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Low cost and eco-friendly green synthesis of silver nanoparticles using *Citrus reticulata* leaf extract: Analysis of their antioxidant properties

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Abstract

This study explores the green synthesis of silver nanoparticles (AgNps) utilizing *Citrus reticulata* (mandarin) leaves as a sustainable and eco-friendly approach. In the current study, three varieties of *Citrus reticulata* leaves such as heen naran, yaki naran, and nas naran were used and AgNps were formed through bioreduction of silver nitrate to a colloidal solution of AgNps. Water was used as the solvent for AgNp synthesis. Nanoparticle formation was identified with colour change in samples from yellowish-brown to reddish-brown and by measuring the absorbance at 320-560 nm range in UV visible spectroscopy. Optimization was carried out at 90ºC and 60ºC for 15 minutes, 30 minutes, 45 minutes, and 1 hour, also at room temperature for 72 hours. Room temperature was considered as the optimum temperature for silver nanoparticle synthesis. Optical properties were analyzed by calculating the bandgap energies. All nanoparticles from three varieties showed semiconducting properties. The antioxidant capacity of AgNp was determined with antioxidant assays such as Total Flavonoid Content (TFC), Total Phenolic Content (TPC), and Total Antioxidant Capacity (TAC) assays. Silver nanoparticles showed higher TFC, TPC, and TAC, compared to water extracts. Statistical analysis was performed with single-factor ANOVA analysis. Based on the results, a significant difference was observed between AgNp and water extracts in TFC and TAC while no significant difference was observed between AgNp and water extracts in TPC. TAC was found to be strongly correlating to TPC while a weak positive linear correlation was observed between TAC and TFC, and in between TFC and TPC assays.

Keywords: Silver nano particles, *Citrus reticulata* leaves, Green synthesis, Antioxidants

1. Introduction

The world is facing a major problem associated with human health due to free radicals induced diseases such as neurodegenerative disorders, cardiovascular diseases, diabetes mellitus, and various respiratory diseases. Free radical is a molecule that is capable of independent existence with a free unpaired electron in the outer shell, which causes the molecule to become highly reactive and attack the macromolecules such as lipids, proteins, and nucleic acids, leading to homeostatic disruption and cell damage.[1]

Reactive Oxygen Species (ROS) can be produced due to various factors such as smoking, pollution, radiation, processed food, heavy metals, and UV light. Antioxidants can defend against ROS by donating an electron and inhibit oxidative damage due to their free radical scavenging property. Antioxidants are available as flavonoids, enzymatic antioxidants, unprocessed food, and vitamins.[2] .

Natural antioxidants are mainly present in medicinal plants. Polyphenols act as the major antioxidants in plants as they are good hydrogen donors, and these are accepted by highly reactive radicals to become stable.[3] . Major polyphenols in plants are flavonoids, anthocyanins, chalcones, stilbenes, lignans and phenolic acids (Figure 1).

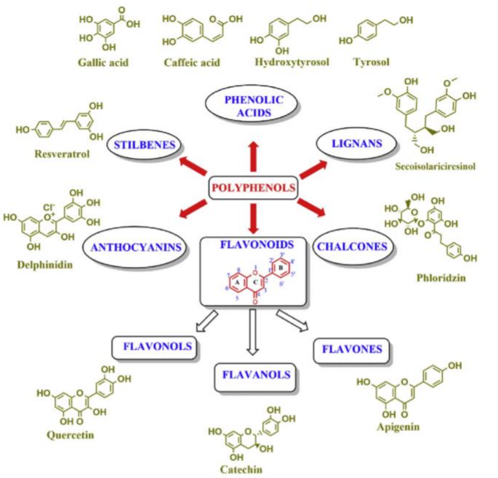


Figure 1. Classification of Polyphenols containing antioxidant property.[4]

Synthetic antioxidants, such as BHT and BHA, are widely used in the food industry, therapeutics, and cosmetics, but they pose potential risks to human health. As a result, there is a high demand for an alternative for synthetic antioxidants. Several studies have recently been reported about the use of AgNp synthesized from natural sources that can reduce the use of synthetic antioxidants.[5]

