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Green synthesis of silver nanoparticles using *Oryza sativa* leaves extract and analysis of antioxidant property

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Abstract

Oxidative stress by free radicals can damage the intracellular compartments leading to a large variety of diseases. A therapy for these diseases can be identified by preventing oxidative stress through antioxidants. Plant mediated nanomaterial synthesis is a revolutionary method for extracting natural antioxidants due to the eco- friendly nature and cost-effectiveness. Silver (Ag) plays a vital role in formation of nanoparticles from plant extracts. In this study, the silver nanoparticles (AgNPs) were synthesized through water extracts of five varieties of Sri Lankan traditional *Oryza sativa* leaves include in 'Paduwas Siwuru', 'Herath Banda', 'Madathawalu', 'Mutu Samba', and 'Murungakayen'. This method allows AgNPs formation following an optimization process, which was carried out for 15, 30, 45, and 60 minutes at 60 °C and 90 °C. The formation of AgNPs was indicated by the color change of the water extracts and further characterized using a UV-vis spectrophotometer. It was further confirmed by determining the morphology analysis of AgNPs using a SEM. The observed AgNPs were spherical shaped, and the size were around 40 nm. The band gap energy was measured to confirm the nanoparticles were semiconductors. The AgNP samples were subjected to the characterization of antioxidant properties. Thus, Total Antioxidant Capacity (TAC), Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) assays were performed. It was demonstrated a higher significant for TPC and TAC and lower significant for TFC over the respective water extracts. Comprehensively, the *Oryza sativa* leaves can be used as an effectual way to find the therapeutic approaches for free radical-induced diseases.

Key words: *Oryza sativa*, silver, nanoparticles, Antioxidant activity

Introduction

Atherosclerosis, cardiovascular diseases, inflammatory diseases, cancer, and aging are associated with the damaging of biological molecules such as DNA, proteins, lipids and changing their structural and functional features. This is mainly due to oxidative stress.[1] An imbalance between the generation and scavenging of free radicals leads to oxidative stress. The free radicals bind with biological molecules in a healthy human cell by electron pairing when they exceed the scavenging capacity.[2] It results in lipid peroxidation, protein oxidation, and DNA fragmentation leading to multiple diseases. The studies have shown that experimenting with synthetic antioxidants exhibited an adverse effect on biomolecules.[3]

Antioxidants are stable molecules that can donate an electron to an excited free radical and inhibit free radical formation leading to reduce oxidative stress. Research on Vitamin E as a synthetic antioxidant for experimenting reproduction of mice obtained the ability to prevent lipid peroxidation by reducing oxidative stress.[4] There are a variety of synthetic antioxidants such as BHT, phenolic antioxidants, and BHA (Figure 1) which are used in the food, cosmetics, and medical industry. Due to the high-temperature instability and their carcinogenic nature at higher concentrations, studies have been shifted into finding natural antioxidants derived

from plant sources as an alternative to synthetic antioxidants. The strong natural antioxidants such as flavonoids, polyphenols, lignans, quercetin and curcumin (Figure 1) can be mainly found in berries, olives, cherries, and citrus.[5]

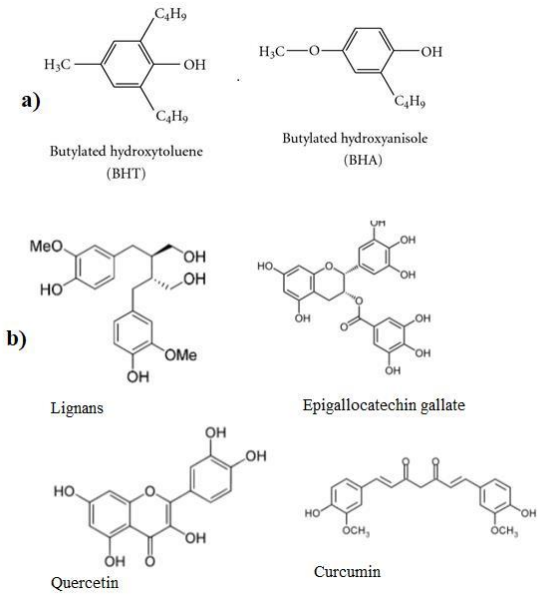

Figure 1. Aromatic structures of antioxidants. a) Synthetic antioxidants.[6] b) Natural antioxidants.[7]

