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Original Research Article

Characterisation, Metabolite Profile, and Antioxidant Activity of Silver Nanoparticles Synthesised Using the Algae *Palmaria palmata*

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ABSTRACT

Palmaria palmata contains phytochemicals with potential antioxidant properties, such as polyphenols and mycosporins (MAAs). Phycoerythrin and phycocyanin are pigments found in the red algae species *Palmaria palmata*. It is believed that *P. palmata* derived silver nanoparticles have excellent antioxidant activity. This study aims to determine the morphological, metabolite profile and antioxidant activity of silver nanoparticle compounds synthesised using *P. palmata*. The green synthesis of silver nanoparticles was performed using *P. palmata* as a bioreductant. The morphological characteristics observed were the size and shape of the particles, which were observed using the particle size analyser (PSA) and scanning electron microscopy (SEM). Biochemical characteristics studied were secondary metabolite profiles and antioxidant activity of silver nanoparticle compounds using *P. palmata*. The metabolite profile was tested using High-Performance Liquid Chromatography (HPLC). The antioxidant activity was tested using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method with ascorbic acid as the positive control. The study showed that *P. palmata* AgNPs are spherical, with a size of 185.5 nm. *P. palmata* AgNP contains phenolic group metabolites of gallic acid at a retention time of 2.74 minutes with a peak height of 89.248 mAu and a relative peak area of 98.73%. The antioxidant activity of AgNP synthesised using *P. palmata* possesses an IC_{50} value of 17.113 ± 1.584 ppm, indicating a more robust antioxidant activity compared to the extract of *P. palmata*, which had an IC_{50} value of 33.875 ± 11.238 ppm

Keywords: Antioxidant, characterisation, *Palmaria palmata*, profile metabolite, silver nanoparticles.

Introduction

Nanoparticles are solid colloidal particles with a diameter of 1-1000 nm. They are macromolecular materials and can be used for treatment as a drug delivery system whose active compounds have been dissolved, entrapped, and encapsulated. Nanoparticles are widely used because of their many benefits, including their unique qualities in particle size, surface area, surface reactivity, charge, and shape, allowing them to be used in various industries, including cosmetics, pharmaceuticals, and medicine.[1]Silver nanoparticles are one type of nanoparticle used in cosmetics ingredients. They were chosen because they provide superior material properties for cosmetic products and also act as antimicrobials.[2.]

Nanoparticles from the red algae species like *Kappaphycus* sp., *Gelidiella acerosa*, *Gracilaria dura*, and *Gelidium amansii*, have shown that green synthesis is a more effective and environmentally friendly way to create nanoparticles.[3, 4]

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Green synthesis method for nanoparticles is more efficient than physical and chemical methods. It can be done on a large scale without high temperatures and pressure, and it has a shorter incubation time and process simplicity.[2] In addition, since this method does not involve the use of hazardous chemicals, the synthetic residues that are disposed of do not pose a threat to the environment.[5]

P. palmata is red, and this is due to the pigment content, which is the phycobiliprotein pigment group consisting of r-phycoerythrin and phycocyanin. The dominant pigment of red algae is r-phycoerythrin.[6] Phycoerythrin pigment can act as an antioxidant by donating protons and chelating metal ions on the hydrophobic side.[7] The apoprotein and prosthetic components of the phycobiliprotein structure may stabilise Reactive Oxygen Species. The apoprotein component can reduce hydroxyl radicals and hypochlorous acid radicals through reactions with cysteine and methionine residues. Phycocyanin can stabilise single and double-bond oxygen oxidation.[8] This study aims to determine the characteristics, metabolite profile and antioxidant activity of silver nanoparticle *P. palmata* compounds.

Material and Methods

Plant collection and identification

The red algae *P. palmata* used in this study was obtained from the Saumlaki coast of Maluku, Indonesia, 7°58'48.9 "S 131°20'26.6"E. (Figure 1). It was collected on May, 2023 and identified by S. Prabaningtyas and M. Sapta Sari 2024 at the Department of Biology, Universitas Negeri Malang. *Palmaria palmata* (Linnaeus) F.Weber & D. Mohr 1805.

