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Nitric Oxide Scavenging Activity, Total Phenolic and Flavonoid Content of *Persea americana* Fruit Peel Mediated Silver, Gold and Alloy Nanoparticles

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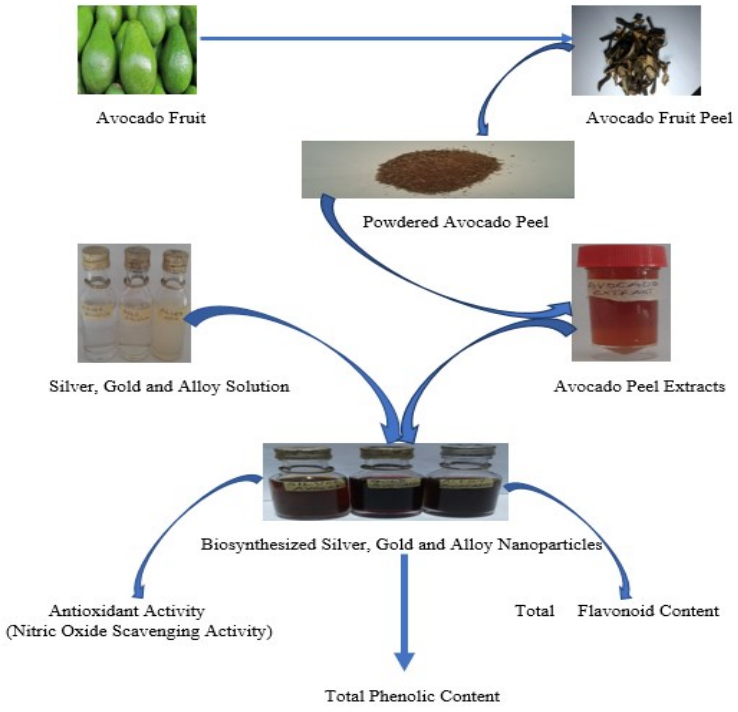
Highlights

- This study established the potential of *Persea americana* for the synthesis and characterization of nanoparticles.
- It also established the potential of synthesized PA-AgNPs, PA-AuNPs and PA-Ag-AuNPs as nitric oxide scavengers.
- The antioxidant activity of the PA-AgNPs, PA-AuNPs and PA-Ag-AuNPs using phenolic and flavonoid contents was established in this study.

Graphical Abstract



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Abstract

Green synthesis of nanoparticles which involves using biological materials such as plant extracts has attracted a great attention of recent. In the current study, *Persea americana* (PA) fruit peel were used to synthesis silver nanoparticles (PA-AgNPs), gold nanoparticles (PA-AuNPs) and alloy bimetallic nanoparticles (PA-Ag-AuNPs) and their antioxidant activity using nitric oxide scavenging assay alongside with total phenolic content (TPC) expressed in microgram gallic acid equivalent per gram of extract (µg GAE/g) and flavonoid content (TFC) expressed in microgram quercetin equivalent per gram of extract (µg g^{-1} QE) were evaluated. The study showed that, the nitric oxide scavenging activity, total phenolic and total flavonoid contents of the synthesized nanoparticles increased in a dose dependent manner as compared to the standard. PA-Ag-AuNPs have the highest nitric scavenging power of 68.5%, followed by PA-AuNPs (66%) and the least value of 48.9% obtained in PA-AgNPs at concentration of 100 µg/ml. PA-Ag-AuNPs and PA-AuNPs showed same value of total phenolic content (250.19 µg GAE/g), while PA-AgNPs gave 249.43 µg GAE/g as least value at concentration of 100 µg/ml. The highest total flavonoid content of 129.73 µg g^{-1} QE was displayed by PA-AgNPs with the least value of 82.10 µg g^{-1} QE obtained in PA-Ag-AuNPs. The results obtained confirmed *P. americana* fruit peel as a potential biomaterial for nanoparticle synthesis which can be exploited for its antioxidant activity. In summary, these fascinating bioactivities exhibited by the synthesized nanoparticles in this study showed their prospect in therapeutic biomedical applications.