Determination of natural antioxidants is commonly done by nanotechnology-based methods through synthesizing nanoparticles from plant materials. Nanoparticles contain nanoscale dimensions (1-100 nm) and a large surface area to volume ratio. Thus, they have enhanced catalytic reactivity, non-linear optical performance, thermal conductivity, and steadiness to chemicals.[8] There are top-down and bottom-up approaches for synthesizing metallic nanoparticles (Figure 2). The top- down approach involves in fractionation of large-scale particles into nanoparticles which is comparatively costly and time-consuming. Furthermore, the bottom-up approach is more advantageous since it produces nanostructures with fewer defects and homogenous chemical composition. Physical, chemical, and biological are the methods of bottom-up approach. Requirement of high temperature, energy, pressure, and the utilizing toxic chemicals suggested as the limitations for physical and biological methods. Therefore, the technology has shifted into eco-friendly methods. Considering these, biological methods are preferable over physical or chemical methods due to their eco-friendly, non-toxic, and inexpensive properties. The nanoparticles can be synthesized with controlled size, shape, and structure by controlling the reaction conditions including reducing agent, stabilizer, and different synthetic methods.[9]

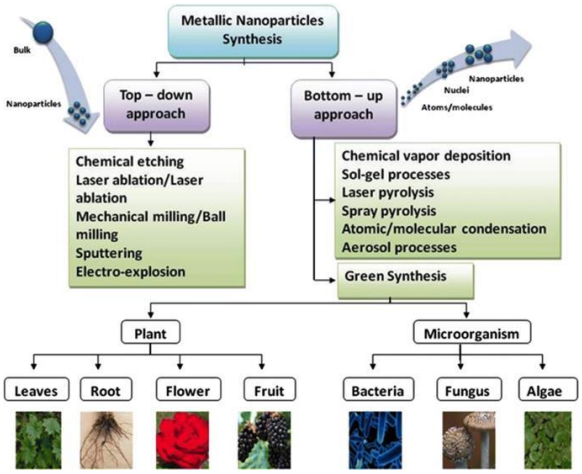

Figure 2. Synthesis approaches to the preparation of metal nanoparticles.[10]

Green synthesis is a plant or microorganisms-based production technique of biological methods. Microorganisms such as bacteria, fungi, and actinomycetes are used for the green synthesis but due to high maintenance and requirement of complex steps including sampling, isolating, and culturing plant-mediated synthesis has preferred.[11]

Generally, plant-mediated synthesis produces metal nanoparticles by the process of bioreduction. As Figure 3, secondary metabolites such as phenols, tannins, alkaloids, and minerals produced during the biological processes of the plant, results in the AgNPs synthesis.[12]

The metallic nanoparticle synthesis is explained by three steps such as activation phase, growth phase, and termination phase.[14] During the activation phase, the metal ions are reduced

into metal atoms by the secondary metabolites available and undergo nucleation. The resulting stabilized metal atoms and metabolite complex give rise to small nanoparticles which turn the separate smaller nanoparticles into larger particles through the coarsening process with increasing thermodynamic stability of nanoparticles during growth phase. This is carried out by Ostwald ripening where the nucleation and growth of nanoparticles and further metal ion reduction occurs. The shape of the synthesized nanoparticles is determined during the termination phase (Figure 4). At the termination phase, the nanoparticles gain a stronger and more energetically favourable consistency, and this process strongly influences the ability to stabilize metal nanoparticles by the plant extract.[10]

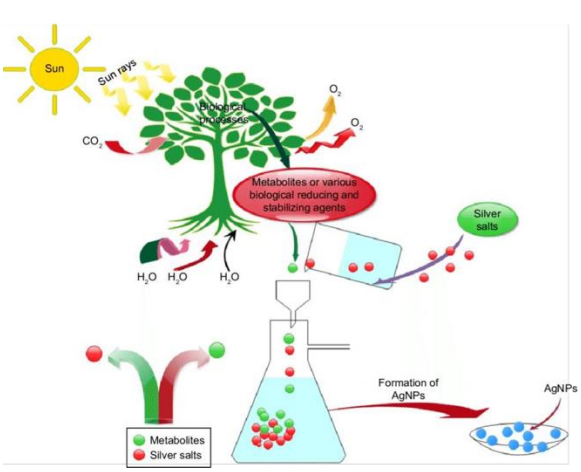

Figure 3. The process describes the formation of AgNP.[13]

