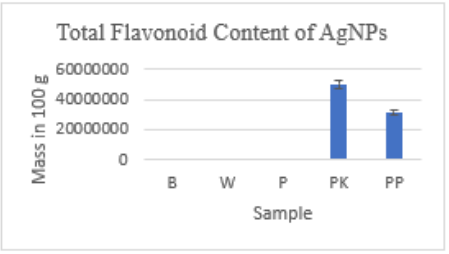

Figure 11: TFC of the five varieties of Petunia leaf AgNPs.

The TFC is higher in AgNPs compared to water extracts. Among the two AgNP samples TFC was observed higher in PK.

Table 5: Single-factor ANOVA for TFC of five varieties of Petunia leaf water extracts and AgNPs.

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	5	6309722	1261944.44	4.50138E+11
Column 2	2	8.2E+07	41020833.3	1.73445E+14

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	2E+15	1	2.2582E+15	64.43067315	0.00049	6.60789
Within Groups	2E+14	5	3.5049E+13			
Total	2E+15	6				

ANOVA was performed to test for significance between water extracts and AgNPs.

Determination of Total Phenolic Content (TPC)

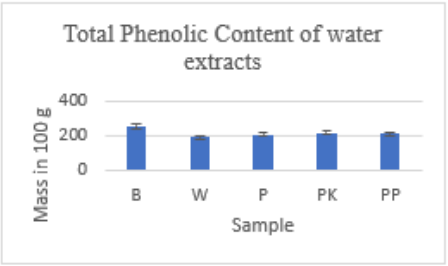

Figure 12: TPC of five varieties of Petunia leaf water extracts.

B shows the highest TPC out of all the water extracts.

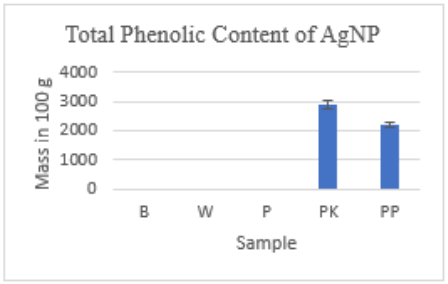

Figure 13: TPC of five varieties of Petunia leaf AgNPs.

The TPC is higher in AgNPs compared to water extracts. Among the two AgNPs, TPC was observed higher in PK.

Table 6: Single-factor ANOVA for TPC of five varieties of Petunia leaf water extracts and AgNPs

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	5	1090.714286	218.1428571	549.847
Column 2	2	5081.428571	2540.714286	225408

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	7706197.2	1	7706197.201	169.287	4.78238E-05	6.60789
Within Groups	227607.551	5	45521.5102			
Total	7933804.75	6				

ANOVA was performed to test for significance between waters extract and AgNPs.

Determination of Total Antioxidant Capacity (TAC)

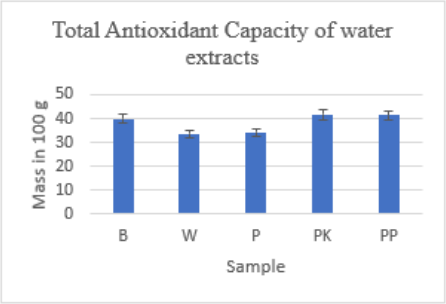

Figure 14: TAC of five varieties of Petunia leaf water extracts.

PK is the highest in TAC compared to the other water extracts

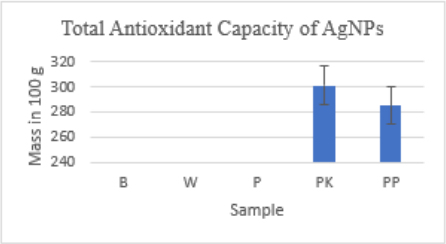

Figure 15: TAC of five varieties of Petunia leaf AgNPs.

The TAC is higher in AgNPs compared to water extracts. Among the two, AgNPs TAC was comparatively higher in PK.

Table 7: Single-factor ANOVA for TAC of five varieties of Petunia leaf water extracts and AgNPs.

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	5	190.739	38.1477	15.55785124
Column 2	2	587.045	293.523	126.5495868

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	93166.3	1	93166.3	2467.575561	6.24804E-08	6.607890974
Within Groups	188.781	5	37.7562			
Total	93355.1	6				

ANOVA was performed to test for significance between water extracts and AgNPs.