1. Introduction

A great number of scientific researchers had documented the pathogenesis of uncountable number of human diseases traceable to the oxidative stress. The building up of highly free radicals such as hydroxyl (OH˙**),** peroxyl (ROO˙), singlet oxygen, hydrogen peroxide, superoxide and nitric oxide (NO) accompanied by unpaired lone of electron bring on oxidative stress. This is a player in the pathogenesis of various physiological conditions such as cellular injury, cancer, aging [1, 2], renal disorders, hepatic, cardiovascular, neurodegenerative [3, 4].

Free radicals originate from different sources within the cell and its environment. Free radicals are generated by aerobic organisms during normal metabolic processes [5]. They are produced in the plasma membrane, cell respiration and within mitochondria membrane through the electron transfer. However, mitochondria has been well established as the leading source of the oxidative damage due to the fact that free radicals such as superoxide can break out of the electron transport chain [6, 7].

However, in order to neutralize the intracellular damage caused by these free radicals, necessitates the use of antioxidants [8]. Antioxidants could be natural or synthetic materials that safeguard or protect the cell from damage caused by free radicals (ROS, free radical, RNS other unsteady molecules) [9]. The synthetic antioxidants viz butylated hydroxyl toluene (BHT) and butylated hydroxyl anisole (BHA) have been documented by several researchers to bring about a lethal effect like cancer on human health [10, 11]. Therefore, the search for the natural compounds with antioxidative potential that has little or no side effect, has been increased in recent years due to their effectiveness, non-toxicity and safety related issues [12, 13].

Plant parts which are not consumed and regarded as waste (underutilized parts) have remained one of the interesting natural sources of antioxidants such as Avocado peel with diverse biological properties [14, 15]. Avocado

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(*Persea americana*) is an indigenous plant to central and southern Mexico and it is consumed around the world. The presence of the bioactive compounds such as carotenoids, vitamins, flavonoid and phenolic compounds in the avocado fruit has made it a functional fruit with great health benefits [16, 17]. Studies have shown that avocado peel are rich in some phytochemicals including phenolic and flavonoid compounds which serve as potential source of antioxidants [16] and capable of preventing several diseases [18, 19].

The persistent exposure to nitric oxide radical give rise to copious carcinomas and inflammatory situation which encompass multiple sclerosis, arthritis, juvenile diabetes and ulcerative colitis. The reaction of the superoxide radicals with nitric oxide leads to the formation of highly reactive peroxynitrite anion ($ONOO^-$) which as well increase greatly the toxicity of the NO [20]. Nitric oxide has been well established to be directly scavenged by the presence of flavonoids [21]. Natural antioxidants are regarded as safe and bioactive agents [22]. The natural source of antioxidants has been regarded to be the main alternatives to the synthetic antioxidants in scavenging the free radicals associated with several side effects [23]. The phenolic compound antioxidant activities are more pronounced as a result of their redox properties. This allows them to function as reducing agents, act as hydrogen donors, and as a singlet oxygen quencher in addition to their metal chelating potential. The antioxidant activity of phenolic compounds render a significant important role in the neutralization of free radicals [24]

In recent decades, green nanotechnology which involves the use of the biological systems like plant extracts for the synthesis of nanoparticles has gained an appreciable recognition. However, in the synthesis of nanoparticles, plant extracts function as reducing and stabilizer agent in the green synthesis of nanoparticles [25]. The bio-reduction process that occurs in the biological synthesis of the nanoparticles is sequel to the presence of the compounds like proteins, sugars, flavonoids, and phenolics among others. These compounds have been reported as the molecules that contribute to the biochemical pathways attributed to the biosynthesis of noble metal nanoparticles [26 -28].

Green nanoparticles therefore spontaneously play an essential role in human life and well-being sequel to their great biomedical applications like antioxidants [29, 30], antimicrobial [29, 30], medical imaging, cancer treatment, treatment of infectious diseases, treatment of neurodegenerative disorder, drug delivery including Parkinson disease and so on [31-34]. Moreover, the robust antioxidant potential exhibited by some nanomaterials has arose interests to develop novel regimens or procedure accompanied by an enriched targeted actions.