For green synthesis method, a suitable plant source should be selected that is rich in metabolites. Therefore, for this study, the plant *O. sativa* was chosen, which is commonly known as rice. It is reported the seeds, husk, and bran of *O. sativa* are a rich source of natural antioxidants, but scientific information is limited for the leaves of Sri Lankan traditional *O. sativa* varieties. According to the studies of the Department of Agriculture Sri Lanka, there are over 2000 traditional varieties can be found in the country. The use of *O. sativa* leaves is reported but for feeding, roof thatching, and broom straws, but and not for biological applications. A study of Adak *et al*., 2020 revealed rice leaves are consisting of high silica content and the AgNP synthesis was avoided earlier.[15] Antioxidants such as phenols, flavonoids, and phytosterols can be found in *O. sativa* leaves.[16] The leaves of the *O. sativa* can be found in many colors as purple rather than normal green color. According to Bianca *et al*., 2021 the reason for the color variation is due to the presence of an increased number of antioxidants.[17] In this study, pigmented Paduwas Siwuru leaves (purple genotype) (Figure 5) were introduced to synthesize AgNPs.

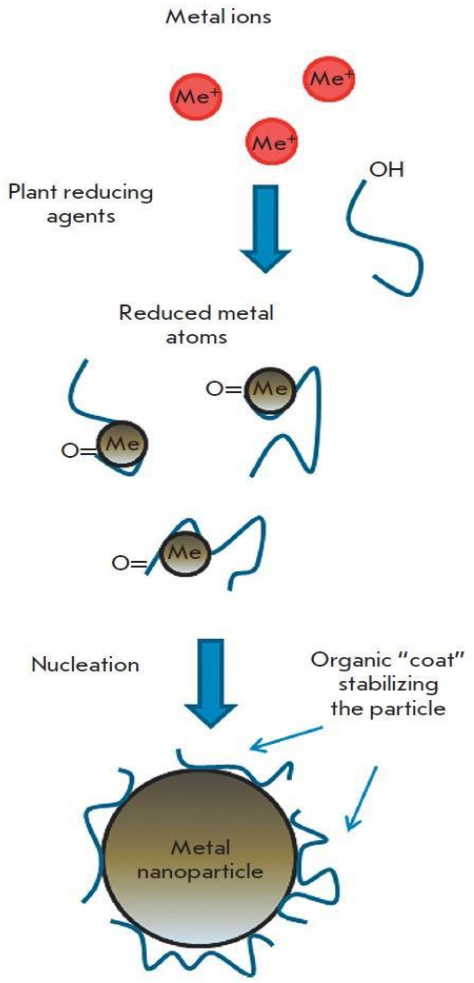

Figure 4. The metal nanoparticle synthesis in a plant extract.[14]

Figure 5. The field where Paduwas Siwuru variety was taken with purple pigmented leaves.

Therefore, this project concerns using leaves of five different Sri Lankan traditional rice varieties include in Paduwas Siwuru, Herath Banda, Madathawalu, Mutu Samba, and Murungakayen, for synthesizing AgNP using water extracts and discovering their antioxidant capacities. To determine antioxidant properties, assays such as TFC, TPC, and TAC and to characterize the synthesized AgNPs SEM analysis will be performed. Hence, the synthesized AgNPs could be explored against human diseases induced by free radicals.

2. Methodology

2.1. Sample collection. Leaves of *Oryza sativa* were collected from the Regional Rice Research & Development Centre, Bombuwala, Sri Lanka. There were five traditional varieties; Paduwas siwuru, Herath banda, Madathawalu, Mutu samba, and Murungakayen.

2.2 Extraction method. The water extract and $AgNO_3$ were prepared according to Srisawat *et al*., 2010 with some modifications. The entire leaf with leaf sheath of each sample was collected from the field and wiped using cotton to remove external contaminants.[18] The samples were air-dried by giving the same conditions for all five varieties at room temperature. 2 g of leaves in each type were measured using the balance and homogenized. 50 mL of distilled water was added to each sample and boiled for 20 minutes at 60 °C. The extracts were filtered using a muslin cloth followed by a Whatman filter paper No. 1. The prepared water extracts were kept at 4 °C until further use.

2.3. AgNP synthesis. A 9 mL of 1 mM prepared $AgNO_3$ solution was mixed with 1 mL of the prepared water extracts of each sample inside the test tubes to synthesize AgNPs. These AgNP samples were subjected to incubation. The absorbance of the synthesized AgNPs were measured using a UV- Vis spectrophotometer for the range of 320-560 nm to identify the nanoparticles. Distilled water was used as the blank for the readings. Samples were kept at 4 °C until further use.