Nanobiotechnology has emerged as integration between biotechnology and nanotechnology, offering various applications in the biological field including green synthesis of nanoparticles.[6] Due to the tremendous growth in nanobiotechnology, so far various noble metal nanoparticles have been synthesized using Au, Cu, Zn, Pt, and Ag. However, more than other noble metal nanoparticles, AgNp has gained attention due to their unique properties such as good conductivity and chemical stability and most importantly non-toxic, cost-effective and ecofriendly. Thus, biocompatible AgNps have extensive applications in many fields such as nanomedicine, cosmetics, food and feed, optics, drug-gene delivery, catalysis and photo-electrochemical applications.[7]

Nanofabrication occurs in two major ways. They are the top-down approach and bottom-up approach. In the bottom-up approach, nanoparticles are fabricated from atomic-scale using chemical and biological methods while in the top-down approach the bulk material is broken down into fine particles to produce nanoparticles using the physical method which includes milling, grinding, and arc discharge method. Among these, chemical methods have been frequently used in large-scale production. Unfortunately, most of the physical and chemical methods for nanoparticle synthesis use various hazardous chemicals and high energy requirements.[8]

The need for an eco-friendly, non-toxic, and cost-effective method for the synthesis of nanoparticles leads to increasing demand for biological approaches. Thus, a method called ''Green synthesis'' was used by scientists to synthesize metal nanoparticles using microorganisms and plant extracts. Several parts of the plant such as the leaf, fruit, stem, and seed can be utilized in plant extract mediated synthesis (Figure 2). This method is potentially advantageous over microorganisms to reduce the cost when isolating and culturing microorganisms, and use of microbes can be hazardous. The rate of reduction of metal ions is much faster, and stable nanoparticles can be formed in plant extract-mediated synthesis.[9] Thus, in the current study *Citrus reticulata* leaves were used to synthesis AgNp from green synthesis. *Citrus reticulata* is commonly called mandarin orange, belonging to the Rutaceae family. Citrus leaves are rich in bioactive components such as flavonoids, ascorbic acid, phenolic compounds, and citric acid which are involved in the bio-reduction of metal ions and stabilization of synthesized nanoparticles by adhering to the surface of AgNPs (Figure 2) and results in high antioxidant capacity in AgNp

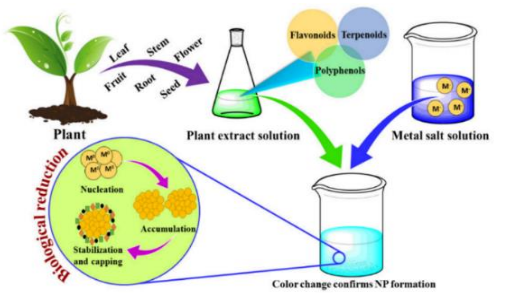


Figure 2. Mechanism of plant extracted mediated green synthesis.[10]

Green synthesis of nanoparticles was first reported in a study conducted by Gardea-Torresdey and his colleagues.[11] Many researchers have focused on the green synthesis of nanoparticles from plant extracts in the past decade. Scientists have also used fruit peel of *Citrus limon, Citrus sinensis,* and *Citrus tangerina* for the synthesis of nanoparticles and to examine their antibacterial activities,[12] and also AgNps have been synthesized from *Citrus reticulata* juice[13] and peel extracts to analyze antioxidant and antibacterial properties.[14] However, less research has been carried out for AgNp from *Citrus reticulata* leaf extract.

More than 60 types of flavonoids have been identified in citrus fruits. Flavonoids are polyphenols with a C6-C3-C6 structure containing two phenolic rings which carry one or more hydroxyl groups connected by a chain with 3 carbons. Citrus flavonoids can be classified as flavanols, flavanones, flavones, and isoflavones. [15]

Citrus plants consist of mainly glycosylated flavanones and poly-methoxylated flavones.[16] A study carried out by Dalia and others in 2016, identified that *Citrus reticulata* leaf oil contains linalool (21.20%) and sabinene (23.10%) as a major component and in the fruit peel oil, limonene (79.64%) was found to be the most abundant component. Limonene is a major flavonoid contributing to antioxidant activity while linalool and sabinene are also bioactive components that possess antioxidant activity.[17]

This study aims to synthesize AgNp in an ecofriendly and cost-effective manner using five varieties of *Citrus reticulata* leaf extracts. *Citrus reticulata* leaves were chosen because synthesis of nanoparticles with leaves can be used as an effective tool in management of waste. In Sri Lanka, *Citrus reticulata* leaves are utilized in ayurvedic medicine due to their extensive medicinal properties and nanoparticles were synthesized from these leaves to increase the efficiency of the medicinal value of *Citrus reticulata* leaves.[18] In the current study, antioxidant activity was determined by using TFC, TAC, and TPC assays. Statistical analysis was performed with SPSS and ANOVA.