Other materials

These include a laboratory blender, test sieve with 100 mesh, incubator (Memmert), analytical balance (Sartorius), particle size

analyser (Microtrac, Burlington Canada), scanning electron microscope, TM 3000 Hitachi with SwiftED 3000 X-Ray Microanalysis (Hitachi TM3000, Japan), cuvette, micropipette (Biorad), micropipette tip (OneMed), hot plate (Thermo Scientific), glass beaker (Iwaki), spatula, mortal and pistil, magnetic stirrer, Erlenmeyer (Iwaki), glass funnel, 15 mL centrifuge tube (OneMed), test tube (Iwaki), UV-Vis spectrophotometer (Biorad Smart spec plus), micropipette (Biorad), micropipette tip (OneMed), centrifuge (Thermo Scientific), High-Performance Liquid Chromatography (HPLC) (Thermo Scientific).

Morphological characterization

Morphological characterization was performed using Particle Size Analyzer (PSA), Scanning Electron Microscopy (SEM), and Energy Dispersive X-ray (EDX). The characterization Particle Size Analyser (PSA) aims to determine the diameter and distribution of the nanoparticles.[9] Characterization by a Scanning Electron Microscope (SEM) and Energy Dispersive X-ray (EDX) is used to determine the nature of particle morphology, composition, and distribution of elements contained in nanoparticles.[3]

Preparation of plant material

The plant material (*P. palmata*) was dried under shade for a week, crushed with a mechanical blender and filtered with a 100-mesh sieve to obtain the powder material used for this study.

Nanoparticles Synthesis

A slightly modified green method for synthesising silver nanoparticles using algae was adopted.[11] Briefly, a 10 mL solution of 1 mM AgNO3 was mixed with 1 g of P. palmata powder (1:10 w/v). The mixture was covered with aluminium foil and stirred for four hours at a speed of 400 rpm in the dark, at 28°C, using a magnetic stirrer and hotplate. It was then incubated for 1 hr. A shift in the colour of the solution, from clear yellow to brownish yellow or brownish red, indicates the formation of silver nanoparticles. After that, the solution was centrifuged for 30 minutes at 20°C at 4000 rpm. The pellet or sediment from centrifugation was dried in an incubator at 45°C for 24 hours. The dried nanoparticle powder was then crushed until smooth using a mortar and pestle.[11]

Morphological Characterisation of Nanoparticles

The morphological characterisation of the nanoparticles was performed using PSA and SEM tests. For the particle size analysis test, 1 mg of silver nanoparticle powder was dissolved in 10 mL of distilled water, and a particle size Analyzer (PSA) was used to examine the solution. Parameters determined include particle size, zeta potential value, and nanoparticle distribution. The SEM test was performed by analysing 10 mg of nanoparticle powder using a Scanning Electron Microscope (SEM). The shape and size of the nanoparticles were determined.

Metabolites Profiling Test by High-Performance Liquid Chromatography (HPLC)

The metabolites profile of *P. palmata* extract and AgNP (silver nanoparticle) solution were analysed using a C-18 HPLC column (250 mm x 4.6 mm, 5 µm). The AgNP solution and *P. palmata* extract were tested for the presence of phenolic compounds using a gallic acid solution as standard. The AgNP, extract, and standard gallic acid solutions were prepared at a concentration of 20 ppm. Each solution (20 µL) was then injected into the HPLC system. The mobile phase solvents consist of acetonitrile (solvent A), 9% glacial acetic acid (solvent B), and methanol (solvent C). The mobile phase gradient elution program was 0-10 minutes (5% A: 95% B), 10-20 minutes (10% A: 80% B: 10% C), and 20-30 minutes (20% A: 60% B: 20% C). With a 30-minute analysis period, the flow rate was set at 1 millilitre per minute. Chromatograms at a wavelength of 275 nm were captured using a UV-Vis detector at room temperature. By comparing the sample's peak retention time with standards to identify bioactive compounds.[12]