2.3.1 Optimisation. A 9 mL of 1 mM $AgNO_3$ was mixed with 1 mL of each plant extract in separate test tubes. The resulted samples were incubated for 15, 30, 45, and 60 min at 90 $^{\circ}$C and 60 $^{\circ}$C temperatures. The absorbance was measured using a UV- vis spectrophotometer for the range of 320-560 nm to determine the synthesized AgNPs. Distilled water was used as the blank. Samples were kept at 4 °C until further use.

2.4. Dilution. A 1 mL of each plant extract and AgNP samples were diluted with 14 mL of distilled water separately and kept at 4 °C until further use.

2.5. Antioxidant Assays. Diluted samples of water extract and AgNPs were used for the antioxidant assays.

2.5.1. Total Antioxidant Capacity (TAC). TAC was determined according to Rao *et al*., 2010 with some modifications.[1] Phosphomolybdenum assay was performed to analyze TAC. 0.5 mL of prepared solution (0.6 M H_2SO_4, 28 mM Na_3SO_4,

and 4 mM $[NH_4]_6Mo_7O_{24}.4H_2O$ in 1:1:1 ratio) was added to 1.5 mL of each diluted sample separately and incubated at 90°C for 90 mins. This assay was carried out in triplicates. The absorbance was measured using the UV- vis spectrophotometer at 695 nm using distilled water as blank. The results were expressed in g Ascorbic Acid Equivalents per 100 g (g AAE/100 g).

2.5.2. Total Phenolic Content (TPC). Determination of TPC was conducted as per the method in Srisawat *et al*., 2010 with some modifications.[18] Folin- Ciocalteu assay was executed to analyze phenolic content. 0.2 mL sample was added to the mixture of 1 mL of 10% Folin- Ciocalteu reagent and a 0.8 mL of 7.5 % Na_2CO_3 solution. This assay was carried out in triplicates. The samples were incubated by giving dark conditions for 1 h. The absorbance of the incubated samples were measured using UV- vis spectrophotometer at 750 nm using distilled water as the blank for readings. The results were expressed in g Gallic Acid Equivalents per 100 g (g GAE/100 g).

2.5.3. Total Flavonoid Content (TFC). TFC was analysed along with several modifications. An aluminium colorimetric method was performed to analyze TFC. 1.25 mL sample was mixed with 0.15 mL of 10 % w/v $AlCl_3$ and 0.075 mL of 5% $NaNO_2$ and incubated for 30 min at room temperature. The assay was carried out in triplicates and the absorbance was measured using the UV- Vis spectrophotometer at 510 nm. Distilled water was used as the blank for the readings. The results were expressed in µg Quercetin equivalents per 100 g (μg QE/100 g).[18]

The graphs that indicate absorbance vs wavelength for each assay (Total Antioxidant Capacity, Total Phenolic Content, and Total Flavonoid Content) were analyzed using Microsoft Excel software. The optimization of AgNPs was carried out both at 60 °C and 90 °C for 15, 30, 45, and 60 min by using Microsoft Excel software. One-way ANOVA tables were performed to obtain statistical information of the samples using Microsoft Excel software. The correlation of TPC vs TFC, TPC vs TAC, and TAC vs TFC were analyzed by using IBM® SPSS® software.

3. Results and Discussion

There are different approaches such as medical, pesticides, and food industry that have been occupied with the parts of *O. sativa* include in the husk, bran, and seeds by synthesizing nanoparticles due to antioxidant activity.[19] But there are limited researches that have been carried out on synthesizing nanoparticles using leaves of Sri Lankan traditional varieties of *O. sativa*. Since the leaves of this plant are not used for any purpose other than being used as straws, it may have various biological properties. Hence, this study was carried out to synthesize AgNPs using *O. sativa* leaves extracts and determine their antioxidant capacity.

To extract the bioactive compounds of *O. sativa* leaves, water was used as the solvent. Despite the other solvents of extraction including alcohol, chloroform, ether, and ionic liquid,[20] water is the most polar, nontoxic, and non-flammable solvent. It allows the dissolution of large varieties of polar organic compounds such as phenols, flavonoids, and phytochemicals.[21] To concentrate the extracts, 60^{o}C temperature was used to avoid hydrolysis and degradation of the samples. It was observed by the study of Ngo *et al*., 2017 the highest extractable solid was obtained by absolute methanol followed by ethanol, methanol, and acetone.[22] The results of water extract were determined as half of the extractable solids compare to the absolute methanol. It also suggested that a yield with a high level as possible when using 50% (v/v) water with ethanol, methanol, and acetone.

