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Comparative analysis of the *in-vitro* free radical scavenging properties of silver nanoparticle and *Hibiscus Sabdariffa* extract

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Abstract

Free radicals may be attributed to the cause of degenerative diseases and aging. Accumulation of these unstable molecules brings about oxidative stress which leads to oxidative damage. Nanomedicine offers a new approach in the diagnosis and treatment of various infections and diseases. The *in-vitro* free radical scavenging properties of Hibiscus Sabdariffa extract and Silver Nanoparticle was carried out. Nanoparticle was synthesized using biological method and the synthesized nanoparticle was characterized for further confirmation using methods such as UV-Visible Spectroscopy, Scanning electron microscopy (SEM), Transmission electron microscopy (TEM), Energy Dispersive Spectroscopy (EDX) and X-ray diffraction (XRD). The result of the Uv-Visible of the nano extract showed maximum light absorption at a wavelength of 420 nm. The EDX revealed 79.52% Ag (silver) and 20.48% O (oxygen). The microscopic studies gave agglomerated spherical silver nanoparticle with average size of 65.9 nm. The XRD result shows that the silver nanoparticle exhibited a polycrystalline face centered cubic (FCC) structure with an average crystallite size of 65.9 nm. The result of the total antioxidant capacity showed a higher antioxidant capacity of the nano extract compared to the crude extract. The crude extract recorded an IC50 of 0.42 mg/ml for DPPH, 0.38 mg/ml for Nitric oxide, 0.33 mg/ml for hydroxyl radical and 0.67 mg/ml for superoxide radical. The nano extract recorded an IC50 of 6.6 mg/ml for DPPH, 0.72 mg/ml for nitric oxide radical, 0.28 mg/ml for hydroxyl radical and 0.66 mg/ml for superoxide radical. These findings suggest that the nano extract has a unique antioxidant profile compared to the crude extract,

Keywords: Antioxidant; Free radicals; *Hibiscus sabdariffa*; Silver Nanoparticle

1. Introduction

Reactive oxygen species (ROS) and free radicals are unstable molecules that can cause oxidative stress leading to cellular damage, various degenerative diseases and aging.[1]

Free radicals once formed can bring about chain reactions. The first free radicals pulls an electron from a molecule, destabilizes the molecule and turns it into a free radical and this effect can go on and on which will eventually disrupt and damage the whole cell. It may lead to broken cell membranes, change the structure of a lipid. The damaged molecules may mutate and grow tumors as well as changing the DNA code.

All radicals share some common properties due to their unpaired electrons such as having unique species and being present under special and limited conditions. They are highly reactive and very unstable and can donate or accept an electron from other molecules. They behave as oxidants or reductants. Their common examples are superoxide anion

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radical (O^{2-}) hydrogen peroxide (H_2O_2), hydrogen oxide (OH), Nitric oxide radical (NO), peroxynitrite radical (ONOO). Free radicals can be generated internally through the mitochondria, through inflammation, exercise, phagocytosis and peroxisomes while they can be generated externally through the following sources: environmental pollution, cigarette smoke, radiation, drugs and pesticides and ozone layer.

Antioxidants, which neutralize free radicals play a crucial role in maintaining health and preventing oxidative stress-related disorders.[2] In recent years, researchers have explored the antioxidant properties of nanoparticles and plant extracts including silver nanoparticles (AgNPs) and *Hibiscus sabdariffa* extract (HSE).

The importance of green synthesis and the use of plants in the synthesis of nanoparticles cannot be overemphasized. According to [3] and [4], green synthesis eliminates the need for harsh chemicals, making the process more environmentally friendly. The use of plant extracts provides a sustainable and renewable source of materials. Green synthesis method can produce nanoparticles with unique properties such as increased biocompatibility and stability. Plant based synthesis demonstrates the potential of plant extracts as reducing and stabilizing agents [3]. Plant based synthesis methods can provide a simple, cost effective and eco-friendly approach to nanoparticle production [3,4]

AgNPs have shown promising antioxidant activity due to their small size and high surface area [5], while HSE has been traditionally used for its medicinal properties, including antioxidant and anti-inflammatory effects [6]. However, a direct comparison of the in vitro free radical scavenging properties of AgNPs and HSE is lacking.