Antioxidant Test

Antioxidant tests were performed using the DPPH method.[13] Tests were performed on nanoparticle sample solutions (Test Solution I), extract sample solutions (Test Solution II), and positive control solutions. The test solution I (4.5 mL) was transferred into a test tube, followed by 1.5 mL of 0.2 mM DPPH solution. The same was done for Test Solution II and the positive control solution. Subsequently, the mixture was mixed thoroughly and incubated for half an hour at 37°C. The absorbance of the solutions was then measured at 517 nm. The determination was done in triplicates. The absorbance data obtained from each concentration of each test solution and positive control (ascorbic acid) were calculated as a percentage value of antioxidant activity using the following formula (1).[13,14]

$$\%\ inhibition = \frac{Abs\ control - Abs\ sample}{Abs\ control} x\ 100\% \quad (1)$$

Statistical Analysis

All data were computed and expressed as mean ± standard deviation (SD). The data were analysed using SPSS Analysis of Variance (ANOVA). Significant differences between the means were determined using Duncan's multiple-range tests. $P<0.05$ was regarded as significant.

A

B

Figure 1: The algae *P. palmata* (a) dry sample (b) fresh sample

Results and Discussion

The Scanning Electron Microscope (SEM) is an electronic microscopy used to view the surface of an image of a material. In addition, it can provide information related to the chemical composition of a substance, both conductive and non-conductive. This ability makes SEM widely used for research and industrial purposes. This type of microscope uses electromagnetic and electrostatic mechanisms as alternatives to light to control the incoming light and the visualisation of the resulting images. SEM has a large field of view (FOV), can enlarge objects up to one to two million times, and guarantees a much better image resolution than a light microscope. The SEM of the AgNP was observed at 3000x and 4000x magnifications. The size of *P. palmata* extract ranges from 2.2 to 3.3 µm with a spherical particle shape. *P. palmata* powder showed random shapes (Figure 2). The size of AgNPs observed at 3000x and 4000x magnification was 119 nm – 422 nm. The largest size distribution is in the range of less than 400 nm. The results of the size distribution of *P. palmata* AgNPs tested using the Particle Size Analyzer (PSA) are shown in the 100-1000 nm image. The average diameter size of *P. palmata* AgNPs with the highest volume (67.8%) was 185.5 nm, while the diameter size with the lowest volume (32.2%) was 746 nm (Figure 3).