Ag is a toxic and strong antibacterial that can damage the cell structure, growth, and metabolism. It inhibits protein synthesis by binding with DNA and proteins of the cell leading to cell death. Nanoparticles can be synthesized using Ag. Zn and Cu are alternative, and the reactivity is higher in Ag than Zn and Cu.[23]

UV-Vis spectrophotometry was used to gain the structure of the AgNPs based on the surface plasmon resonance (SPR). When the AgNPs are excited by a specific wavelength, the conduction electrons undergo collective oscillation which is known as SPR. The SPR peaks of Figure 6 determine the yield and the distribution by height and width respectively. The peaks with shorter wavelengths indicate the decreased size of the AgNP.[24] Since the peaks of the samples centered in 460-500 nm wavelength and the lower height of the peak denoted the increased size and the and the lower AgNP yield respectively. As Makarov *et al*., 2014 reported, the formation of high AgNP yield is possible when increasing the reaction temperature.[14] The ratio of 9:1 of $AgNO_3$: water extract is the most appropriate for achieving hydrodynamic size while the dilution has increased the lead to slow AgNP formation. It also can increase the AgNP yield by increasing the concentration of the extraction. [15]

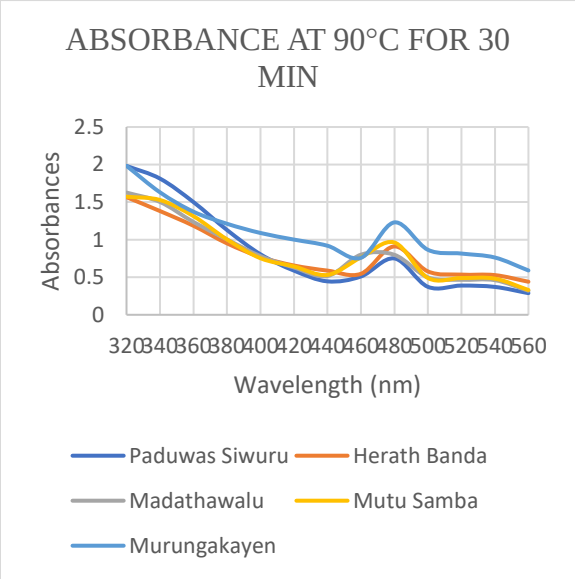

Figure 6. Absorbance at optimization temperature using UV-vis spectrophotometry. The peaks centered in 460-500 nm wavelength for all samples.

The AgNPs were optimized to obtain accepted temperature and duration. It exhibited a color change and the peaks for absorbance, indicating the formation of AgNP at 90°C for 30 min and 45 min for all samples. The color of the samples was changed after incubating the plant extracts with $AgNO_3$ from colorless (Figure 7.a) to yellowish brown due to excited surface plasmon vibration (Figure 7.b). The reduction of Ag atoms into Ag^+ ions on the effect of heat leading to the formation of AgNPs.[25] The study of Adak *et al*., 2020 indicated that between 36-48 h period of incubation leads to constant absorbance of AgNPs and when increasing the temperature improves the ability to the formation of AgNPs.[15] Hence, 90°C was the optimum temperature for the AgNP synthesis and the samples were taken for further study. The samples which were incubated for 48 h at room temperature obtained AgNPs for Paduwas Siwuru.

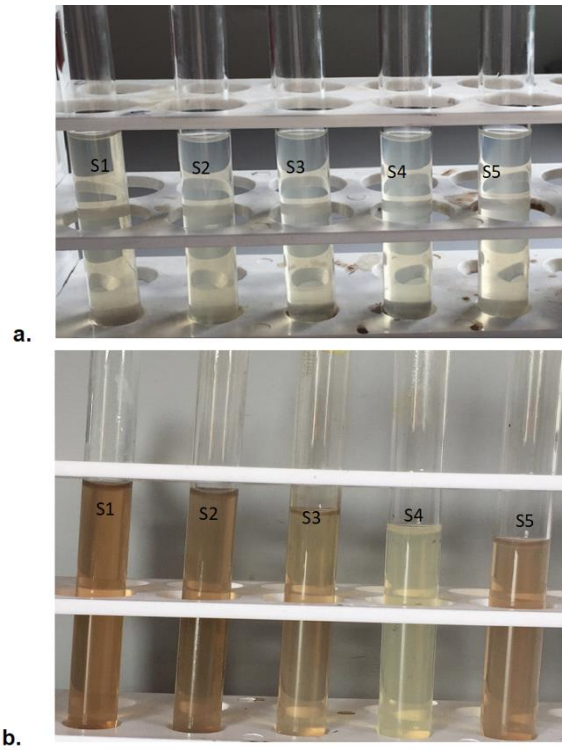

Figure 7. Multi paneled figure (a, b) showing five varieties of *O. sativa* before and after incubation with $AgNO_3$. a) Leaf extracts mixed with $AgNO_3$ before incubation. Absorbance at optimization temperature. b) Leaf extracts mixed with $AgNO_3$