This study aims to investigate and compare the antioxidant activities of AgNPs and HSE using in vitro assays such as 2,2-diphenyl-1-picrylhydrazyl (DPPH), Nitrous oxide, hydroxyl, superoxide radical scavenging methods and total antioxidant capacity. It will portray the relevance of *Hibiscus sabdariffa* silver nanoparticle as having antioxidant properties and being crucial for disease prevention, diagnosis and treatment. Combining *Hibiscus sabdariffa* with its silver nanoparticle for enhanced antioxidant activity brings about the novelty in the study and the interdisciplinary approach which combines botany, nanotechnology and pharmacology which will lead to potential applications in food, pharmaceuticals and cosmetic industries. The nanoparticle may improve enhanced extract bioavailability and promote standardization of extraction methods. However, the limitations of the study are quite enormous, requiring expertise in multiple fields such as nanotechnology, botany and pharmacology, cost effective and time consuming. It has a limited scope which focused on *Hibiscus sabdariffa* extract and nano extract limiting generality of other medicinal plants and synthesizing nanoparticle in large scale may be quite challenging. It has been the interest of clinicians to develop safe drugs with increased efficacy, high performance and unique properties that conventional medicine could not provide. Drugs that can reach the target sites with 100% bioavailability and increased therapeutic effects, hence the need to explore nanotechnology through nanomedicine to serve in diagnosis, drug delivery and treatment. Silver which is a metallic nanoparticle possesses some medicinal qualities as was described by previous studies which when combined with medicinal plant such as *Hibiscus sabdariffa* will bring a better therapeutic effect. The biocomponents in the medicinal plant will act as in situ reducing and capping agent [7]. *In vitro* free radical scavenging analysis of DPPH, nitric, hydroxy, superoxide dismutase and total antioxidant capacity were used in the method in other to ascertain the antioxidant capacity of the crude and nano extract respectively. The findings in this study will provide insights into the potential of these agents as antioxidants and their potential applications in pharmaceutical and biomedical fields.

2. Materials and Method

2.1. Materials

2.1.1. Extraction Materials

Hibiscus sabdariffa flower, whatman no1 filter paper, muslin cloth.

2.1.2. Chemical /Reagents

Absolute Ethanol, Deionized water, Silver Nitrate (Koch – Light Laboratories England), grinded flower of *Hibiscus sabdariffa*, Chloroform, Diethyl Ether, Hexane all of analytical grade from Merck Germany.

All other chemicals and reagents used were from varied sources and of analytical grade.

2.1.3. Glass Wares

Measuring Cylinder, Filteration Funnel, Cornical Flask, Beakers, petri dishes, Test tubes, Round bottom flasks, Volumetric flasks, pipettes, microplates, curvettes, spectrophotometer cells, etc.

2.1.4. Equipment/ Apparatus

Digital pH meter (Labtech, India), Incubator, UV-visible spectrophotometer model D_{20} (Bausch and Laumb, Germany), Digital spectrophotometer model 390 (Turner®, USA), Rotary microtome, Digital Camera (Minolta, Japan), Hot air oven (Gallenpkam, England), Water bath (Grant, England), Digital weighing balance-Mettler PT 320 (Mettler-Wagen, Switzerland), Rotary shaker (Marrienfeld, Germany), Vaccum dessicators, Deep freezer (Freshpoint FDF-196), Bench centrifuge (Clay adams, USA), Automatic micro-pipettes (TECO® diagnostics, USA), No 1 Whatman Filter paper, Magnetic Stirer (Searchtech Instruments, British Standards), Muslin Cloth, Gc-Ms 2010 QP Schimazu Japan

2.2. Methods

2.2.1. Preparation of Plant Extract

Fresh flower of *Hibiscus sabdariffa* obtained from Eke Ukwu market Owerri, Imo State, Nigeria were picked and removed of debris, dried at room temperature. The dried leaves were ground to fine powder using a mill (BL-335Kenwood) and stored in airtight container. Four hundred (400g) of the powdered flower were soaked in 2.0L, 80% ethanol. The solution was left to stand for 4 days with occasional agitation and later filtered through a qualitative filter paper (Whatman, No. 1). The crude leaf solution was rotor evaporated at 49^0C (Buchi Rotavapour, Japan) and the extract was stored in airtight container and kept in a refrigerator for further analysis.

2.2.2. Preparation of Nano Extract

The method of Jayachandran et al, (2021) was used with slight modifications.

Fifty (50g) of the powdered *Hibiscus sabdariffa* flower were soaked in 250ml deionized water and heated in a water bath at 60 ^{0}C for 1 hour. It was allowed to cool, filtered with a muslin cloth and re-filtered gradually with filter paper. Silver nitrate ($AgNo_3$) (1M) was added to the filtrate and heated again while stirring with a magnetic stirrer for 30 "min". Solution was cooled and centrifuged at 1000 rpm for 15 minutes. The sediment was washed twice with deionized water and evaporated to dryness with a magnetic stirrer at controlled temperature. Sample was kept in the refrigerator for further analysis.