DPPH Radical Scavenging Activity

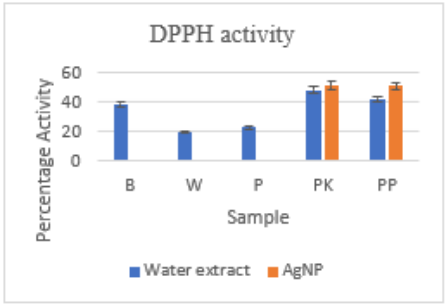

Figure 16: DPPH radical scavenging activity of five varieties of Petunia leaf water extracts and AgNPs.

The AgNPs were observed to have slightly higher values than water extracts.

Table 8: Single-factor ANOVA analysis of DPPH radical scavenging activity of Petunia leaf water extracts and AgNPs

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	5	2578.097345	515.619469	35089.95
Column 2	2	15420.35398	7710.17699	244.7333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	73945225.63	1	73945225.6	2629.546	5.33E-08	6.607891
Within Groups	140604.5501	5	28120.91			
Total	74085830.18	6				

ANOVA was performed to test for significance between water extracts and AgNPs

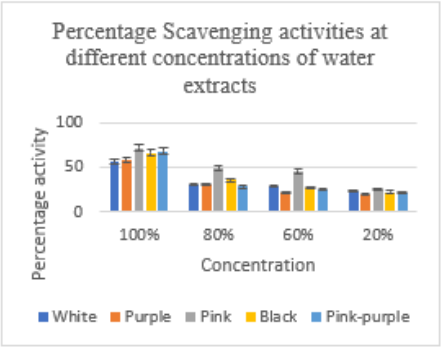

Figure 17: IC50 of five varieties of Petunia leaf water extracts.

All water extracts percentage scavenging activity decreased with the concentration decrease.

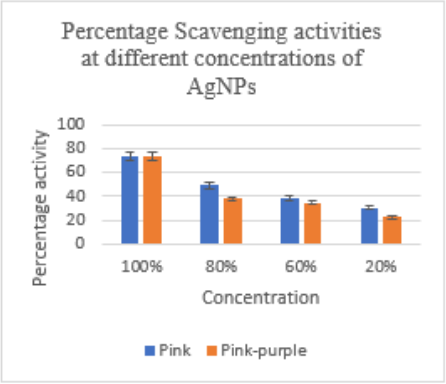

Figure 18: IC50 of five varieties of Petunia leaf AgNPs.

Both AgNPs percentage scavenging activity decreased with the concentration decrease. Out of which PP AgNPs showed a higher reduction.

Table 9: IC50 values of water extracts and AgNPs

Sample	IC_{50} water	IC_{50} AgNPs
White	146.4887	-
Purple	142.8205	-
Pink	91.58221	104.6844
Black	100.6337	-
Pink-purple	103.0585	86.62678

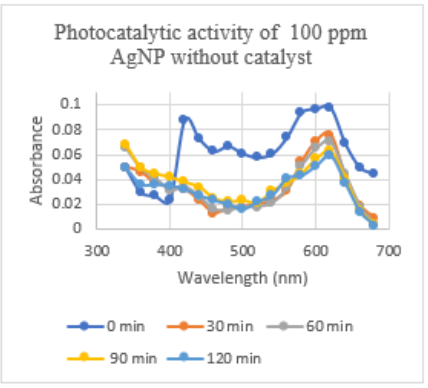

Figure 19: Photocatalytic activity of 100 ppm AgNPs without catalyst

The curve gradually decreases with time.

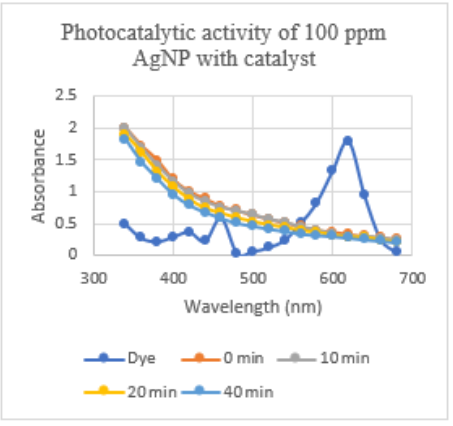

Figure 20: Photocatalytic activity of 100 ppm AgNPs with catalyst

A rapid degradation of the dye is observed

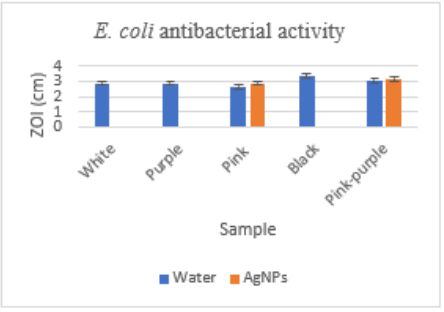

Figure 21: Antibacterial activity against E. coli

AgNPs show comparatively higher ZOI values than water extracts.