Several studies have been carried out to examine the antioxidant activities of the avocado fruit, its part and its synthesized nanoparticles. Adebayo *et al.* [29] evaluated the antioxidant activity of avocado peel extract using DPPH and ABTS assay. Calderon-Oliver *et al.* [35] reported antioxidant activity of avocado peel extract using radical scavenging assay and Oxygen Radical Absorbance Capacity (ORAC) and Ferric Reducing Antioxidant Power (FRAP). Antioxidant activities of seed and peel were also evaluated by Daiuto *et al.* [36].

Therefore, this study evaluates the antioxidants activities of AgNPs, AuNPs and Ag-AuNPs alloy nanoparticles using nitric oxide (NO) scavenging, total phenolic and flavonoid contents of the *Persea americana* peel extract. To the best of our knowledge, this study will be the first time when the evaluation of nitric oxide (NO) scavenging activity, total phenolic and flavonoid contents of the green synthesis of the nanoparticles NPs by

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Persea americana fruit peel extract would be reported.

2 Materials and Methods

2.1 Reagents

All reagents used in this study were obtained from Sigma-Aldrich Chemical, Germany and are of analytical grade.

2.2 Preparation of Plant extract

Persea americana (PA) fruits (Avocado pears) were procured from Aroje Village, Ogbomoso, Oyo State (South-West Nigeria) of latitude (8.113^o N) and longitude (4.2340^o E). The identification was done at the Department of Pure and Applied Biology at Ladoke Akintola University of Technology Ogbomoso with the Herbarium No LHO 584. The fruits were washed, peeled off, air-dried for 4 days under ambient temperature, and then macerated for 10 s using an electric blender (Binatone 1.5 Litre Blender with Grinder BLG-452). Ten gram of the pulverized avocado peel was suspended in a clean bottle containing 150 ml of distilled water and kept in a dark cupboard for 24 h as described by Adebayo *et al*. [29]. The suspension was filtered by using Whatman No. 1 filter paper while the filtrate was centrifuged at 4000 rpm for 20 min. The supernatant was used without further purification.

2.3 Biosynthesis and Characterization Silver, Gold and Alloy nanoparticles

Silver nanoparticles (AgNPs), Gold nanoparticles (AuNPs) and Silver-Gold Alloy nanoparticles (Ag-AuNPs) were synthesized using the avocado fruit peel extracts and characterized as reported by Adebayo *et al.* [29]. Summarily, 1 ml of the sample extract was added to a reaction vessel that contains 24 ml of 1 mM silver nitrate ($AgNO_3$) and Chloroauric acid ($HAuCl_4$) solution. 1 mM Silver ($AgNO_3$) and 1 mM Chloroauric acid ($HAuCl_4$) in the 4:1 (v/v) respectively were mixed together to get silver-gold solution. Synthesis of the alloy nanoparticles were made by adding 1 ml of the sample extract to a reaction vessel containing 24 ml of silver-gold solution[37]. The reaction was completed under static conditions at room temperature (30 ± 2ºC) for 2 h. All the materials were kept at ambient temperature (30 ± 2ºC) to discover the change in colour as a result of nanoparticles formation.

Simultaneous biogenic reductions of silver and gold ions in the reaction mixtures to form nanoparticles were discovered through changes in colour and quantitatively surveyed by measuring the absorbance spectra of the reaction mixtures, using UV-visible Spectrophotometer, Fourier Transform Infrared (FTIR) spectroscopy, Transmission Electron Microscopy (TEM) Micrograph, Energy Dispersive X-ray (EDX) Spectroscopy, X-ray Diffraction (XRD) and Selected Area Electron Diffraction (SAED).