2.2.3. Invitro Free Radical Scavenging Assay

Nitric oxide radical scavenging assay

The scavenging effect of extract on nitric oxide was measured according to the method of [8] as described by [9]. The assay was carried out in 4 ml volume consisting of varying concentrations of extract (0 - 2000 µg/ml) and quercetin stock solution (0-1000 µg/ml) in phosphate buffer (pH. 7.2). To the tubes, 1ml of 5 mM sodium nitroprusside solution in phosphate buffer (pH. 7.2) was added and incubated at 29°C for 2 hours. Nitrite formed was measured by reacting 2 ml of the incubation solution with 1ml Griess reagent (equal volume of 1% sulphanilic acid and 0.1% N-(1-naphthyl) ethylene diamine dihydrochloride (NED). The absorbance was measured in a spectrophotometer at 550 nm. Inhibition of nitrite formation by extract or quercetin was calculated relative to the control which was void of extract or quercetin.

$$\% \, Inhibition = \frac{Absorbance\ control - Absorbance\ test}{Absorbance\ control} \times 100$$

2.2.4. Hydroxyl radical scavenging assay

Hydroxyl radical scavenging ability of extracts was measured by assessing 2-deoxyribose degradation by hydroxyl radicals generated from Fe^{3+} / ascorbate / EDTA / H_2O_2 system as described by [10]. The reaction mixture consisted of deoxyribose (2.8mM), $FeCl_3$ (0.1mM), EDTA(0.1mM), H_2O_2 (1mM), ascorbic acid (0.1mM), KH_2PO_4/K_2HPO_4–KOH buffer (20mM, pH 7.4) and the extract (0 – 3000 µg/ml) in a final volume of 1.0 ml. After incubation for 1 hr at 37^oC, deoxyribose degradation was measured as thiobarbituric acid reactive substances (TBARS) by the method of [11], as modified by [12]. The test set up was further treated with 1.5ml of 20% acetic acid, 1.5ml of 0.8% thiobarbituric acid (TBA), 0.2ml of 8.1% sodium dodecyl sulphate (SDS) and the mixture heated at 100^oC for 1 hr. This was cooled to room temperature and 2ml of trichloroacetic acid added, vortexed vigorously and centrifuged at 3000 rpm for 10 minutes. The absorbance of the supernatant was measured in a spectrophotometer at 532nm wavelength. Inhibition of

deoxyribose degradation which gives an indication of hydroxyl radical scavenging action and iron chelating activity [13] was calculated as follows:

$$\%\ Inhibition = \frac{Absorbance\ control - Absorbance\ test}{Absorbance\ control} \times 100$$

2.2.5. 2, 2-diphenyl-1-picryl-hydrazyl (DPPH) radical scavenging assay

The scavenging activity of extracts for the radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH) was determined according to the method of [14] as described by [15]. The test consisted of 1.0ml extract (0 – 500 mg/ml) dissolved in methanol and mixed with 2 ml of 0.02 mg/ml DPPH (Fluka Chemie, Switzerland) in methanol. The set-up was incubated for 15 min at room temperature and its absorbance measured at 517 nm. Tannic acid (0 – 500 mg/ml) was used as positive control. The radical-scavenging activity was calculated as follows:

$$\%\ DPPH\ radical\ scavenging = \frac{Blank\ absorbance - Absorbance\ test}{Blank\ absorbance} \times 100$$

The scavenging data was fitted into mathematical equations with highest correlation coefficient and used to evaluate IC_{50} (concentration causing 50% inhibition) values of the extract and standard.

2.2.6. Determination of Superoxide dismutase

This assay was carried out according to the procedure of [16]. In the assay, the reaction mixture comprised of 1.1ml extract (0-4000µg/ml) in 50mM Phosphate buffer (pH=7.4), 0.075ml of 20mM L-Methionine, 0.4ml of 1%(v/V0 Triton X-100, 0.075ml of 10MM hydroxylamaine and 0.1ml of the sample was incubated at 30^{o}C for 5min. This was followed by the addition of 80ul of 50uM riboflavin and the tubes were exposed to incandescent light (200watts Lamp) for 10minutes. Thereafter, 1ml of 1% sulphanilamide and 1ml of 0.1% NED were added and the absorbance of the colour formed measured at 543nm.

The SOD radical-scavenging activity was calculated as follows:

$$\%\ \boldsymbol{SOD\ scavenging} = \frac{\boldsymbol{Absorbance_{Control} - Absorbance_{Test}}}{\boldsymbol{Absorbance_{contol}}}$$

2.2.7. Total antioxidant capacity

The total antioxidant capacity of extract was determined by using phosphomolybdenum as described by [17]. An aliquot of 0.4 mL of extract dissolved in methanol (1 mg/mL) was mixed with 4 mL of phosphomolybdenum reagent (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were incubated in a waterbath at 95º C for 90 minutes, cooled and the absorbance was measured at 695 nm. The total antioxidant activity of the sample was determined using a standard curve prepared for ascorbic acid. The total antioxidant capacity of the extract was expressed as mg of ascorbic acid equivalents (AAE)/g of extract.