after incubation at 90°C for 30 min. (S1 - Paduwas Siwuru, S2 – Herath Banda, S3 – Madathawalu, S4 – Mutu Samba, S5 – Murungakayen).

According to the Zielinska *et al*., 2009 different reducing agents including $NaBH_4$ can be used to obtain the AgNP with controlled size. Also providing Polyvinyl pyrrolidone (PVP) and polyvinyl alcohol (PVA) as stabilizing agents can protect the synthesized colloids of Ag.[26]

The optical properties were analyzed by performing UV–Visible absorbance spectra. The conductivity of the nanoparticles was determined to characterize them by measuring the bandgap energy. The energy difference between the conduction band and the valance band of the molecular orbitals is indicated by bandgap energy. The transfer of electrons from conductance band to valance band required minimum bandgap energy. When the bandgap <3 eV is referred to as semiconductors while >4 eV is referred to as insulators.[27] According to the calculation the synthesized AgNPs at optimum temperature were classified as semiconductors (table 1).

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

E=Band gap Energy

h= 6.626×10^{-34} Js

c_{light}= Speed of light= 3×10^{8} ms^{-1}

λ= Wavelength peak of AgNP synthesis (nm)

Table 1: AgNP classification according to band gap energies.

Synthesized AgNP	**Band Gap Energy (eV)**	**Classification**
Paduwas Siwuru	2.48	Semiconductor
Herath Banda	2.03	Semiconductor
Mutu Samba	1.78	Semiconductor
Madathawalau	1.87	Semiconductor
Murungakayen	1.25	Semiconductor

It is well known that free radicals play a specific role in various pathogenic expressions. To avoid these free radical activities antioxidants, can act against them. Both scavenging activity and the defence mechanisms perform a vital role for them. The phosphomolybdate method was used to obtain the TAC where the reduction of Mo (VI) into Mo (V) green color complex (Figure 8) was determined by antioxidant compounds at acidic pH which give a maximum absorption at 765 nm. They neutralize the free radicals forming decomposed peroxides.[28]

Figure 8. Reduction of Mo during antioxidant activity.[29]

As Figure 9 indicated, AgNPs of Madathawalu express higher antioxidant capacity while Paduwas Siwuru expresses lower antioxidant capacity. It was observed a significant difference ($p \leq 0.05$) between the water extract and the AgNPs of the leaves samples during this study (Table 2). According to Abeysekara *et al*., 2012 the bran extract of *O. sativa* revealed a significance ($p \leq 0.05$) between water extract and AgNPs for TAC.[30] Also, Rao *et al*., 2010 was revealed the TAC content from methanolic extracts of rice bran showed a significant difference from water extracts and it can be changed by increasing the concentration of the extracts.[1]

Phenols are involved in the inhibition of different oxidative enzymes and the stabilization of lipids against peroxidation. Hence, they are increasingly used in the food industry, to improve the quality of the food. Phenolics compounds obtain a scavenging ability by their hydroxyl group. The modified Folin– Ciocalteu reagent

technique was used to determine the TPC in *O. sativa*.[15]

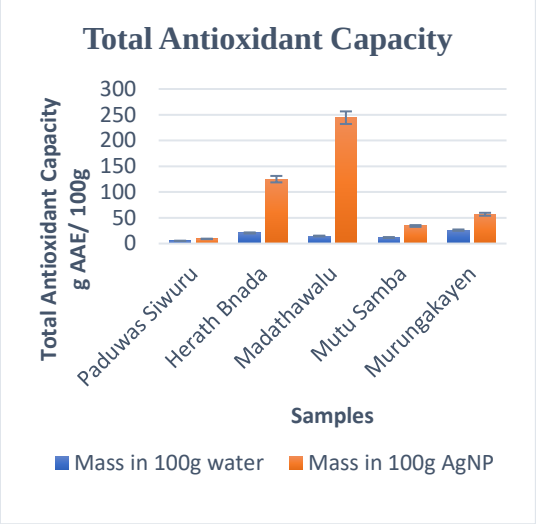

Figure 9. TAC of water extract and AgNP samples expressed as the g AAE/100 g. AgNPs of all five varieties exhibited a higher antioxidant capacity than their respective water extracts.