2. Methodology

2.1 Sample collection. Citrus reticulata leaves of all five varieties were collected from Fruit Crops research and Development Centre, Horana, Sri Lanka. They are Heen naran, Yaki naran, Nas naran, Jama Naran and Maha naran.

2.1 Methodology.

COSHH forms and ethical consideration forms were prepared according to the reagents and materials used to carry out the research.

2.1.1 Preparation of aqueous leaf extracts. Five varieties of fresh mandarin leaves were air-dried under the same conditions for several days. Dried leaves were finely cut into small pieces and grounded with a motor and pestle. Leaves were weighed using an analytical balance and 2 g from each leaf variety was taken. To 2 g of each grounded sample, 50 mL of distilled water was added, and leaves were immersed in beakers containing distilled water. The samples were incubated at 80ºC for 20 min in the dry oven. The samples were filtered using a Whatman filter paper No.1 into 50 ml falcon tubes. The water extracts obtained were stored at 4ºC in the refrigerator until further use.

2.1.2Preparation of silver nitrate solution. Using an analytical balance, 0.0425 g of silver nitrate was measured. Measured silver nitrate was mixed with distilled water in a 250 mL volumetric flask. Distilled water was added to top up. The volumetric flask was covered with aluminum foil to maintain dark conditions.

2.5.1 *Synthesis of silver nanoparticles by green synthesis.* 1 mL of water extract was mixed with 9 mL of 1 mM aqueous $AgNO_3$ solution in a 250 mL volumetric flask and was kept in the dry oven at 60ºC and 90ºC for 1 hr, 45 min, 30 min, and 15 min under dark conditions. Dark conditions were maintained by covering the test tubes and with aluminum foil. The same procedure was followed at room temperature for 72 hrs.

2.5.2 *Characterization of silver nanoparticles.* A UV visible spectrophotometer was used to identify the formation of silver nanoparticles. Distilled water was used as the blank. The absorbance of samples was measured within the 320-560 nm range for samples obtained during optimization temperatures with varied time intervals.

2.5.4.1 Determination of antioxidant activity. Diluted silver nanoparticle samples and aqueous extracts were prepared by adding 14 mL of distilled water to 1 mL of each silver nanoparticle and aqueous extract sample. The diluted samples were stored in the refrigerator at 4ºC until further use. Each analysis was carried out in triplicates.

2.5.4.2 Determination of total flavonoid content (TFC). The aluminum chloride colorimetric method was used to analyze total flavonoid content. From each of the previously prepared 10 % w/v aluminum chloride and potassium acetate solutions, an amount of 0.1 mL was added into each 2.8 mL of dilute aqueous samples separately and incubated at room temperature for 40 min. The same procedure was performed on each diluted silver nanoparticle sample. Subsequently, the absorbance of the samples was measured at 415 nm using distilled water as the blank. The total flavonoid content was expressed in µg Quercetin equivalents per 100g (µg QUE/100 g).

2.5.4.3 Determination of Total Phenolic Content (TPC). Folin-Ciocalteu reagent was diluted by adding 10 mL of the reagent with 90 mL distilled water. To 0.5 mL of the diluted aqueous extract samples, a mixture of 2.5 mL of Folin-Ciocalteu reagent and 2 mL of 7% Na_2CO_3 were added. The same procedure was followed for diluted silver nanoparticle samples. The samples were incubated at room temperature for 30 min under dark conditions. The absorbance was measured at 765 nm using distilled water as the blank. The results were expressed in g Gallic acid equivalents per 100g (g GAE/100g).

2.5.4.4 Determination of total antioxidant capacity (TAC). Phosphomolybdenum reagent was prepared by using equal volumes of 28 mM sodium sulfate, 4 mM ammonium molybdate and 0.6M sulfuric acid. To 1 mL of phosphomolybdenum reagent, 3 mL of diluted aqueous extract sample was added.

The samples were incubated at 90°C for 90 min. The same procedure was followed for diluted silver nanoparticle samples. The absorbance was measured at 695 nm by using distilled water as the blank and total antioxidant capacity was expressed as g ascorbic acid equivalents per 100 g (g AAE/100g).