3. Results

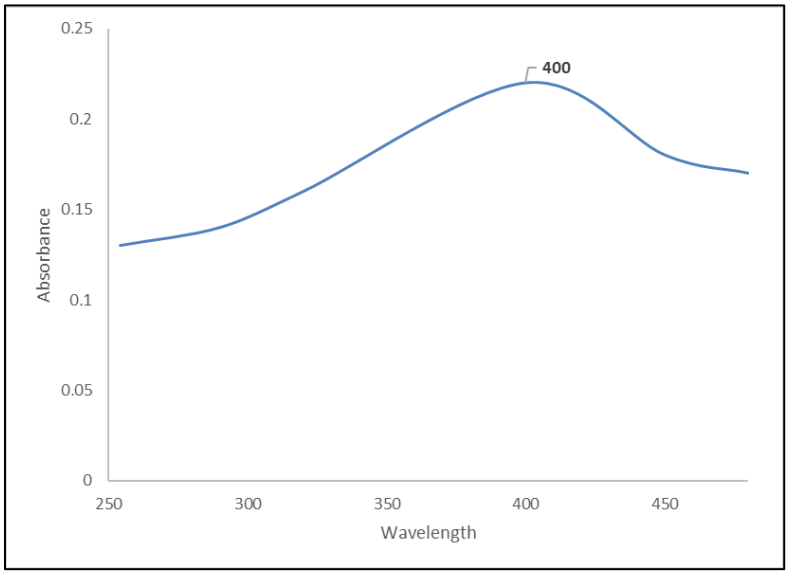


Figure 1 Result of the characterization analysis of *Hibiscus sabdariffa* nano extract using Uv-Visible spectrophotometer

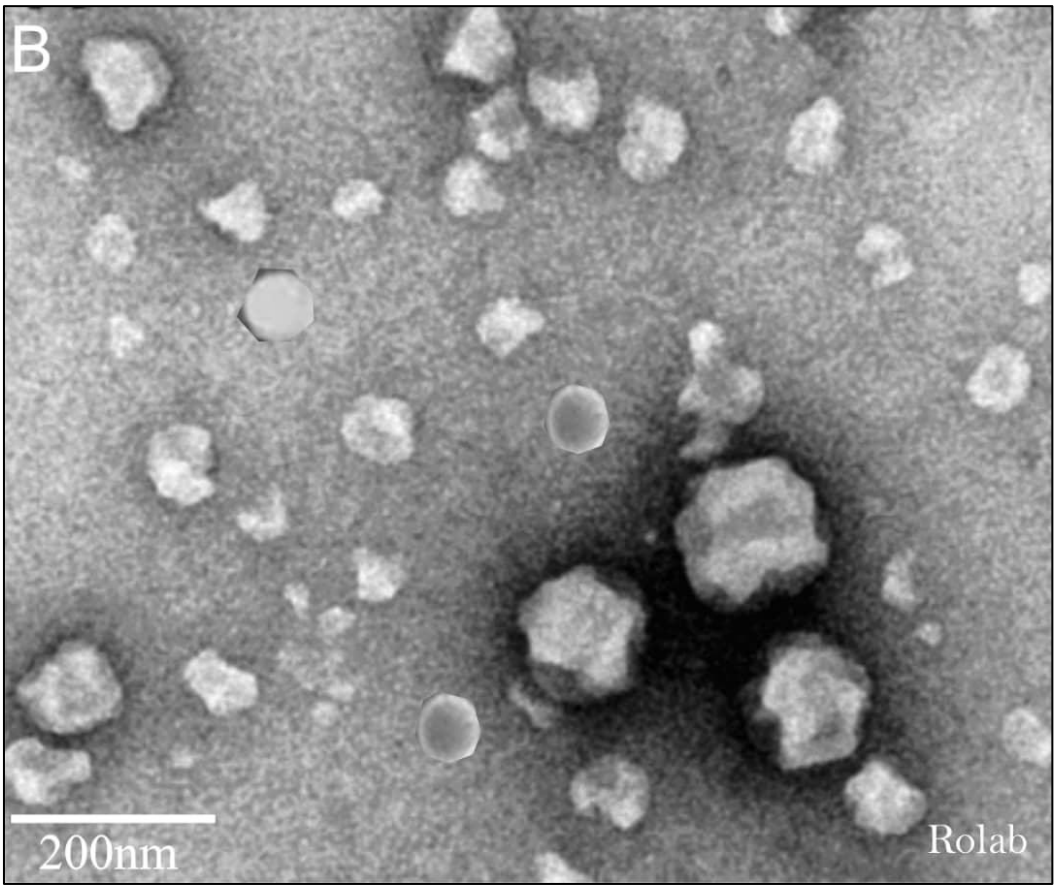


Figure 2 200 nm resolution of TEM analysis of *Hibiscus sabdariffa* silver nanoparticle