During the mechanism, phenols act as hydrogen donors and a reducing agent that undergo oxidization. The blue color phosphotungstic acid and phosphomolybdic acid complex were produced after the reduction reaction of the yellow color Folin-Ciocalteu reagent (Figure 10). The conditions are given by the Na_2CO_3 form phenolate by disassociation of phenolic compounds.[31]

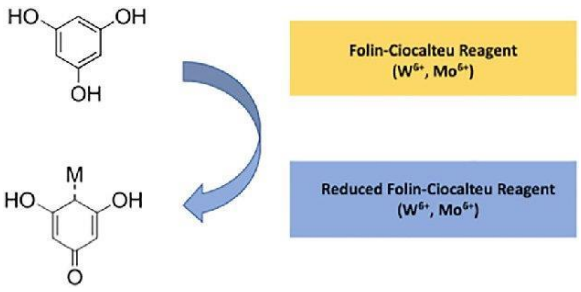

Figure 10. Reduction of Folin-Ciocalteu reagent during phenol oxidation.[32]

The phenol yield of the AgNP was higher than the water extract as given in Figure 11. Among, AgNP samples, the highest phenol content was obtained by Madathawalu whereas the lowest was obtained in Murungakayen. The phenolic yield was comparatively lower and equal in water extracts. A study by Panunto *et al*., 2010 revealed the water extract of rice bran was obtained a significant difference ($p \leq 0.05$) for samples showing the probability to have heat-liable phenols, and concatenation of the initial extract could be affected to the phenolic yield of the leaves extract.[3] It can be increased when increasing the incubating temperature.

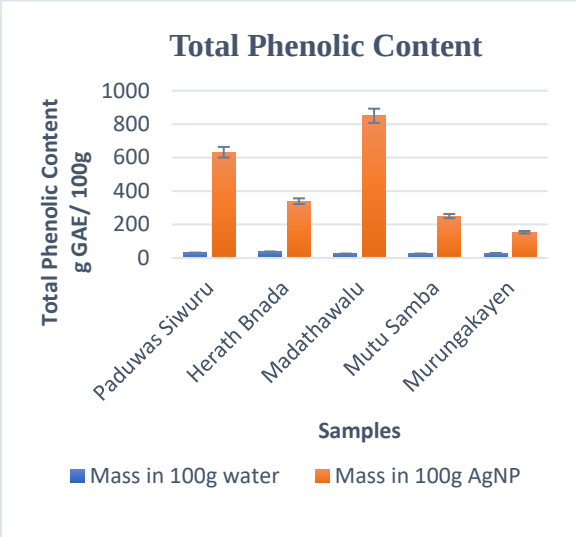

Figure 11. TPC of water extract and AgNPs expressed as g GAE/100 g. The phenolic yield was comparatively much higher in AgNPs than in the respective water extracts.

Flavonoids contribute antioxidant activity as same as phenols. They provide a positive effect on health via anti-inflammatory, antibacterial, antiviral, and anticancer activities. They perform as a scavenger for free radicals and oxidizing molecules.[28] During the mechanism of the Aluminum Chloride Colorimetric method (Figure 12) Al^{3+} reacts with OH groups of flavonoids result in the formation of acid-stable flavonoid-Al^{3+} yellow color complex. It contains flavonoids with the C-4 keto group and either C-3 or C-5 hydroxyl group of flavonoid maximum abruption at 510 nm. Besides, it contains an acid

liable complex with the ortho-dihydroxyl groups in the A- or B- ring of flavonoids. The absorption is not affected by the other phenolic content. The intensity of the flavonoid-Al^{3+} the complex is directly proportional to the flavonoid concentration.[14] The research of Amorim *et al.*, 2008 revealed that using this method can identify flavonoids and tannins and it helps to use plants for pharmacological studies.[33]

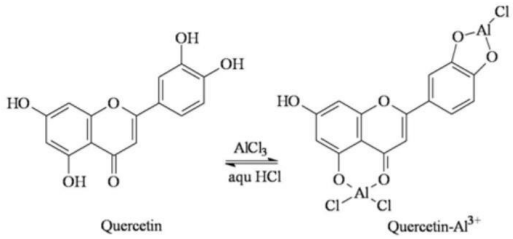

Figure 12. Mechanism of action of Aluminium chloride colorimetric method.[33]