2.5.4.5 Statistical analysis. Microsoft Excel 2013 software was used to generate graphs for total flavonoid of water extracts and silver nanoparticles expressed as Quercetin equivalents, total phenolic content of water extract and silver nanoparticles expressed as gallic acid equivalents, and total antioxidant capacity of water extracts and silver nanoparticles expressed as ascorbic acid equivalents.

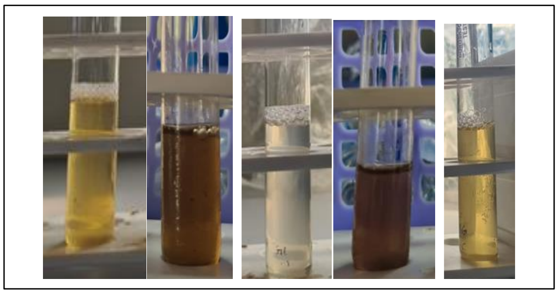
Single factor ANOVA was performed to identify the statistical significance difference between silver nanoparticles and aqueous extracts in total flavonoid content, total phenolic content, and total antioxidant capacity. Using SPSS, correlation tables were generated and correlations between antioxidant assays were identified with Pearson coefficient.

3. Results

3.1 *Identification of* AgNp.

In this study, AgNps were synthesized from *Citrus reticulata* leaf extract by using the green synthesis method to access antioxidant properties. This method acts as a reliable, sustainable, and eco-friendly procedure and aims at reducing the generated waste. In several studies, researchers have shown that mandarin leaves are rich in phenolic compounds, vitamin C, and flavonoids which significantly contribute to the antioxidant capacity.[20]

Synthesis of AgNp using plant leaf extract of *Citrus reticulata* was observed visually with colour change from light brown to dark brown (Figure 3). Several studies have reported that AgNp show striking colours from light yellow to reddish-brown due to the excitation of surface plasmon vibrations in AgNp.[21] A wide range of various nanoparticles can be synthesized by changing several factors like incubation temperature, time, pH, and solvent used in nanoparticle synthesis, and also the yield of nanoparticles can be increased by adjusting the above parameters.[22]



(S1) (S2) (S3) (S4) (S5)

Figure 3. Colour change of aqueous leaves extracts. (S1): Heen Naran, (S2): Yaki Naran, (S3): Nas Naran, (S4): Jama Naran, (S5): Maha Naran.

The polarity index of the reaction medium affects the size of nanoparticles. Smaller and spherical-shaped nanoparticles are produced with a higher polarity index while larger nanoparticles are produced with a lower polarity index. Various organic solvents with high polarity are mainly used in the synthesis of silver nanoparticles, such as water, ethanol, methanol, isopropanol, or mixtures containing water and alcohol.[23] Water was used as the solvent due to its eco-friendly and non-toxic nature and also acts as a reducing agent and non-reactive with any components in the plant extract.

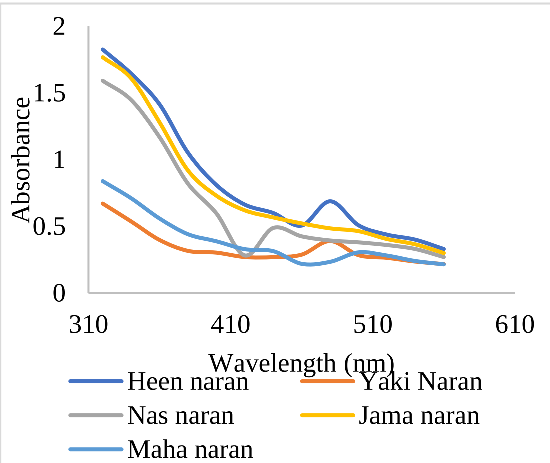


Figure 4. Absorbance at optimization temperature (room temperature) using UV-vis spectrophotometry. Three peaks were observed in heen naran, yaki naran and nas naran.