Table 1 Histogram table for XRD Result

Size Bin (nm)	Frequency
50 – 60	5
60 – 65	15
65 -70	30
70 75	25
75 – 80	10
80 – 85	5
85 – 90	0
90 – 95	0

The histogram showed a peak around 65 – 70 nm which is close to the average size of 65.9 nm.

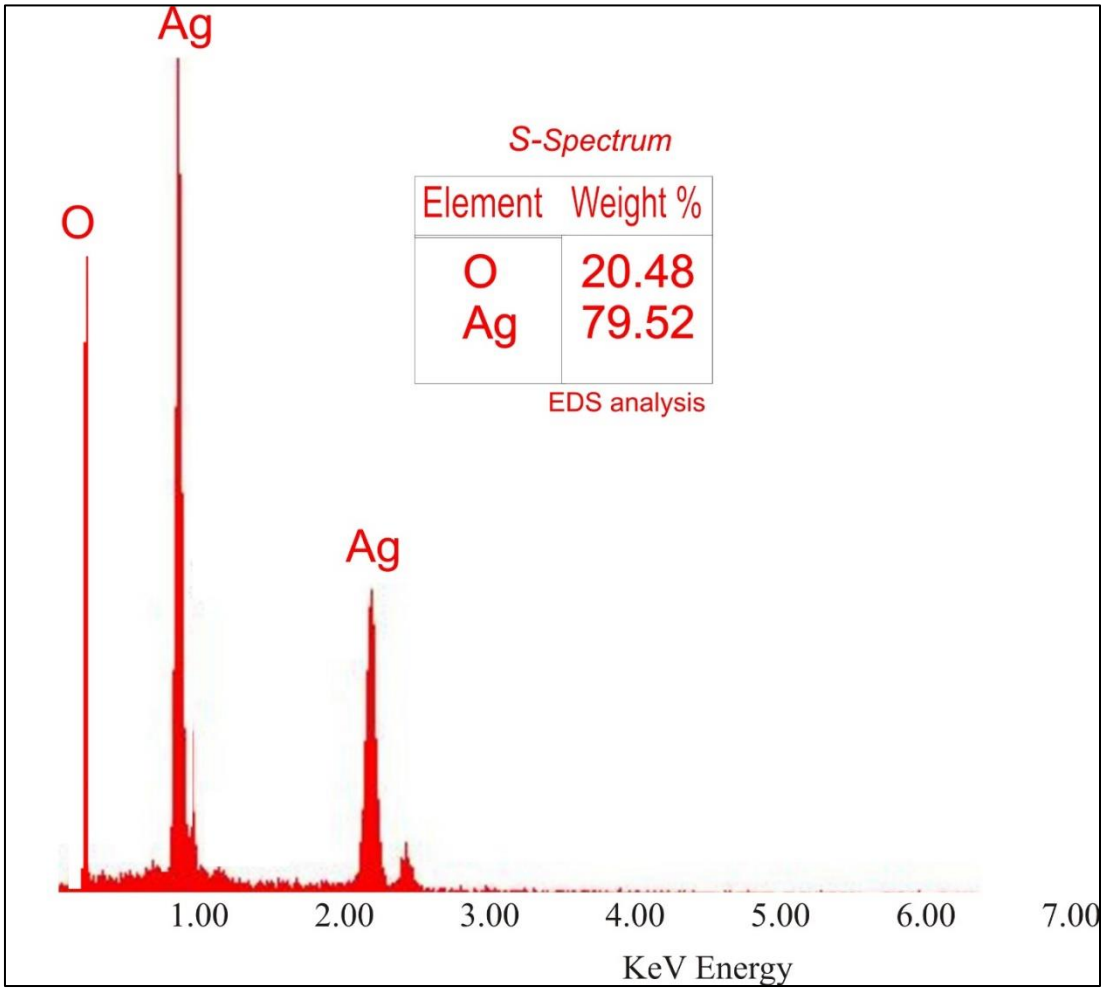


Figure 3 Result of the Energy Dispersive Spectroscopic Analysis (EDX) of *Hibiscus sabdariffa* silver nanoparticle.