As the Figure 13, the extraction of *O. sativa* samples exhibited comparatively higher flavonoid yield for AgNP samples but indicated as significantly lower ($p \geq 0.05$) than water extracts. The highest flavonoid yield was observed in Herath Banda while the lowest was observed in Murungakayen within the AgNP samples. Despite the pigmented variety, (Paduwas Siwuru) non-pigmented varieties (Herath Banda, Madathawalu, Mutu Samba, and Murungakayen) also exhibited AgNPs. According to Wen, 2015 the high flavonoid content can be observed in pigmented varieties and suggested non-pigmented varieties also gain flavonoids due to genetic variations, growth factors, and errors in the extraction method.[34]

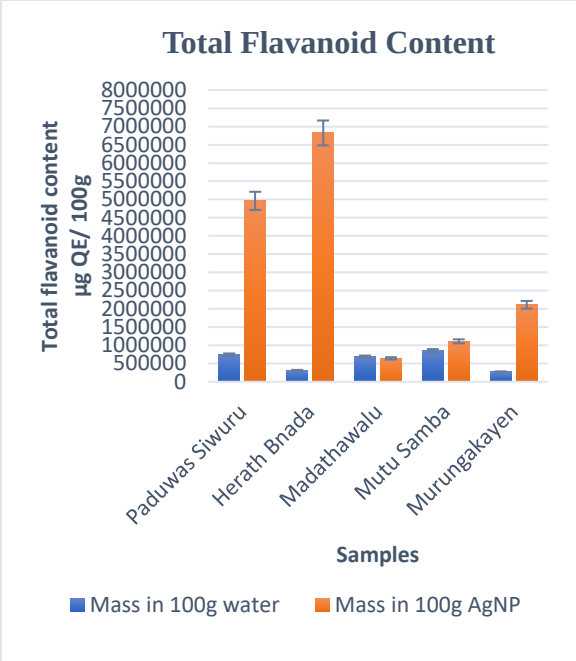

Figure 13. TFC of water extract and AgNP samples determined in g QE/100 g. Flavonoids of AgNPs attain higher compare with the respective water extracts.

Also, the strong correlation of TPC and TAC can be occurred due to antioxidants present in the leaves including phenols and other phytochemicals contribute to the antioxidant activity against reactive oxygen species rather than flavonoids. This can be also provided the weak correlation between TAC and TFC that can be due to the radical scavenging of both antioxidants and flavonoids (Figure 14).

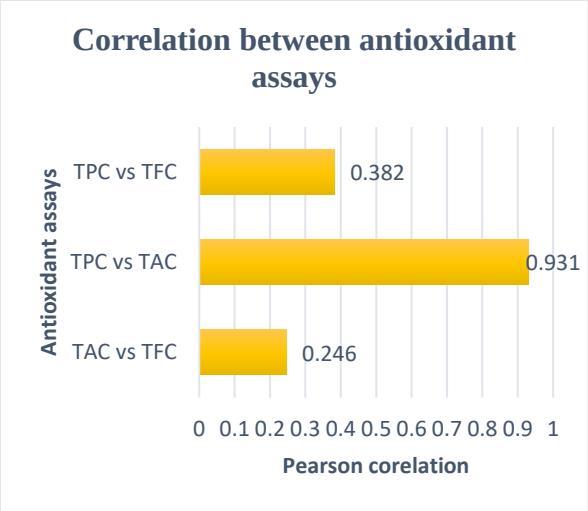

Figure 14. Correlation between antioxidant assays showing a strong positive correlation in TPC and TAC (0.93) while a weak positive correlation in TAC and TFC (0.246) compare with the correlation of TPC and TFC (0.382).

Conclusion

In conclusion, the formation of AgNPs was observed for all five varieties which incubated at 90 °C for 30 mins. The diameter of the AgNPs was obtained around 40 nm and categorized as semiconductors based on the calculated band gap energy.

Antioxidant assays showed decreased flavonoid content and increased antioxidant where the phenolic content for AgNPs over their respective water extracts. Hence, the nanoparticles can be extracted from plant-mediated synthesis methods and can be used for biomedical applications such as discovering drugs for diseases and other industries due to their eco-friendly and non-toxic properties.

Acknowledgements

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