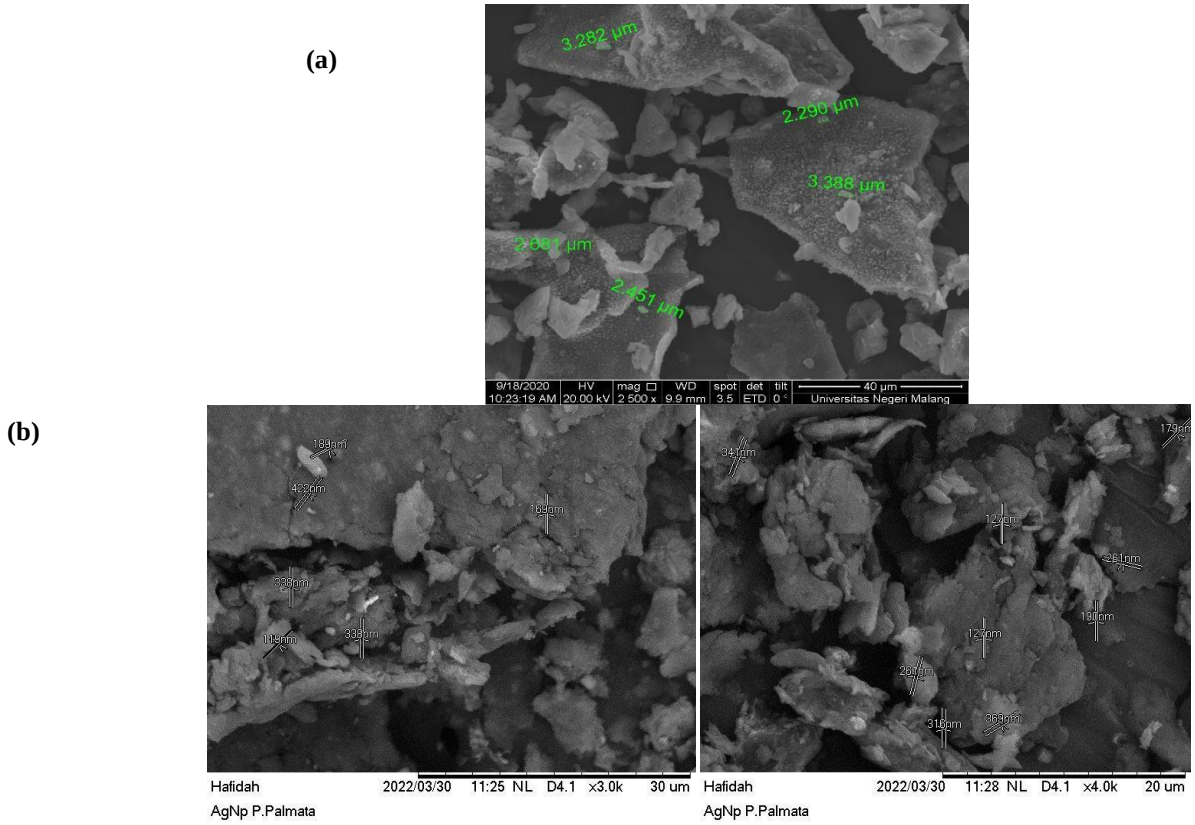

Figure 2: SEM images (a) Powder *P. palmata* (b) AgNP *P. palmata* form using Scanning Electron Microscopy (SEM) 3000x magnification and 4000x magnification

Particles smaller than 1000 nm are included in the nanoparticle category.[13] The recommended nanoparticle size is in the size range of 200-400 nm. The study showed that AgNPs *P. palmata* were successfully formed with the highest average size distribution of 185.5 nm. Variations in particle size and shape are influenced by several factors such as temperature, silver precursor concentration, solution pH, reducing agent, and the method used. Another influencing factor is the aggregation event of the nanoparticle.[16,17] A summary of Particle Size Analyzer (PSA) data for *P. palmata* AgNP is presented in Table 1. The Mean Intensity Diameter (MI) value was 372.0 nm, the Mean Number Diameter (MN) value was 169.3 nm, the MA (Mean Area Diameter) value was 243.7 nm, and the Standard Deviation value was 295.1 nm). The MI value shows the mean intensity diameter calculated from the intensity distribution (signal). It shows the relationship of the detected light signal. The MN value is the mean number diameter value calculated from small particle volume distribution data. The MA (Mean Area Diameter) value displays the particle surface area, which is the mean area diameter value derived from the volume distribution. The width of the observed particle size distribution is explained by the SD (Standard Deviation) value, which does not suggest statistical error in the measurement. The particle size distribution is narrower than the lower the SD value.[18,19,20]

The PDI (Polydispersion Index) value of AgNP of *P. palmata* was 0.628. The function of the PDI value is to indicate the degree of particle size homogeneity.[3] Particle size homogeneity indicates particle stability. The more homogeneous the particle, the more stable it will be. A good PDI value should be < 0.7. Particles with a size < 0.7 have a high degree of homogeneity or are referred to as monodisperse. In contrast, particles with a size > 0.7 have a broad particle size distribution and are less homogeneous. The PDI value of AgNP synthesised using *P. palmata* was 0.628 or < 0.7, so the particle size is classified as homogeneous and stable. [21,22]

Table 1: Result of particle size analysis using PSA.