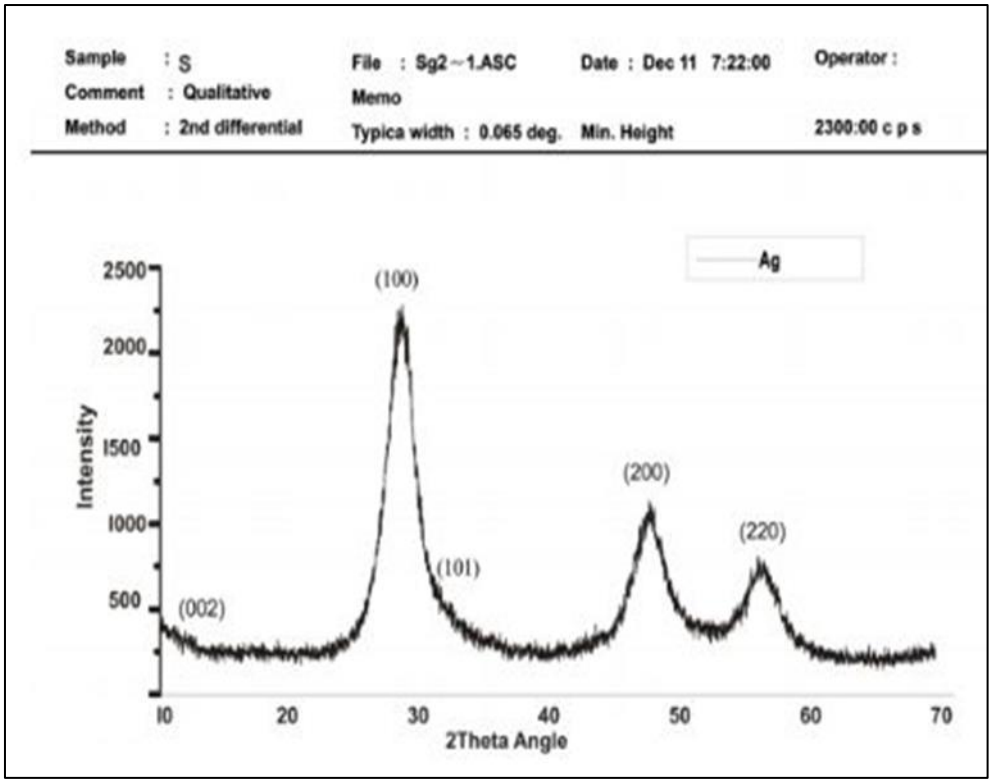


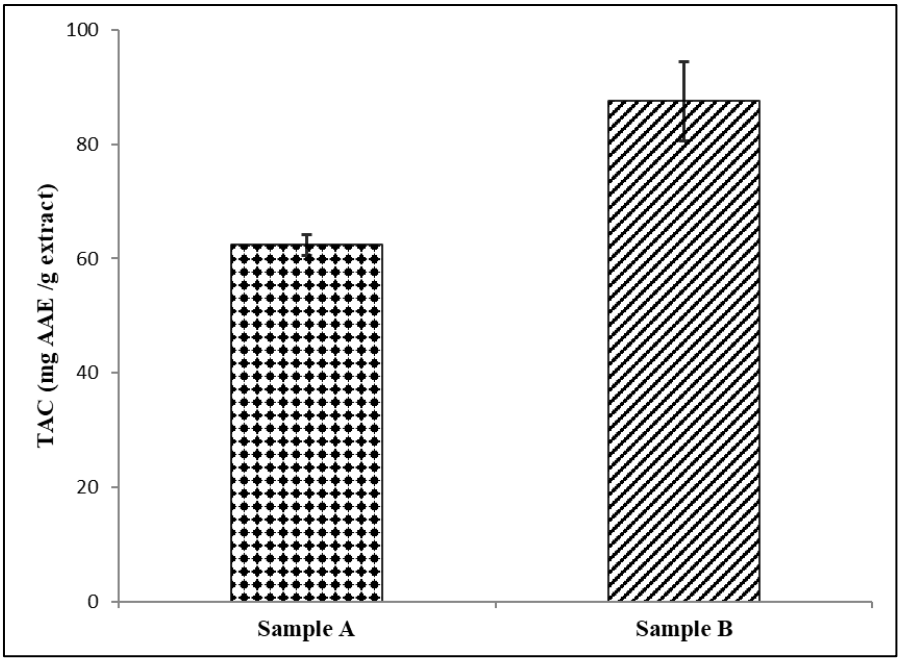
Figure 4 Result of the X-ray Diffraction Graph of *Hibiscus sabdariffa* silver nanoparticle