Colour change from yellowish-brown to reddish-brown indicates the formation of AgNp in samples. UV-Visible spectroscopy was used to further confirm the formation of AgNp. In this study, optimization of AgNp was performed with incubation temperatures such as 60ºC and 90ºC for 15 min, 30 min, 45 min, and 1 hr, and at room temperature for 72 hrs. Higher incubation temperatures were not used as it can degrade the phenolic and other active components in the plant samples.[23] As shown in (Figure 4), the optimized temperature for AgNP formation was at room temperature. AgNPs were not synthesized at 60ºC during any time interval but at 90ºC for 15 min, nanoparticles were formed for two samples: Heen naran and yaki naran. For all five samples, a peak was obtained at 340 nm range when the condition was at 60ºC for 15 minutes. This can be due to the presence of unreacted silver in the solution which can cause an increase in absorbance. Synthesis of AgNp for the highest number of samples was observed at room temperature for 72 hrs with absorption peaks within the 440-480 nm range (Figure 4). Thus, the optimum temperature for the synthesis of AgNPs was considered as room temperature for 72 hrs and these samples were used for further analysis. The formation of nanoparticles took 3 days to complete at room temperature due to the slow bio-reduction process of Ag ions to Ag nanoparticles.[24] In a study by Leenus and his coworkers in 2020, AgNps were synthesized with *Citrus reticulata* leaves at room temperature confirms the results of the current study.[25]

The optical properties of AgNps were analyzed with UV Visible spectroscopy. AgNps have various optical properties such as absorption and emission.[7] The conductivity of nanoparticles can be measured by using the bandgap. The bandgap is the energy difference between the valence band (VB) and conduction band (CB). The minimum energy required for electron transmission from VB to CB is known as the bandgap energy which is specific to each type of nanoparticle. Nanoparticles with bandgap energy less than 3eV are considered semiconductors while nanoparticles with more than 4eV are considered insulators. All three AgNp samples were classified as semiconductors as depicted in table 1. Band gap energies were measured using the following equation,

$$E=\frac{h \times c_{light}}{\lambda}$$

where E= bandgap energy: h= 6.626 x 10^{-34} Js: c_{light}=speed of light= 3 x 10^{8} ms^{-1}: λ= wavelength peak of AgNP synthesis (nm).

Table 1. Classification of silver nanoparticles based on bandgap energies.

Synthesized AgNPs	Band gap energy (eV)	Classification
S1	2.58	Semiconductor
S2	2.70	Semiconductor
S3	2.82	Semiconductor

Bandgap energy is inversely proportional to the size of the nanoparticles. Based on the bandgap energies, AgNPs of S3 is the smallest in size while S1 is the largest of all three AgNPs samples.

3.2 Antioxidant assays

3.2.1 Total Flavonoid Content.

Plants contain polyphenolic compounds which can act as natural antioxidants and function as free radical scavengers and reducing agents.[26] Flavonoids are the most common polyphenolic compounds. Pandey and his coworkers in 2019 carried out research using leaves, fruits, and peels of 3 varieties of citrus plants and showed that leaves of citrus species contain high TPC and TFC compared to citrus peel and fruits.[27] Flavonoid solubility depends on the polarity and chemical nature of the solvent.[28] A study by Khettal and his coworkers in 2016,[18] stated that regardless of the citrus species, TFC and TPC are high in aqueous leaf extract compared to methanolic extracts.

A study by Zhang and others have evaluated the antioxidant capacity of mandarin fruit tissues by determining the TFC and TPC. The highest number of antioxidants were present in the peel when compared to seeds, pulp residues, and juice, with the highest TFC and TPC, suggesting that that the use of fruit waste such as peel is ideal for the eco-friendly synthesis of silver nanoparticles.[29]

TFC was analyzed with $AlCl_3$ colorimetric method. $AlCl_3$ forms acid-stable complexes with the keto group of 4th carbon and either the hydroxyl group of 3rd carbon or 5th carbon of flavanols and flavones. Also, with the ortho-dihydroxyl groups in the A- or B-ring of flavonoids, acid-labile complexes can be formed.[30] The maximum absorption was measured spectrophotometrically at 415 nm. TFC in all AgNp samples was high compared to aqueous extracts. In (Figure 5), there is no flavonoid content in S2. This can be due to the degradation of flavonoids during the extraction procedure. The p-value was 5.41E-05 and the F value was greater than the Fcrit value: 102.4012> 5.987378, indicating a statistically significant difference between AgNp and water extracts. Naz and his coworkers in 2017,[30] conducted research for AgNp synthesis from diluted and concentrated kinnow peel extract, which is a variety of *Citrus reticulata*, and stated that TFC was higher in AgNp when compared to the peel extract.