2.4 Antioxidant activities

2.4.1 Nitric oxide scavenging (NOS) activity

The radical scavenging capacities of the synthesized nanoparticles were determined using nitric oxide as described by Green *et al.* [38]. The NO was induced by sodium nitroprusside which was then reacted with oxygen to give rise to nitrite ions, assayed by Griess reaction with slight modification. The nitric oxide (NO) scavenging activity of the synthesized AgNPs, AuNPS and Ag-AuNPs were evaluated by incubating sodium nitroprusside (5 mM, in Phosphate buffer saline) with various concentrations (20, 40, 60, 80 and 00 µg/ml) of the synthesized AgNPs, AuNPs and Ag-AuNPs at 25 °C. Griess reagents of 0.5 mL was added after 2 h and the absorbance was measured by using spectrophotometer at 550 nm. The percentage inhibition of NO was calculated by using the formula:

$$1 - \frac{a_s}{a_c} x100$$

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Table 1: Nitric acid scavenging activity (%) of biosynthesized nanoparticles (AgNPs, AuNPs and Ag-AuNPs)

Samples	Concentrations µg/ml				
	20	40	60	80	100
PA-AgNPs	31.1	32	35.5	44.1	48.9
PA-AuNPs	47	60.7	62	64	66
PA-Ag-AuNPs	34.5	45.5	51.8	57.9	68.5
Ascorbic acid	72.3	74.3	77	78.5	80

Where a_s is absorbance of the sample; a_c is absorbance of control.

2.4.2 Determination of Total phenol content

Total phenol content of the synthesized AgNPs, AuNPS and Ag-AuNPs were measured by the Folin-Ciocaltaeu method as described by Zhou [39]. Exactly 150 µl of synthesized nanoparticles was mixed with 2400 µl of the ultrapure water with 150 µl of 0.25 N Folin-ciocalteu reagents. The mixture was well stirred and allowed to react on stand for 3 min. Then, 300 µl of 1 N sodium carbonate solution were later added to the mixture and were well stirred. The solution was then incubated at room temperature in the dark for 2 h. The absorbance of the solution was measured spectrophotometrically at 516 nm and the results are expressed in mg of GA equivalents per gram of the extract fraction. The phenolic content was calculated as gallic acid equivalents GAE/g of the extracts on the basis of a standard curve of gallic acid ($Y = 0.0026x + 0.2695$, $R^2 = 0.9742$). All experiments were performed in triplicate.

2.4.3 Determination of Total flavonoid content

The total flavonoid of the synthesized nanoparticles was evaluated by a method described by Edewor *et al.* [40]. Exactly 10 ml of 30% (v/v) ethanol was mixed with 0.7 ml of 5% (w/w) sodium nitrite and 1 ml of each concentration of the particles (20 µg/ml, 40 µg/ml, 60 µg/ml, 80 µg/ml and 100 µg/ml). It was stirred for 5 min and 0.7 ml of 10% aluminium chloride (w/w) was added. The mixture was stirred again and then 5 ml of 1 mol/l sodium hydroxide was added. The mixture was diluted with 5 ml of 30% (v/v) of ethanol and left to stand for 10 min. The absorbance of the mixture was then measured at 500 nm using spectrophotometer. Different concentration of Quercetin (Standard) was prepared same way as extract preparation above and the absorbance read as well at 500 nm. This was used to obtain a graph. The concentration of total flavonoid content in the test sample was calculated from the calibration plot ($Y = 0.0005x + 0.0051$, $R^2 = 0.9919$) and expressed as µg quercetin equivalent (QE)/g of the extracts. All the tests were conducted in triplicate and the total flavonoid content determined from the graph by extrapolation. Three independent tests were conducted.

3. Results and Discussion

3.1 Characterization of Synthesized nanoparticles

The biosynthesized nanoparticles (AgNPs, AuNPs, and Ag-AuNPs) showed absorbance (UV-Vis spectrum) values of 455.5, 538 and 540.5 nm respectively, with the existence of -NH_2 and -OH functional groups displayed by FTIR absorption spectra. The broad peaks 3503–3358 and 1735–1750 cm^{-1} correspond to the existence of OH of phenol/alcohol –NH_2 of amide of proteins with C=O of carbonyl groups, respectively, as well as deformation atoms. The broadness is as a result of the overlap of both O–H and N–H bonds stretching of primary (1°) and second-