Table 2 Result of the *in-vitro* free Radical Scavenging Properties of *Hibiscus sabdariffa* crude and nano extract

Parameters	**Sample**	**Threshold Inhibitory concentration (IC_{50}) (mg/ml)**	**Model Equation**	**R^2**
DPPH radical Scavenging	A (Crude)	0.422 ± 13.92^{a}	Sigmoid, 3 Parameter	0.967
	B (Nano)	6.160 ± 49.28^{b}	Logistic, 3 Parameter	0.992
	Gallic acid	0.214 ± 1.71^{c}	Logistic, 3 Parameter	0.992
Nitric oxide radical scavenging	A (Crude)	0.382 ± 4.20^{d}	Logistic, 3 Parameter	0.989
	B (Nano)	0.721 ± 10.81^{e}	Logistic, 3 Parameter	0.985
	Quercetin	0.609 ± 10.98^{f}	Logistic, 3 Parameter	0.982
Hydroxyl radical scavenging	A (Crude)	0.327 ± 3.92^{g}	Logistic, 3 Parameter	0.988
	B (Nano)	0.282 ± 3.10^{h}	Logistic, 3 Parameter	0.989
	Quercetin	0.041 ± 0.78^{i}	Logistic, 3 Parameter	0.981
Super oxide radical scavenging	A (Crude)	0.669 ± 3.34^{J}	Logistic, 3 Parameter	0.995
	B (Nano)	0.664 ± 2.65^{J}	Logistic, 3 Parameter	0.996
	Quercetin	0.088 ± 1.14^{k}	Logistic, 3 Parameter	0.987

Values are mean ± standard deviation of 3 determinations. Mean values with different superscript are significantly different ($p<0.05$)

Table 1: shows the comprehensive result of the invitro free radical scavenging activities of the crude and nano extract of *Hibiscus Sabdariffa,* with different radical parameters. The threshold inhibitory concentration (IC50) expressed in micro gram per mL (µg/ml) was converted to milli gram per mL (mg/mL) for ease of discussion.



Sample A – *Hibiscus sabdariffa* crude extract. Sample B – *Hibiscus sabdariffa* nano extract

Figure 5 Total Antioxidant Capacity of the crude and nano extract of Hibiscus sabdariffa flower.

4. Discussion

4.1. Result of the characterization analysis of the nano extract of *Hibiscus sabdariffa* using Uv-Visible spectrophotometer:

The result of the Uv-Visible of the nano extract showed maximum light absorption at a wavelength of 420 nm as could be seen in fig 1, which shows the graph of the Uv-Visible spectrophotometry. This indicates that the silver nanoparticle exhibits a strong surface plasmon resonance (SPR) at 420 nm. This could be as a result of the collective oscillations of electrons on the surface of the nanoparticles which are excited by the incident light. The initial roughness of the curve could be as a result of solvent or impurity effects which affected the lower wavelength region while the smoothing out of the curve at higher wavelengths suggests that the nanoparticle absorption dominates. This uv analysis result is related to the uv result obtained by [18] which reported that the formation of silver nanoparticle was confirmed by the appearance of a characteristic surface plasmon resonance (SPR) peak in the Uv-Vis spectrum. This Uv result showed a strong surface plasmon resonance which confirmed that there was formation of nanoparticles.

4.2. Result of the Transmission Electron Microscopy of *Hibiscus sabdariffa* silver nanoparticle:

The microscopic studies gave agglomerated spherical silver nanoparticle with average size of 65.9 nm according to fig 2 which shows the 200 nm resolution of the TEM analysis. This indicates that the silver nanoparticles are in the nanoparticle range, which is consistent with the expected size range for nanoparticles. It also suggests that the nanoparticles are relatively uniform in size, which is desirable for many applications. This size of the nanoparticles can also influence their properties such as the optical properties – exhibiting strong surface plasmon resonance (SPR) absorption, which is consistent with the UV-VIS result (420 nm). it can influence catalytic activity because nanoparticle of this size can have high surface areas, making them suitable for catalytic applications. It can influence its biological interactions because nanoparticle of this size can interact with cells and biomolecules, making them suitable for biomedical applications.

4.3. Result of the Energy Dispersive Spectroscopic Analysis (EDX) of *Hibiscus sabdariffa* silver nanoparticle

The EDX revealed 79.52% Ag (silver) and 20.48% O (oxygen) as was shown in fig 3 which gave the energy dispersive spectroscopic spectrum. This shows the presence of elemental silver. The high percentage of silver confirms the presence of silver nanoparticles. The presence of oxygen suggests that the silver nanoparticles are oxidized, possibly due to surface oxidation, silver oxide formation, residual solvent or moisture, possible silver oxide phases, surface modification or less likely contamination from environment or sample handling.

