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Article

Antioxidant Activity and Biochemical Modulation of *Moringa oleifera*-Synthesized Silver Nanoparticles in Phenylhydrazine-Induced Anemia in Rats: An *In Vitro* and *In Vivo* Study

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Abstract

The study evaluated the*in vitro* and *in vivo* antioxidant properties and biochemical regulation of *Moringa oleifera*-synthesized silver nanoparticles (Mo-AgNPs) silver nanoparticles in anaemic rats. Method: Mo-AgNPs were biogenically synthesized and *in vitro* antioxidant properties against 1,1-diphenyl-2-picrylhydrazyl (DPPH), nitric oxide (NO) including total antioxidant capacity (TAC) was determined. Thirty male Wistar rats were induced with anemia using 40 mg/kg. b.wt of phenyl hydrazine and were randomly assigned into six groups (n=5): 1: normal control; 2 received 10 mg/kg of Mo-AgNPs exclusively;3 received 40 mg/kg of phenylhydrazine (PHZ) only; 4 received 40 mg/kg of PHZ with 100 mg/kg of ferrous sulfate; 5 and 6 received 40 mg/kg of PHZ with 5 mg/kg and 10 mg/kg of Mo-AgNPs, respectively. Result: The percentage inhibition of Mo-AgNPs against DPPH was not significant ($p>0.05$) compared with 15.63 µg/ml of ascorbic acid. Subsequently, the percentage inhibition of Mo-AgNPs on NO at various concentrations was significant ($p<0.05$) against the standard. The TAC of Mo-AgNPs exceeded 100 in ascorbic acid equivalent (AAE) at the minimal concentration of 15.63 µg/ml.Haemoglobin level decreased significantly ($p<0.05$) in group 3 against group1. Significant increased ($p<0.05$) in Hb level was recorded in groups 2, 4, 5 and 6 relative to group 3. Malondialdehyde (MDA) level increased ($p<0.05$) in group 3 compared to group 1. Groups 4, 5, and 6 exhibited a significant reduction ($P<0.05$) in MDA levels relative to group 3. Activities of superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) were reduced ($P<0.05$) in groups 3 and 4 relative to group1. Total protein and high-density lipoprotein significantly decreased ($P<0.05$) in group 3 against group 1. Cholesterol, triacylglycerol, low-density lipoprotein, and albumin levels showed non-significant reductions ($p>0.05$) in groups 4, 5, and 6 relative to group 3.The antibacterial activity of Mo-AgNPs against the test organisms was confirmed. Conclusion: Mo-AgNPs show promise as potent antioxidants, particularly at lower concentrations, suggesting potential for oxidative and haematological recoveries.

Keywords

Moringa oleifera, Antioxidant, Silver nanoparticles, Phenylhydrazine, Anaemia

Article History

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1. Introduction

Anaemia can be referred to as a decrease in red blood cells or haemoglobin level, which results in insufficient oxygen transport to tissues, causing weariness, weakness, and diminished cognitive performance. The repercussions of anaemia are extensive, bearing considerable health and economic ramifications. The disorder may result in impaired cognitive and motor development in children, negative pregnancy outcomes, and a significant financial strain on people, families, and society [1]. The management of anaemia is frequently costly, rendering it unattainable for vulnerable groups in low- and lower-middle-income nations, which experience the highest prevalence of anaemia, especially among rural populations, impoverished households, and individuals lacking formal education [2]. Moreover, existing therapies for anaemia, like iron supplements, blood transfusions, and bone marrow transplants, are costly, underscoring the necessity for novel therapeutic alternatives for anaemia.

Since antiquity, individuals have utilised various plant parts in diverse forms to treat and control ailments. The importance of herbal medicines in primary healthcare is increasing worldwide, especially in low-income nations, where plant-based remedies have been utilised for centuries to address various health issues, harnessing the therapeutic potential of phytochemicals, which are abundant in plant-derived compounds with possible health advantages [3] Phytochemicals have demonstrated potential in the prevention and management of metabolic diseases, among other health concerns [4]. Nonetheless, the administration of these therapeutic phytochemicals to target locations is complicated by their inadequate solubility and substantial molecular size, thereby restricting their therapeutic effectiveness [5]. Researchers have employed nanotechnology to improve medication characteristics, safeguard them during circulation, and facilitate targeted delivery. Encapsulating phytochemicals within nanoparticles has benefits compared to conventional drug delivery techniques, enhancing medication stability and solubility, hence augmenting the therapeutic efficacy of plant extracts [6-8]. Moreover, nanoparticles can selectively target certain cells or tissues to enhance the therapeutic index of phytochemicals [8].