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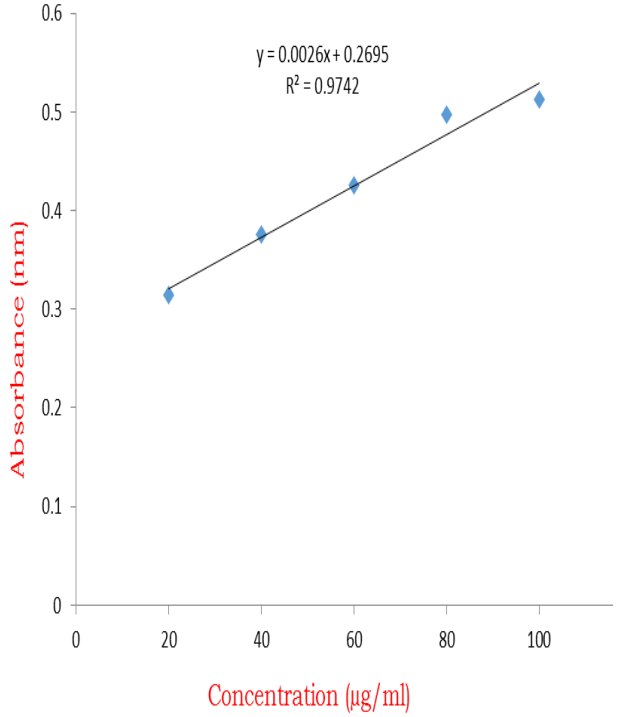

Fig. 1: Total Phenolic Content Standard (gallic acid) Curve

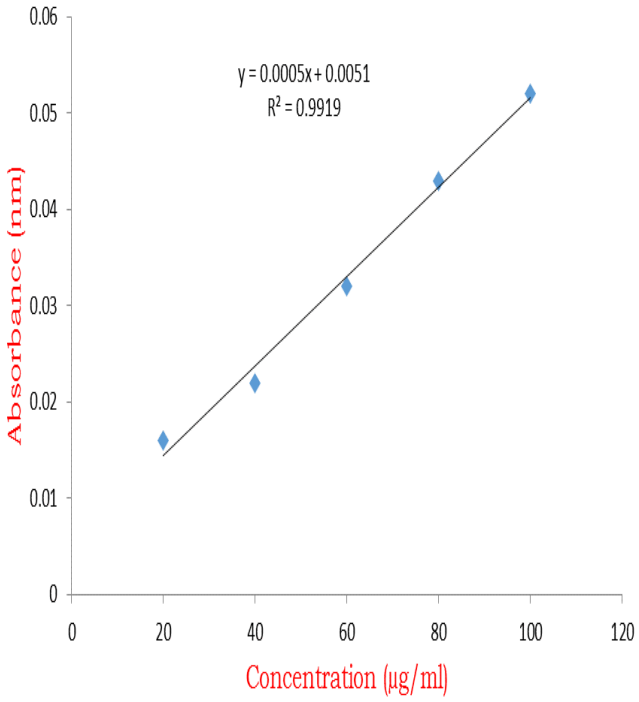

Fig. 2: Total Flavonoid Content Standard (Quercetin) curve

ary (2°) amines. The peaks 1181–1184, 2150–2198 and 2409–2423 cm^{-1} are assigned to the C–N stretch of aliphatic amines, C≡C stretch of alkynes and CO_2 absorption, respectively. The indication is that biomolecules are rich in amine (N-H) and hydroxyl (O-H) groups which accounted for the reduction of the metal ions (Ag^{+} and Au^{3+}) and capping of AgNPs, AuNPs, and Ag-AuNPs [29, 30]. It is therefore evident that proteins present in the avocado fruit peel extract accounted for the capping and stabilization of the AgNPs, AuNPs, and Ag-AuNPs. Furthermore, the TEM images of the biosynthesized AgNPs, AuNPs, and Ag-AuNPs showed that the nanoparticles formed were anisotropic, mostly spherical in shape with some occasional aggregation to form rod-like structure. The size range of 18–80 nm for PA-AgNPs, 16–71 nm for PA-AuNPs and 44–55 nm for PA-Ag–AuNPs [29, 30]. Adelere and Lateef [41] reported the wide use of agrowastes that include peels of fruits to produce different types of metal nanoparticles.