Data	Value
MI (*Mean Intensity Index)*	372.0 nm
MN (*Mean Number Diameter)*	169.3 nm
MA (*Mean Area Diameter)*	243.7 nm
SD (*Standard Deviation)*	295.1 nm
PDI (*Polydispertion Index Zeta Potential)*	0.628 + 113.9 mv

The zeta potential value of AgNP synthesised using *P. palmata* was +113.9 mv. The function of the zeta potential is to determine the nature and magnitude of the particle charge that interacts with the electrostatics of the nanoparticles dispersed in the dispersion medium. Electrostatic interactions allow aggregation and repulsion to occur.[23] Zeta potential values < -30 mV or >+30 mV have high stability. Low zeta potential values indicate that the particles are susceptible to aggregation caused by Van der Waals forces in particle interactions. The zeta potential value of AgNP synthesised using *P. palmata* (+113.9 mV) was more than +30 mV, indicating the stability of the charge of the prepared particles.[24]

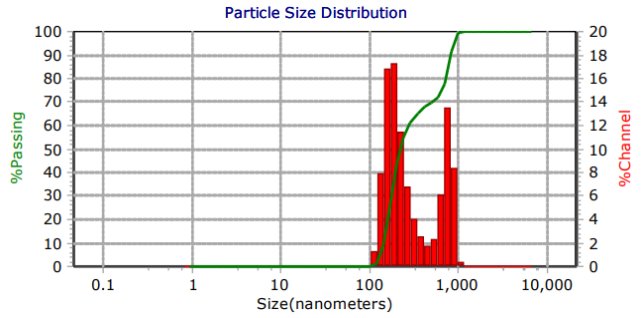

Figure 3: AgNP of *P. palmata* size distribution using Particle Size Analyzer (PSA)

The constituents contained in the AgNP were synthesised using *P. palmata* and were observed with SEM-energy dispersive X-ray (EDX) (Figure 4). The function of EDX is to analyse the elements contained in particles. *P. palmata* AgNP contains Ag, Cl, Ca, C, O, Fe, Si, Zr, Br, and Pt. The existence of these elements functions to ensure the success of AgNP formation. Carbon (C) and oxygen (O) can interact with AgNP metabolites. AgNP formation is influenced by phytochemical components contained in *P. palmata*, which contain carbohydrates, proteins, lipids, pigments (phycoerythrin, chlorophyll a, carotenoids), and phenolic compounds. Several metabolites, such as carbonyl groups of flavonoids, terpenoids, carbohydrates, and phenolics, act as reducing agents which can trigger the bioreduction of Ag+ ions to AgO in AgNPs.[2]

The plant extract and AgNPs of *P. palmata* were analysed using HPLC to detect the presence of secondary plant metabolites using gallic acid as a standard. The result of the metabolite profile testing is shown in Figure 5. The results showed that *P. palmata* extract and AgNP samples contained phenolic compounds in the form of gallic acid. *P. palmata* extract tested using the Folin-Ciocalteu method was reported to contain gallic acid.[25] Gallic acid, also known as trihydroxybenzoic acid or 3,4,5-trihydroxybenzoic acid, is a phenolic compound. It contains only one benzene ring structure with a molecular formula of $C_7H_60_5$. Gallic acid has been reported to be present in most plants. It is biosynthesised from 3-dehydroshikimate catalysed by shikimate dehydrogenase to produce 3,5-didehydroshikimate, which tautomerises to form gallate.[26, 27]

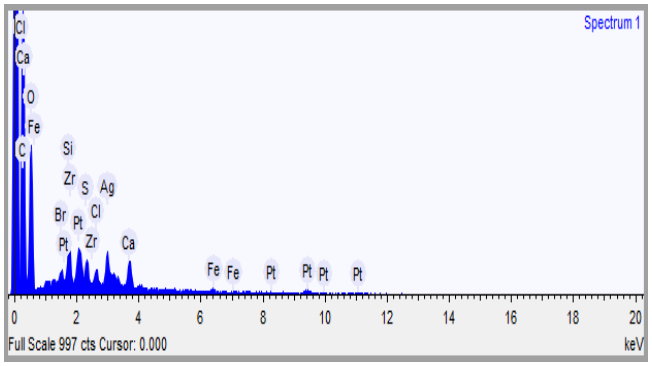

Figure 4: Identification of key elements of *P. palmata* AgNP using energy dispersive X-ray (EDX)