Oxidative stress contributes significantly to anaemia pathogenesis, leading to erythrocyte membrane damage, impaired erythropoiesis, and disrupted iron metabolism [9]. The antioxidant property of medicinal plants contributes in mitigation of oxidative stress, potentially improving anaemia outcomes by neutralizing reactive oxygen species, enhancing endogenous antioxidant defenses, reduce erythrocyte membrane damage and protect erythroblasts from oxidative damage as well as improve iron metabolism [10]. *Moringa oleifera* (*M. oleifera*) has historically been utilised to address numerous diseases, and its extracts have antioxidant, anti-inflammatory, and therapeutic characteristics, including anticancer, antidiabetic, antifungal, antibacterial, and anti-infertility actions [9-11].The experimental data on *M. oleifera* and electro-spun mats loaded with oil ensure that they exhibit strong antibacterial, antioxidant, and anti-inflammatory activities [12].A study had shown that *M. oleifera* aqueous extract posses hepatoprotective capability against phenylhydrazine (PHZ)-induced hepatotoxicity [11]. The prospective health advantages of *M. oleifera* are ascribed to its abundant phytoconstituents, which encompasses flavonoids, phenolic acids, and glucosinolates [12]. Recently, *M. oleifera* has been utilised to synthesize silver nanoparticles with prospective therapeutic applications [13,14]. Silver nanoparticles exhibit antibacterial, anti-inflammatory, and antioxidant capabilities, rendering them a promising instrument for disease prevention and treatment.

Despite the established link between oxidative stress and anemia as well as the increasing trends in nanomedicine, the therapeutic potential of silver nanoparticles synthesised from *M. oleifera* in the treatment of anaemia remains significantly underexplored; PHZ-induced anaemia involves oxidative stress-mediated erythrocyte damage and impaired erythropoiesis, hemce therapeutic strategies targeting oxidative stress may offer a promising approach for managing anaemia. The antioxidant properties and biochemical effects of *oringa oleifera*-synthesized silver nanoparticles (Mo-AgNPs) in PHZ-induced anaemia needs to be thoroughly investigated, necessitating an examination of the *in vitro* and *in vivo* antioxidant activities and biochemical modulation of Mo-AgNPs in PHZ-induced anaemic rats. The objective is to offer significant insights into the prospective therapeutic uses of Mo-AgNPs in the treatment of anaemia and other illnesses associated with oxidative stress.

2. Materials and Methods

2.1 Materials

Fresh leaves of *M. oleifera*, male albino rats, animal cages and feed, oven, beakers, needles and syringes, water bath. Scissors, forceps, and more dissection instruments. Filter paper, cotton wool, test tubes, centrifuge, manual blender, gloves, ethylenediaminetetraacetic acid bottles, oral gavage, spectrophotometer (HMG Labtech SPECTRO star), electronic balance, Acu-Answer Hb meter, distilled water, PHZ, silver nitrate ($AgNO_3$), ferrous sulphate (100 mg). All utilised compounds were of the highest commercially accessible analytical grades.

2.2 Plant Material / Identification

Fresh leaves of *M. oleifera* were procured from a local farmer in Ndoro Ikwuano LGA, Abia State.Based on the scientific literature, *M. oleifera* leaves were chosen over seeds for studies for their superior nutritional profile, higher antioxidant content, and greater safety profileThe plant was verified by a botanist at the Department of Plant Science

and Environmental Management, Michael Okpara University of Agriculture, Umudike, in conjunction with the University of Benin. The voucher specimen UBH-M340 was deposited in the departmental herbarium for reference.