3.2 Antioxidant activity

The antioxidant activities of the synthesized PA-AgNPs, PA-AuNPS and PA-Ag-AuNPs were assessed through nitric oxide scavenging assays. The ascorbic acid was used as the control (standard). The nitric oxide scavenging abilities of the PA-AgNPs, PA-AuNPs and PA-Ag-AuNPs are displayed in Table 1. According to the results, the biosynthesized nanoparticles exhibited robust potentials in scavenging free radicals in a dose-dependent manner. The PA-Ag-AuNPs showed a higher inhibition of nitric oxide (68.5%), followed by PA-AuNPs (66%) and the smallest value of 48.9% was obtained from PA-AgNPs. In the present study, based on nitric oxide scavenging, bimetallic silver-gold alloy nanoparticles (PA-Ag-AuNPs) showed highest antioxidant activity compared to PA-AgNPs and PA-AuNPs. The antioxidative action exhibited in the biological systems are due to the presence of different bioactive molecules such as phenolic and fla-

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Table 2: Total phenolic content (TPC) of the biosynthesized nanoparticles

Samples	Concentrations µg/ml				
	20	40	60	80	100
PA-AgNPs	114.82±0.01^{c}	122.13±0.01^{c}	145.97±0.01^{c}	177.53±0.00^{c}	249.43±0.01^{ab}
PA-AuNPs	154.04±0.01^{b}	157.89±0.01^{b}	165.58±0.01^{b}	223.28±0.01^{b}	250.19±0.00^{a}
PA-Ag-AuNPs	179.43±0.01^{a}	196.36±0.01^{a}	207.89±0.01^{a}	242.88±0.00^{a}	250.19±0.00^{a}

Values are expressed as the mean ± standard deviation. All superscripts indicated a significant difference ($P < 0.05$) between the means ($n = 3$). Values in the same column with different superscripts are significantly different.

Table 3: Total flavonoid contents of the biosynthesized nanoparticles

Samples	Concentrations µg/ml				
	20	40	60	80	100
PA-AgNPs	25.43±0.97^{b}	31.43±0.64^{bc}	49.47±0.94^{c}	76.43±1.28^{b}	129.73±1.27^{a}
PA-AuNPs	15.73±0.69^{c}	34.03±1.02^{b}	64.07±0.99^{ab}	88.10±0.96^{a}	118.07±0.99^{b}
PA-Ag-AuNPs	34.13±0.94^{a}	59.80±0.64^{a}	68.13±0.94^{a}	74.10±0.96^{bc}	82.10±0.96^{c}

Values are expressed as the mean ± standard deviation. All superscripts indicated a significant difference ($P < 0.05$) between the means ($n = 3$). Values in the same column with different superscripts are significantly different.

vonoid compounds acting as scavengers of singlet oxygen and free radicals [42, 43]. The ability of the flavonoids and phenolic compounds to scavenge nitric oxide had been well documented [44]. We can speculate that these constituents might be accountable for the observed nitric oxide scavenging activity. Many pathophysiological processes in human body are caused by excessive production of nitric oxide which resulted mainly by the action of nitric oxide synthase (NOS) and downstream mediation process in human metabolic processes. Hence, nitric oxide production inhibition could be of therapeutic advantages. The inhibition of nitric oxide exhibited by the synthesized nanoparticles is achieved via inhibition of nitric oxide synthase and inhibition of downstream mediators (45). However, we can say the nitric oxide scavenging activity exhibited by the synthesized nanoparticles are due to the presence of the phytochemical constituents in the avocado fruit peel extract which had been previously established for their antioxidant potential [46]. The same constituents might be responsible for the synthesized nanoparticles as capping and stabilizing agents [47-49]. This work agreed with the results reported by Adebayo *et al.* [29] where antioxidant activity of the PA-AgNPs, PA-AuNPS and PA-Ag-AuNPs synthesized from *Persea americana* fruit peel were determined by using DPPH and ABTS assay. This work also agreed with the work documented by Unuofin *et al.* [50] where antioxidant activity of the silver-platinum bimetallic nanoalloy synthesized from *Vernonia mespilifolia* were determined using DPPH and ABTS.