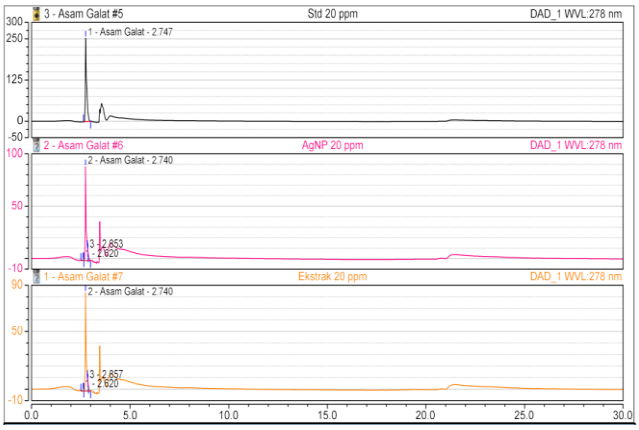

Figure 5: High-performance liquid Chromatography (HPLC) chromatogram of extract and AgNP of *P. palmata* and gallic acid standard.

The content of gallic acid compounds in *P. palmata* extract and AgNP samples was characterised by a compound peak at a sample retention time almost equal to the standard retention time. The definition of retention time is the time of the chromatographic component from injection to peak. Retention time is a valuable starting point for qualitative chromatography because, under specific conditions, every compound has a corresponding retention time. The analyte's interaction with the stationary phase determines how long the retention period is. Longer retention times are associated with stronger interactions.[26] The identification of gallic acid using High-Performance Liquid Chromatography (HPLC) is shown in Table 2. The retention time for the standard gallic acid compound was 2.747 minutes, while the retention time for the *P. palmata* extract and AgNP samples was 2.740 minutes. Therefore, it can be said that the *P. palmata* extract and AgNP samples contained the same compounds as the standard compound. However, a study reported that the peak of gallic acid compounds in AgNP of *Sambucus ebulus* extract appeared at a retention time of 4.3 minutes.[28] The AgNP sample showed a gallic acid peak height of 89.248 mAu and an area of 98.73%, while the extracted sample had a gallic acid peak height of 85.257 mAu and an area of 98.23% (Table 2).

Table 2: High-Performance Liquid Chromatography (HPLC) Identification of extract, AgNP, and gallic standard.

Peak of compound	Retention time (minutes)	Height of Peak (mAu)	Relative Area Size (mAu*min) (%)
Gallic acid (standard)	2.747	252.615	100
Gallic acid (AgNP)	2.740	89.248	98.73
Gallic acid (extract)	2.740	85.257	98.23

This indicated that the amount of gallic acid compound in AgNP synthesised using *P. palmata* was greater than that of the extracted sample. The area value suggests the absorption of the analyte and can be used to determine its concentration. The area and peak height in the chromatogram can be used as quantitative information about a compound.[29] A study of the AgNP synthesised from the leaves of *Lythrum salicaria* L showed an increase in levels of metabolites in the AgNPs expressed, which was greater than those in the extract.[30] Flavonoid and phenolic metabolites are more abundant in AgNP than

in the plant extract. The AgNP has a quercetin content of 0.14 mg/g, greater than that of the extract at 0.05 mg/g. The increase in the content of phenolic compounds in nanoparticles is believed to be caused by the presence of quinoid compounds produced by the oxidation of phenol (OH) groups in phenolics adsorbed on the surface of the nanoparticles.[23] Secondary metabolite plays a vital role in the formation of AgNPs. Carbonyl and hydroxyl functional groups found in plant extracts can act as reducing agents and stabilisers of nanoparticles by preventing aggregation.[31] Direct interaction between Ag^+ ions and algae powder in solution results in Ag^+ ions being reduced to Ag^0. The reduction mechanism involves a phenolic group (-OH), which binds to the Ag^+ ion, which is then oxidised to quinone, producing Ag^0.[32] Gallic acid is reported to act as a reducing agent and stabiliser of silver nanoparticles. Apart from that, gallic acid also has antioxidant, anti-ageing, anticancer, antiviral and antibacterial activity.[27]