Table 10: Single-factor ANOVA analysis of the antibacterial activity against E. coli of Petunia leaf water extracts and AgNPs

ANOVA was performed to test for significance between water extracts and AgNPs

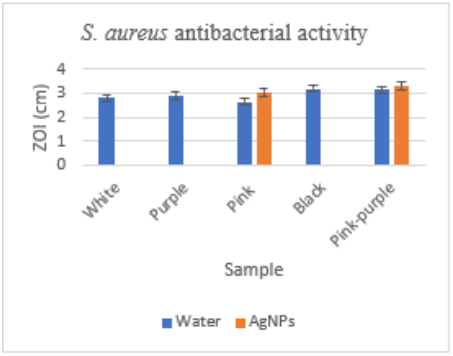

Figure 22: Antibacterial activity against S. aureus

AgNPs show comparatively higher ZOI values than water extracts.

Table 11: Single-factor ANOVA analysis of the antibacterial activity against S. aureus of Petunia leaf water extracts and AgNPs.

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	5	14.7	2.94	0.05425
Column 2	2	6.35	3.175	0.03125

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.078893	1	0.078893	1.58898	0.263096	6.607891
Within Groups	0.24825	5	0.04965			
Total	0.327143	6				

ANOVA was performed to test for significance between water extracts and AgNPs

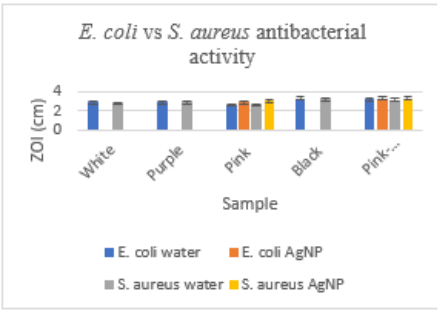

Figure 23: Antibacterial activity against E. coli and S. aureus

E. coli shows higher ZOI values than S. aureus.

Table 12: Single-factor ANOVA analysis of the antibacterial activity against E. coli and S. aureus of Petunia leaf water extracts and AgNPs

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	5	15	3	0.07625
Column 2	2	6.25	3.125	0.10125
Column 3	5	14.7	2.94	0.05425
Column 4	2	6.35	3.175	0.03125

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.104071	3	0.03469	0.53003	0.671785	3.708265
Within Groups	0.6545	10	0.06545			
Total	0.758571	13				

ANOVA was performed to test for significance between water extracts and AgNPs

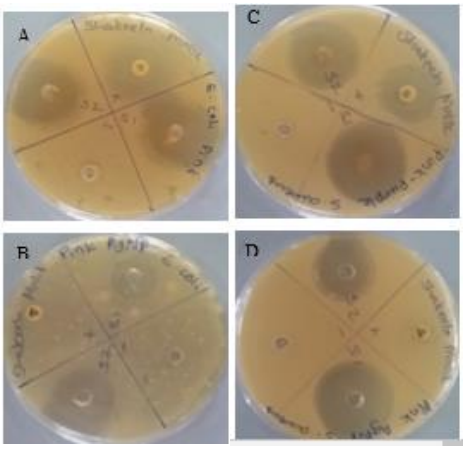

Figure 24: Antibacterial activity of PK water extract (A) and AgNPs (B) against E. coli and PP water extract (C) and AgNPs (D) against S. aureus.