3.2 Phytochemical constituents

Phytochemicals like flavonoids, phenolics, anthocyanins and others belonging to a family known as polyphenolic compounds have become exceptionally popular as sources of natural antioxidants. These compounds have been established to have the ability to donate an electron, act as metal chelators and as singlet and triplet oxygen quenchers [45, 51]. Many reports have documented different natural antioxidant compounds in plants [52-54]. They are regarded as the secondary plant phenols with strong

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antioxidant potential and as the bulk important oxidative components of the plants [55, 56]. The antioxidant activity of phenol is based on their redox properties which play a significant role in scavenging and neutralizing free radicals.

In this present study, the total phenolic results were obtained from a calibration curve fig 1. ($y = 0.0026x + 0.2695$, $R^2 = 0.9742$) of gallic acid (20–100 µg/mL) and expressed in gallic acid equivalents (GAE) per g of sample (Table 2). The concentration of the phenolic compounds increased with increasing concentration of the sample. The phenolic contents of PA-AgNPs ranged from 114.82-249.43 µg GAE/g, PA-AuNPs ranged from 154.04-250.19 µg GAE/g while that of PA-Ag-AuNPs ranged from 179.43-250.19 µg GAE/g. PA-AuNPs and PA-Ag-AuNPs have the greatest phenolic contents (250.19 µg GAE/g) and PA-AgNPs gave the lowest phenolic content (249.43 µg GAE/g) at a concentration of 100 µg/ml.

The phenolic content values obtained in this current study differ slightly compared to those in the literature. This could be ascribed to the presence of different quantity of sugars, carotenoids, ascorbic acid, solvents used, plant age, extraction time, varied geographical location or methods of extraction, which may change the quantity of phenolic [57, 58]. However, the nucleophilic nature of said phytochemicals, which play a very significant role in the reduction and chelation of transitional metals also assist in stabilizing them [59, 60].

The TFC of the biosynthesized nanoparticles are shown in Table 3. The results were obtained from a calibration curve fig 2 ($y = 0.0005x + 0.0051$, $R^2 = 0.9919$) of quercetin (20–100 µg/g) and expressed in quercetin equivalents (QE) per g of sample (Table 3). The concentration of the flavonoid compounds increased with increasing concentration of the sample. The flavonoids content of the PA-AgNPs ranged from 25.43-129.73 µg/g QE, PA-AuNPs ranged from 15.73-118.07 µg/g QE, while that of the PA-Ag-AuNPs ranged from 34.13-82.10 µg/g QE. PA-AgNPs has the greatest flavonoids contents (129.73 µg/g QE) followed by PA-AuNPs (118.07 µg/g QE) and the least value of 82.10 µg/g QE was obtained from PA-Ag-AuNPs at 100 µg/ml. Flavonoids are said to be a secondary metabolite with antioxidant activity, the potency of which rely on the amount and position of free OH groups [61]. At a concentration of 100 µg/ml, the highest value was obtained in PA-AgNPs and the order of increased flavonoid content potential are as follows: PA-Ag-AuNPs<PA-AuNPs<PA-AgNPs. The flavonoids content of biomaterials had been established to be significantly affected by different factors like the genetic diversity, environmental, biological, seasonal and year-to-year variations [62].

4. Conclusion

In the current study, the antioxidant activity of the PA-AgNPs, PA-AuNPs and PA-Ag-AuNPs might be ascribed to their high phenolic and flavonoid content. These compounds are strong antioxidants with high reducing capacity [63]. The ability of avocado fruit peel extracts to reduce silver/gold ions or to form silver and gold nanoparticles by reducing silver and gold ions might be due to the presence of phenolic and flavonoid compounds which are electron donors. Therefore, *Persea americana* are potential sources of natural reducing agents for green synthesis strategies due to the presence of the hydroxyl and carbonyl groups of bioactive compounds which act as natural reducing agent in the formation, capping and stabilizing the nanoparticle synthesis.

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