The results of the antioxidant activity test of *P. palmata* AgNPs using the DPPH method are shown in Table 3. The AgNP of the *P. palmata* sample had an IC_{50} value of 17.113 ± 1.584 ppm, categorised as a very strong antioxidant. The *P. palmata* extract sample had an IC_{50} value of 133.875 ± 11.238 ppm with moderate antioxidant activity. The ascorbic acid sample used as a positive control had an IC_{50} value of 0.002 ± 0.001 ppm, which falls within the very strong antioxidant activity category (Table 3). The IC_{50} value is categorised into four: very strong antioxidant activity (IC_{50} < 50 ppm), strong (50-100 ppm), moderate (100-150 ppm), and weak (150-200 ppm).[34] A study by Cox *et al*. on the antioxidant properties of edible Irish seaweed showed that the extract had an IC_{50} value of 38.525 g/mL and was included in the very strong antioxidant activity category.[10] The IC_{50} value obtained from this study showed that AgNP synthesised using *P. palmata* possesses very strong antioxidant activity compared to the extracted sample. The higher antioxidant activity in nanoparticle samples may be due to different sizes and volume ratios, which are smaller than the previous particle size. Small particles have a large surface area. Therefore, the number of active sites for scavenging free radicals and inhibiting oxidation reactions is higher.[35, 40] The antioxidant activity of the synthesised nanoparticles was higher because of the size and volume of AgNPs and was also influenced by differences in the phytochemical content.

Table 3: Antioxidant activity screening of AgNP of *P. palmata*, extract, and Ascorbic acid standard.

Sample	Concentration (ppm)	IC_{50} (ppm)	Category
AgNP *P.palmata*	50	17.113±1.584	Very strong
	100		
	150		
	200		
	250		
Extract	50	133.875±11.238	Moderate
	100		
	150		
	200		
	250		
Ascorbic acid	50	0.002±0.001	Very strong
	100		
	150		
	200		
	250		

Polyphenolic compounds such as flavonoids, flavonols, proanthocyanidins, and phenolics have potent antioxidant activity that can prevent cell degradation by free radicals. *P. palmata* has phenolic and polyphenol compounds, so the AgNPs synthesised can act as antioxidants.[33] Phenolic compounds work as antioxidants by donating hydrogen atoms, interrupting the cycle that produces free radicals and averting oxidative stress.[34] The OH group on the aromatic ring is linked to polyphenols' antioxidant activity. Antioxidants scavenge free radicals by transferring their H atoms.[39] AgNPs synthesised using *Sargassum* sp had higher reducing power and antioxidant activity (IC_{50} value of 56.6 µg/mL) compared to the extract samples.[36] The total flavonoid content in *Sargassum* sp and *Gelidium* sp AgNP was higher than in the extract and correlated with the high levels of antioxidants.[37,38] The results of other studies show that the red algae *P. palmata* contain bioactive compounds in the form of Mycrosporine-like Amino Acids (MAAs), phycobiliprotein, and ω-3 Eicosapentaenoic Acid (EPA), which have antioxidant potentials.[7, 8, 10, 33]

Conclusion

The morphological characterisation of the AgNP compound synthesised using *P. palmata* revealed a spherical shape with a mean particle size distribution of 185.5 nm, a zeta potential value of +113.9 mv, and a PDI value of 0.628. The HPLC metabolite profiling showed that AgNP synthesised using *P. palmata* contained phenolic secondary metabolites (e.g. gallic acid). It was shown that there was a retention time of 2.740 minutes, almost the same as the standard retention time of 2.747 minutes. AgNP *P. palmata* produced a higher peak of gallic acid compounds than extract, with a peak height of 89.248 mAu and a relative peak area of 98.73%. The AgNP of *P. palmata* showed potent antioxidant activity with an IC_{50} value of 17.113 ± 1.584 ppm, while that of the extracted sample was 133.875 ± 11.238 ppm, indicating moderate antioxidant activity. Further *in vitro* toxicity and anti-ageing activity testing for cosmetic and other pharmaceutical products is recommended.

Conflict of Interest

The authors declare no conflict of interest.

Author's Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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