DISCUSSION

Biological approaches for AgNP synthesis have grown in popularity over the last decade due to the benefits of enabling a one-step synthesis of eco-friendly, non-toxic NPs without the requirement for culture preservation or further maintenance. Several plant species have been used in the biosynthesis of AgNP across the world to date (Shaikh et al., 2021). Petunia plants have medicinal properties and they are mainly used as an ornamental plant. Up-to-date none of the researches have been carried out to analyse antioxidant activities or synthesis of AgNPs using Petunia leaves. Therefore, in this research Petunia leaves have been used to synthesize AgNPs using water as a solvent. Water was used instead of organic solvents throughout the extraction process to support the protocol's eco-friendliness, cheap and readily available (Mihaylova and Lante, 2019). The presence of phytochemicals in the sample resulted in the reduction of AgNPs from Ag+ to Ag0. The solution colour changed from yellow to dark brown after 72 hours, indicating the synthesis of AgNPs (Figure 7). The absorbance peaks were used to detect the presence of reduced AgNPs at 420 nm due to the surface plasmon resonance showing evidence of AgNP synthesis (Logeswari, Silambarasan and Abraham, 2015).

Petunia leaves were reluctant to form NPs at higher temperatures, above 60°C according to the spectrophotometric graphs (Figure 8), this could be due to its active compounds degradation (Nguyen et al., 2021). However, at RT NPs were synthesized readily and with the increased duration of synthesis more AgNPs could have been yielded (Smiechowicz, Niekraszewicz and Kulpinski, 2021).

TEM determines the size distribution of AgNPs due to the variable chemical and physical properties based on their size and shape. The NPs were measured to be 50 nm in size. It is evident that the AgNPs were distinct and spherical (Figure 9). Similar studies also reported analysing the morphological structure of AgNPs using TEM (Santhoshkumar Parvathy and Soniya, 2021). Bandgap measures the energy difference between the valence band and the conduction band where electrons are able to move. The minimum amount of energy required for an electron to move from bands is known as band energy. Nanomaterials are categorized as semiconductors (<3 eV) or insulators (>4 eV) based on their bandgap energy. The band gap energy was calculated to assess optical properties of AgNPs using the Planck's equation (Figure 25).

$$E = h \times \frac{C}{\lambda}$$

h = Planck's constant (6.626×10^{-34} Js)

C = speed of light (3×10^{8} m/s)

Figure 25: Plank's equation

Table 13: Band gap energies for AgNPs

Sample	Bandgap energy (E)	Classification
Pink (PK)	2.621	Semiconductor
Pink-purple (PP)	2.371	Semiconductor

The antioxidant activity of AgNPs and water extracts were measured using TFC, TPC and TAC assays.

The AlCl3 colourimetric technique was used to calculate TFC at 415 nm wavelength where AlCl3 forms acid-stable compounds with the C-4 keto groups and either the C-3 or C-5 hydroxyl groups of flavonols and flavones, and acid-labile compounds with the ortho-dihydroxyl groups in flavonoids A- or B-rings. (Bhaigyabati, Devi and Bag, 2014). The highest flavonoid content was observed in PK AgNPs (Figure 11) and PK water extracts (Figure 10). There is a significant difference between NPs and water extracts in TFC of Petunia. Since there aren't any researches conducted on antioxidant assays, Zia and his coworkers in 2017, stated that the similar family Lycopersicon esculentum Mill. also shows higher TFC values for AgNPs as compared to water extracts. The one-way ANOVA proves the significant difference by indicating $P < 0.05$ (0.00049) and F > Fcrit (F – 64.43067 Fcrit – 6.60789) (Table 5).

Quantitative analysis for phenolic compounds is performed using the colourimetric assay with the use of the Folin-Ciocalteu reagent, a reagent composed of a mixture of phosphomolybdic and phosphotungstic acid, which is reduced to a mixture of blue oxides of molybdenum and tungsten once the phenols are oxidized. The resulting colour has a maximum absorption wavelength of 750 nm and is proportionate to the total amount of phenolic compounds present originally (Ahamed and Iqbal, 2018). As TFC, PK AgNP (Figure 13) was highest among all the extracts while B was highest in water extracts (Figure 12). Attia et al, 2020 stated that the similar family Solanum melongena also shows higher results for AgNPs for TPC. The one-way ANOVA proves the significant difference by indicating $P < 0.05$ (4.78×10^{-5}) and F > Fcrit (F – 169.287 Fcrit – 6.60789) indicating a significant difference (Table 6).