4.4. Result of the X-ray Diffraction Graph of *Hibiscus sabdariffa* silver nanoparticle

The XRD result shows that the silver nanoparticle exhibits a polycrystalline face centered cubic (FCC) structure with an average crystallite size of 65.9 nm as shown in fig 4 which is the X-ray diffraction graph. The nanoparticles show a preferred orientation or texture as indicated by the relative peak intensities and the crystalline structure is relatively defect free with minimal lattice strain. This XRD result provided a complementary information with that of TEM analysis result.

4.5. Result for the *In-vitro* free Radical Scavenging Properties of the crude and nano extract of *Hibiscus sabdariffa*

The crude extract recorded an IC_{50} of 0.42 mg/mL for DPPH while the nano extract recorded an IC_{50} of 6.6 mg/mL for DPPH. The crude extract has higher DPPH radical scavenging activity than the nano extract.

The crude extract recorded an IC50 of 0.38 mg/mL for Nitric oxide while the nano extract recorded an IC50 of 0.72 mg/mL for nitric oxide radical, the crude extract has higher nitric oxide scavenging activity than the nano extract.

The result of the crude extract for hydroxyl radical scavenging activity recorded an IC50 of 0.33 mg/mL for hydroxyl radical while that of the nano extract recorded an IC50 of 0.28 mg/mL for hydroxyl radical suggesting that the nano extract has a slightly enhanced hydroxyl radical scavenging activity which might be due to its smaller particle size and increased surface area.

The result of the crude extract for superoxide radical scavenging activity recorded an IC50 of 0.67 mg/mL while that of the nano extract recorded an IC50 of 0.66 mg/mL suggesting that the nano extract has similar superoxide radical scavenging activity to the crude extract indicting that the nanosization process did not significantly impact this aspect of antioxidant activity as was indicated in table 1.

The result of the total antioxidant capacity showed a higher total antioxidant capacity of the nano extract compared to the crude extract as was shown in fig 5. This could be as a result of: Different antioxidant mechanisms - The nano extract might have a higher capacity to donate electrons or higher atoms, contributing to its higher total antioxidant capacity despite having lower DPPH and nitric oxide scavenging ability. It might be as a result of Synergistic effects: The nano extract might contain a combination of bioactive compounds that work synergistically to enhance total antioxidant capacity even if individual compounds have lower DPPH and nitric oxide scavenging activity. It might be as a result of increased bioavailabilty: the nano extract smaller particle size might enhance its bioavailability, allowing it to interact more effectively with other antioxidants or cellular components, contributiong to its higher total antioxidant capacity. It might also be as a result of different antioxidant assays: The total antioxidant capacity assay might be more sensitive to certain types of antioxidants or mechanisms whereas DPPH and nitric oxide assays are more specific to free radical scavenging. These findings show that the nano extract has a unique antioxidant suggesting its enhanced bioavailability and improved efficacy. The nano extract may be effective against stress related diseases such as cancer, neurodegeneration and inflammation and may be good in prevention of chronic diseases. These results may be compared with the works of [19] in their findings that *Hibiscus sabdariffa* extract exhibited significant antioxidant and free radical scavenging activities which was attributed to its high content of phenolic compounds, flavonoids, and anthocyanins. Their study suggested that *Hibiscus* sabdariffa extract may be useful in preventing or treating diseases associated with oxidative stress. Also another recent publication [20] that silver nanoparticle synthesized from *Livistona chinensis* extract demonstrated potent free radical scavenging activity. A recent work on synergistic effect of *Hibiscus sabdariffa* showed enhanced antioxidant effect when combined, [21], portraying the enhanced antioxidant effect of *Hibiscus sabdariffa* when combined with other medicinal agents such as silver nanoparticles.

5. Conclusion and Recommendation

In conclusion, the comparative analysis of the in vitro free radical scavenging properties of silver nanoparticle and *Hibiscus Sabdariffa* extract revealed significant differences in their antioxidant activities. The nano extract demonstrated enhanced protection against oxidative damage, reduced ability to neutralize lipid peroxidation, reduced potential to mitigate nitrosative stress, comparable ability to combat mitochondrial oxidative stress and enhanced overall antioxidant potential.

These findings suggest that the silver nanoparticle enhanced *Hibiscus Sabdariffa* extract exhibits improved antioxidant properties particularly in hydroxyl radical scavenging and total antioxidant capacity, compared to the crude extract. This nano extract may have potential applications in pharmaceuticals and biomedical fields where enhanced antioxidant activity is desirable. Further studies are necessary to explore its invivo efficacy and potential applications.

Compliance with ethical standards

Disclosure of conflict of interest

The Authors declare no conflict of interest.

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