2.3 Synthesis of *M. oleifera* Silver Nanoparticles

The technique outlined by Meena et al. [15] was utilised with minor adjustments for the production of Mo-AgNPs. The obtained fresh leaves were shade-dried for six days and further milled using a hand mixer. 10g of the fine powder were mixed with 200 ml of distilled water and boiled at 100 °C for 25 minutes. The mixture was filtered with filter paper, followed by centrifugation, and subsequently filtered a second time through a Whatman No. 1 filter. The filtrate obtained was thereafter transferred to a reagent bottle and stored in a refrigerator for future use. Silver nitrate ($AgNO_3$) served as a silver ion precursor. One milimole(1mM) aqueous solution of silver nitrate, $AgNO_3$ (aq), was put into a conical flask, and 20 ml of the previously obtained extract was put dropwise using a micropipette while heating and constantly stirring at 60 °C for 6 hours to enhance reduction rate and capping of Ag^+ ions by bioactive compounds of the plant. Silver ions (Ag^+ ion) is reduced to metallic silver (Ag^0) by phytochemicals present in the *M. oleifera* extract, leading to the formation of silver nanoparticles.The solution transitioned from colourless to brownish, indicating the development of silver nanoparticles. The reaction occurred at higher pH values (pH 8-10) typically accelerated the reduction reaction, producing smaller nanoparticles faster. Alkaline environments provide more hydroxyl ions (OH^-), promoting the ionization of phenolic compounds in plant extracts and enhancing their reducing power. It was subsequently spun at 12,000 rpm for 20 minutes, and the pellets were collected for use.

2.3.1 Characterization of *M. oleifera* Synthesized Silver Nanoparticles

The characterisation of the Mo-AgNPs was conducted using the methodology adopted by Uhuo et al. [16]. Examinations of the morphology of the composite particles and assessment of particle size were conducted using a Quanta FEG 450 scanning electron microscope (SEM) (TESCAN MIRA3,Czech Republic).

2.3.2 *In Vitro* Antioxidant Activity of Mo-AgNPs

The ability of Mo-AgNPs to scavenge free radical using DPPH was conducted by a method described by Olasehinde et al. [17]. The outcome was presented as a percentage of ascorbic acid inhibition. Ascorbic acid served as the reference standard/positive control across all *in vitro* antioxidant assays. The methodology utilised by Chanda and Dave et al. [18] was employed, with minor modifications, to assess the *in vitro* antioxidant scavenging activity of Mo-AgNPs on Nitric Oxide (NO). Concurrently, the total antioxidant capacity (TAC) of Mo-AgNPs was evaluated. The results were presented as ascorbic acid equivalents (AAE) in microgrammes per millilitre (µg AAE/mL).

2.3.3 *In Vivo* Study: Induction of Anaemia and Animal Treatments

In vivo Toxicity evaluation; Previous studies [19] revealed the value of LD_{50} of *M. oleifera* leaves extract and silver M. oleifera leaves nano-extract were about 14,250 and 13,750mg/Kg, respectively.

Thirty male wistar rats were utilised in this study. The animals were purchased from the laboratory animal production facility at the University of Nigeria, Nsukka. The animals were confined in well-ventilated aluminium cages and subjected to regular environmental conditions (Temperature: 20- 26 ℃, relative humidity: 60%, Cycle duration: a 12-hour dark/light cycle) for a two-week, adaptation period with access to feed and water. International protocols for the management and utilisation of experimental animals were rigorously followed (ARRIVE guidelines). The blood haemoglobin concentrations of animals were measured with an Acu-Answer Hb meter prior and after inducing anemia. Phenyl hydrazine (40 mg/kg b.wt) was injected intraperitoneally to the animals once daily for two consecutive days to induce anaemia based on the preliminary study and established literature. Anaemia was verified by a 40% decrease in blood haemoglobin concentration. Rats were randomly allocated into six groups of five.

Group 1 served as the normal control, receiving solely meal and water.

Group 2 received 10 mg/kg only Mo-AgNPs. This group was intended to assess nanoparticle baseline hematological effects and safety.

Group 3 (negative control group) was given solely 40 mg/kg of PHZ.

Group 4 (positive control group) was injected 40 mg/kg of PHZ in conjunction with the conventional medication, ferrous sulphate, at a dosage of 100 mg/kg.

Group 5 received 40 mg/kg of PHZ and 5 mg/kg of Mo-AgNPs.

Group 6 was given 40 mg/kg of PHZ and 10 mg/kg of Mo-AgNPs.

Treatment was administered for a duration of 30 days.

2.3.4 Determination of Biochemical Parameters

All parameters in the study were established utilising standard methodologies with suitable bio-diagnostic kits/methods, and strictly to the manufacturer's instructions. The methodologies employed by Uhuo et al. [20] were utilised to evaluate lipid profile, malondialdehyde (MDA) levels, and antioxidant defense markers, including superoxide dismutase (SOD), Catalase(CAT), and glutathione peroxidase(GPx). Albumin levels were determined using the method outlined by Gremese et al. [21], whereas total serum protein content was evaluated with a protein test kit (Sigma Diagnostics, P 5656, Sigma, *M. oleifera*, USA).