The TAC of water extract and AgNPs were determined using the phosphomolybdenum technique. Antioxidants reduce Mo (VI) to a green phosphate/ Mo (V) compound with an absorption peak at 695 nm at acidic pH (Bhaigyabati, Devi and Bag, 2014). As expected, PK AgNPs (Figure 15) were observed as the highest from all the samples while PK water extract (Figure 14) displayed the highest among the water extracts. Abhayawardena and Kandiah in 2020 stated that the similar family C. annuum showed that AgNPs had higher TAC values than water extracts. The one-way ANOVA proves the significant difference by indicating $P < 0.05$ (6.248×10^{-8}) and F > Fcrit (F – 2467.575 Fcrit – 6.60789) indicating a significant difference (Table 7).

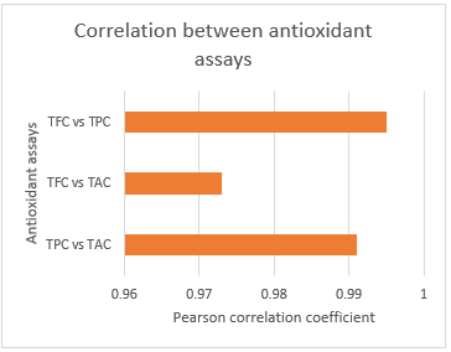

Figure 26: Correlation between antioxidant assays

According to the Pearson correlation coefficient (Figure 26) the highest correlation is observed between TFC-TPC assays (R2=0.995) showing that both flavonoid and phenolic compounds contribute to the antioxidant capacity. Nevertheless, all correlations TPC-TAC (R2=0.991) TFC-TAC (R2=0.973) are strong correlations as R2 is above 0.95. A study on a Solanaceae plant showed a positive correlation of TPC-TAC

indicating that the primary contributor to the antioxidant activity are phenolic compounds and TFC-TAC significance indicating that flavonoid compound was one of the primary contributors to the antioxidant capacities (Leng et al., 2022).

The DPPH (2,2-diphenyl-1-picrylhydrazyl) assay determines free radical scavenging activity. DPPH is a purple, stable free radical which turns yellow indicating the scavenging activity of antioxidants (Jadid et al., 2017). PK AgNP sample shows slightly higher antioxidant activity than PP AgNP while PK water sample is the highest from water samples (Figure 16). The one-way ANOVA proves the significant difference by indicating P < 0.05 (5.33 × 10-8) and F > Fcrit (F – 2629.546 Fcrit – 6.607891) indicating a significant difference (Table 8). Solanum nigrum from Petunia family shows enhanced antioxidant activity in AgNPs compared to water extracts (Ahn and Park, 2020). The IC50 value is the antioxidant concentration required to decrease the initial DPPH concentration from 50%. Scavenging activity is higher when the IC50 value is lower (Loganayaki, Siddhuraju and Manian, 2013). IC50 of PP had the lowest concentration (Figure 18) which indicates its higher antioxidant properties. This can be related to results obtained from TFC, TPC and TAC. According to Abhayawardena, and Kandiah, 2020 a research on C. annuum showed that AgNPs had a lower IC50 than water extracts, indicating stronger antioxidant capacity.

The photodegradation mechanism of MG occurs due to the surface Plasmon resonance effect when electrons collectively oscillate from valence band to conduction band under visible light or UV radiation. Due to the absorption of plasmonic excitation of surface electrons, oxygen present in water is converted into free radicals (O2°). Holes in AgNPs are filled by electrons from MG dye molecules that have been absorbed on the AgNPs surface. This results in the oxidation of dye molecules. Furthermore, produced O2 interacts with H+ ions formed due to water splitting, resulting in the formation of other free radicals including HOo and OHo. These free radicals destroy dye molecules by breaking down complex organic structures into dye intermediates (Kodom et al., 2015). Photocatalysis is used to degrade a variety of pollutants, including dyes, pharmaceuticals, pesticides, herbicides, hydrocarbons, and microorganism inactivation (da Silva et al., 2021).