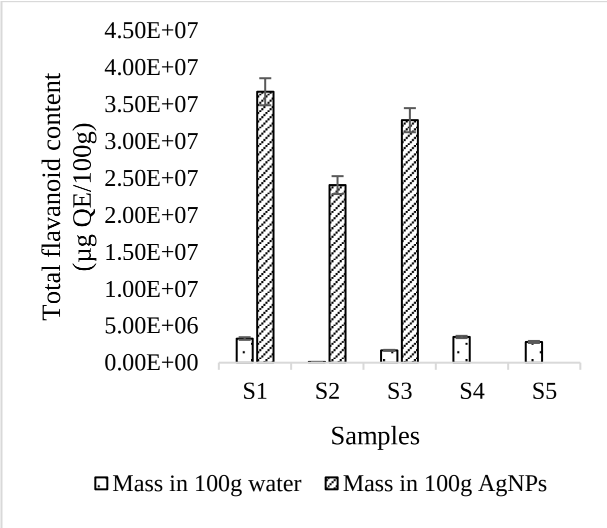


Figure 5. Total flavonoid content of water extracts and AgNPs expressed as Quercetin equivalents (QUE). (S1): Heen Naran, (S2): Yaki Naran, (S3): Nas Naran, (S4): Jama Naran, (S5): Maha Naran.

As depicted in (Figure 5), TFC in AgNp is high compared to the TFC in the water extract. In AgNp extracts, the TFC for S1 and

S3 is the same while S2 has the lowest flavonoid content.

3.2.2 Total Phenolic Content.
TPC was estimated with the Folin-Ciocalteu reagent method.

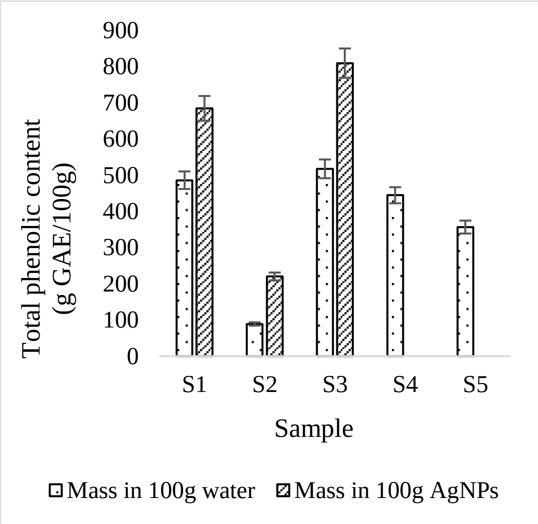


Figure 6. Total phenolic content of water extract and AgNPs expressed as gallic acid equivalents (GAE). (S1): Heen Naran, (S2): Yaki Naran, (S3): Nas Naran, (S4): Jama Naran, (S5): Maha Naran.

In the presence of phenolics, the Folin-Ciocalteu reagent is reduced resulting in the formation of blue oxides of molybdenum and tungsten, with the maximum absorption measured at 765 nm. Even though TPC in all AgNp samples was high compared to their relevant water extract samples, there was no significant difference observed between AgNp and water extracts because the p-value was 0.29185 and F crit value was greater than F value: 5.987378>1.334986. TPC was significantly higher in AgNp when compared to *Citrus reticulata* peel extract.[30] As depicted in Figure 6, AgNp has a high number of phenols when compared to water extract as S3> S1> S2. In the water extract the phenolic content varies as S3>S1> S4 > S5 >S2.

3.2.3 Total Antioxidant Capacity

As depicted in the above (Figure 7), AgNp contained the highest TAC compared to water extracts. When the TAC of AgNp was considered, S3 and S1 were identified with the highest TAC while the lowest TAC was observed in S2. In water extracts, S1, S3, and S4 had the highest antioxidant capacity while S2 was observed as the sample with the lowest antioxidant capacity.