In this experiment, PP AgNPs were used under different conditions to determine MG degradation and the influence of the catalyst. In the absence of a catalyst, a significant decline in the absorbance peak was observed (Figure 19). When the catalyst, cationic NaBH4 was added the absorbance peak declined drastically (Figure 20). Using the (Figure 27) equation the rate constant was calculated.

$$ln\frac{C}{C_o} = kt$$

C = concentration

C_0 = initial concentration

t = time

Figure 27: Rate constant equation

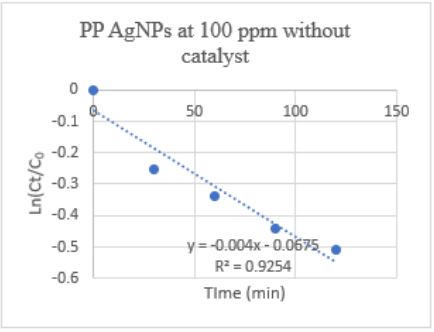

Figure 28: Photocatalytic kinetic graph of PP AgNPs at 100 ppm without catalyst

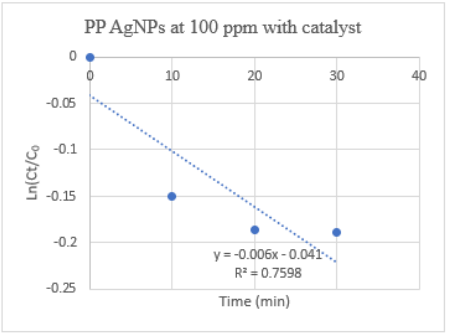

Figure 29: Photocatalytic kinetic graph of PP AgNPs at 100 ppm with catalyst

Table 14: Rate constants of PP AgNPs at different conditions

Conditions	Rate constant (k/min^{-1})
100 ppm without catalyst	0.004
100 ppm with catalyst	0.006

100 ppm with catalyst achieved the highest rate constant with a low duration showing the optimum working conditions of PP AgNPs (Table 14).

AgNPs destroy bacteria by themselves or by continuously generating Ag+. It attaches to the cytoplasmic membrane and cell wall increasing the permeability, leading to bacterial envelope rupture. Cell entry of Ag+ facilitates ROS production creating cell reproduction issues or even death. Furthermore, AgNPs can affect the cell membrane structure causing cell lysis. Moreover, AgNPs may have a role in bacterial signalling pathways. Protein substrates phosphorylation affects signal transduction and tyrosine residues on peptide substrates can be dephosphorylated. If signal transduction disrupts cell apoptosis and multiplication is interrupted (Yin et al., 2020). AgNPs are more toxic to gram-negative bacteria due to their narrower cellular wall compared to gram-positive bacteria. NP penetration into cells is limited when the cell walls are thick hence, the antibacterial effects of AgNPs on gram-positive lower than gram-negative bacteria (Yin et al., 2020).

According to the well diffusion results of E. coli and S. aureus, both PK and PP AgNPs in comparison to their water extracts showed better bacterial antagonism. PP AgNPs had higher antagonism than PK AgNPs. From water samples, B showed the highest antagonism in both E. coli and S. aureus (Figure 23). As results are comparatively higher in E. coli it confirms that gram-negative bacteria have a higher antibacterial effect. The relatively large surface area of the AgNPs allows greater interaction with microorganisms showing higher bactericidal activity (Mousavi-Khattat, Keyhanfar and Razmjou, 2018). Petunia axillaris crude extraction shows higher ZOI on methanolic extracts than water extracts which could be because the active chemicals are more soluble in organic solvents (Kumar, 2015). The following one-way ANOVA shows insignificant difference against antibacterial activity (Table 15).

Table 15: One-way ANOVA against antibacterial activity

Analysis	Results	Inference
E. coli	P > 0.05 (0.622576) F < Fcrit (F – 0.274725 Fcrit – 6.607891)	Not significantly different
S. aureus	P > 0.05 (0.263096) F < Fcrit (F – 1.58898 Fcrit – 6.607891)	Not significantly different
E. coli and *S. aureus*	P > 0.05 (0.671785) F < Fcrit (F – 0.53003 Fcrit – 3.708265)	Not significantly different

In conclusion, AgNPs from two samples PK and PP were successfully synthesized at RT from Petunia leaf extracts showing remarkable antioxidant

properties, antibacterial and photocatalytic properties. Both PK and PP were identified as semiconductors. TEM analysis on PK AgNPs revealed that spherical shaped 50 nm AgNPs were present. AgNPs comparatively showed higher antioxidant properties than water extracts. E. coli showed higher ZOI values than S. aureus. PP AgNPs at 100 ppm have a higher ability to degrade MG dye molecules completely within 40 minutes. Thereby, biosynthesized AgNPs from Petunia varieties could be applied to cure free radical-mediated diseases and to remove toxic dyes by wastewater treatment for a better environment.

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