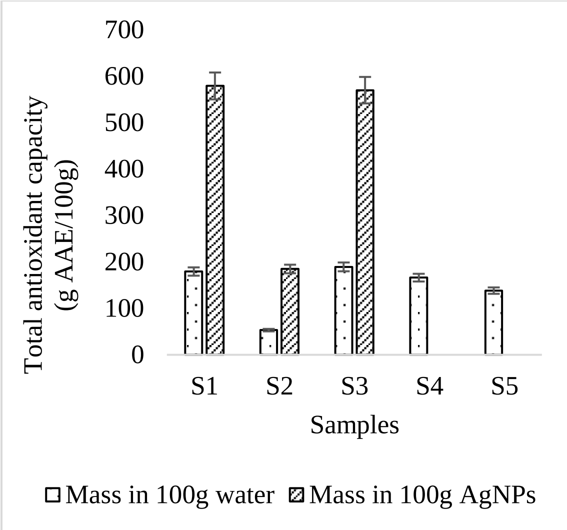


Figure 7. Total antioxidant capacity of water extracts and AgNp expressed as ascorbic acid equivalents (AAE). (S1): Heen Naran, (S2): Yaki Naran, (S3): Nas Naran, (S4): Jama Naran, (S5): Maha Naran.

TAC was estimated with phosphomolybdenum assay. The main principle of the assay is the reduction of Mo (VI) to Mo (V) to form a green phosphate, by the plant extract containing antioxidants with the maximum absorption of 695nm.[31] TAC in all three AgNp was high compared to water extracts. P-value was 0.024558 which indicates a significant difference between AgNp and water extracts. F value was also greater than the F crit value: 8.895163> 5.987378. In a study by Naz and his coworkers stated that TAC was higher in AgNp synthesized from diluted *Citrus reticulata* peel extract than concentrated peel extract and overall, TAC in AgNp was higher than both diluted and concentrated peel extracts.

3.2.4 Correlation
As shown in (Figure 8), the R-value between TAC and TPC was 0.995 which indicates a strong positive linear correlation while the R-value between the weak between TAC and TFC was 0.164, which indicates a weak positive

linear correlation. A weak positive correlation was also observed between TFC and TPC assays with an R-value of 0.131.

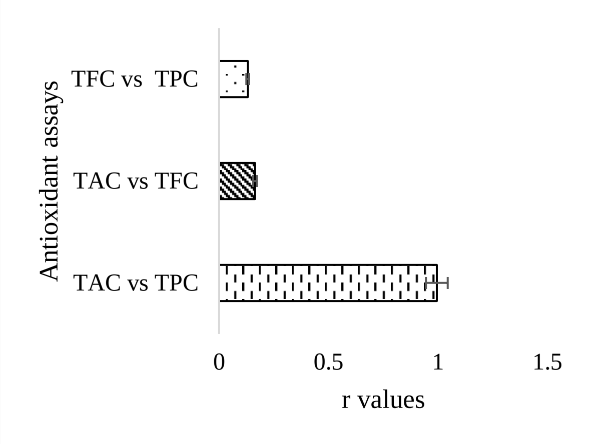


Figure 8. Correlation between antioxidant assays.

Pearson correlation analysis was performed to determine the association between TFC, TPC, and TAC. A higher positive correlation was observed between TAC and TPC while a weak positive linear correlation was shown between TAC and TFC. This can be due to the presence of phenolics as the major component contributing to TAC in *Citrus reticulata leaves.* A study by Khettal and others in 2016,[18] showed that regardless of the solvent used in extraction, the polyphenols in *Citrus reticulata* leaves contribute to 87% of the antioxidant ability. A weak correlation between TAC and TFC indicates that various phenolics and phytochemicals such as tocopherol, ascorbic acid, and other pigments contribute to TAC other than flavonoids. A study by Yang and his coworkers in 2018,[28] showed that flavonoid contribution is less for antioxidant activity in Citrus peels. A weak positive correlation was also observed between TFC and TPC assays due to less flavonoid contribution to polyphenol content in mandarin leaves.

4 Conclusion

In conclusion, AgNps were synthesized from three varieties of *Citrus reticulata* leaves by green synthesis at RT for 72 hrs. The particle size of AgNp was around 40 nm which was analyzed with SEM and bandgap energies. All AgNp samples showed semiconducting properties. The antioxidant property of AgNp was analyzed with TFC, TPC, and TAC antioxidant assays. TAC and TPC showed higher correlation while the lower correlation was in TAC and TFC, also in TFC and TPC. Based on the results, it can be concluded that mandarin leaves are a good source of naturally occurring antioxidants and can be applied in various fields as an eco-friendly, cost-effective, and non-toxic method.

Acknowledgements

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