2.3.5 Antibacterial Activity of Mo-AgNPs

Antibacterial activity of Mo-AgNPs was determined by agar well diffusion assay by Anis et al. [22] with modifications. *E. coli* ATCC 25922 and *S. aureus* ATCC 29213 were adjusted to the 0.5 McFarland standard in sterile saline and swabbed on Mueller-Hinton agar plates in three directions to form a bacterial lawn. Plates were dried for 5 min. Exactly, 6 mm wells were aseptically punched using a sterile cork borer, and 50 µL of Mo-AgNPs at 100%, 50%, and 25% v/v was loaded per well. Moringa aqueous extract, 0.001M $AgNO_3$, and sterile distilled water served as plant, silver ion, and negative controls, respectively. After 30 min of pre-diffusion at room temperature, plates were incubated at 37 °C for 24 h. Zones of inhibition were noted after incubation. All tests were performed in triplicate.

2.3.6 Statistical Analysis

The data produced from the study were analysed via the statistical software IBM-SPSS version 25.0. Analysis of variance (ANOVA) and post hoc testing using least significant difference were conducted for comparisons among various groups. All data were presented as mean ± SEM. P-values below 0.05 were deemed statistically significant.

3. Results

3.1 Characterization of Mo-AgNPs

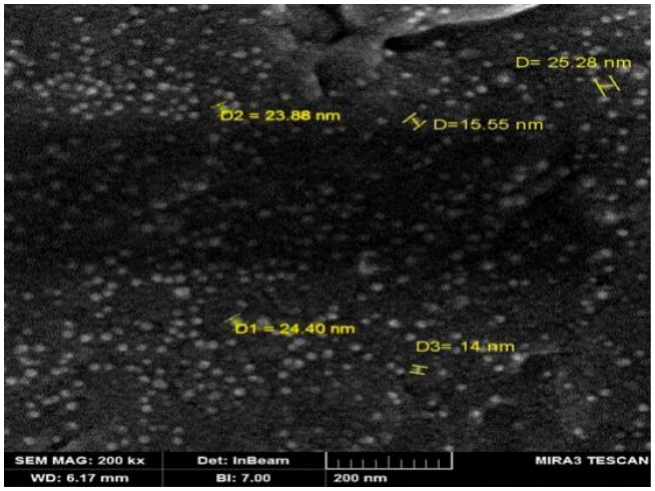


Figure 1. SEM micrograph of Mo-AgNPs.

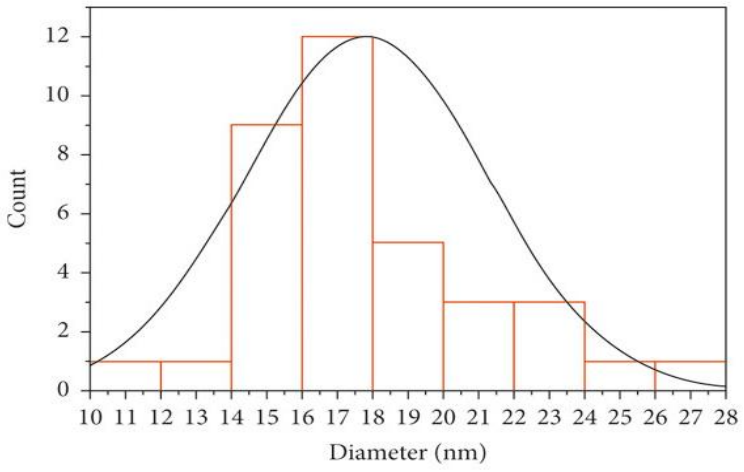


Figure 2. Size distribution of Mo-AgNPs.

3.2 *In Vitro* Antioxidant Scavenging Activity of Mo-AgNPs

3.2.1 2,2-Diphenyl-1-Picrylhydrazyl Radical Scavenging Activity of Mo-AgNPs

Figure 3 shows the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of Mo-AgNPs. The difference in percentage inhibition of Mo-AgNPs on DPPH was not significant ($p>0.05$) against the standard (ascorbic acid) at a lower concentration of 15.63 µg/ml. However, at higher concentrations [31.23, 62.50, 125, 250, and 500 µg/ml], the percentage inhibition of Mo-AgNPson DPPH against the standard was significantly different.

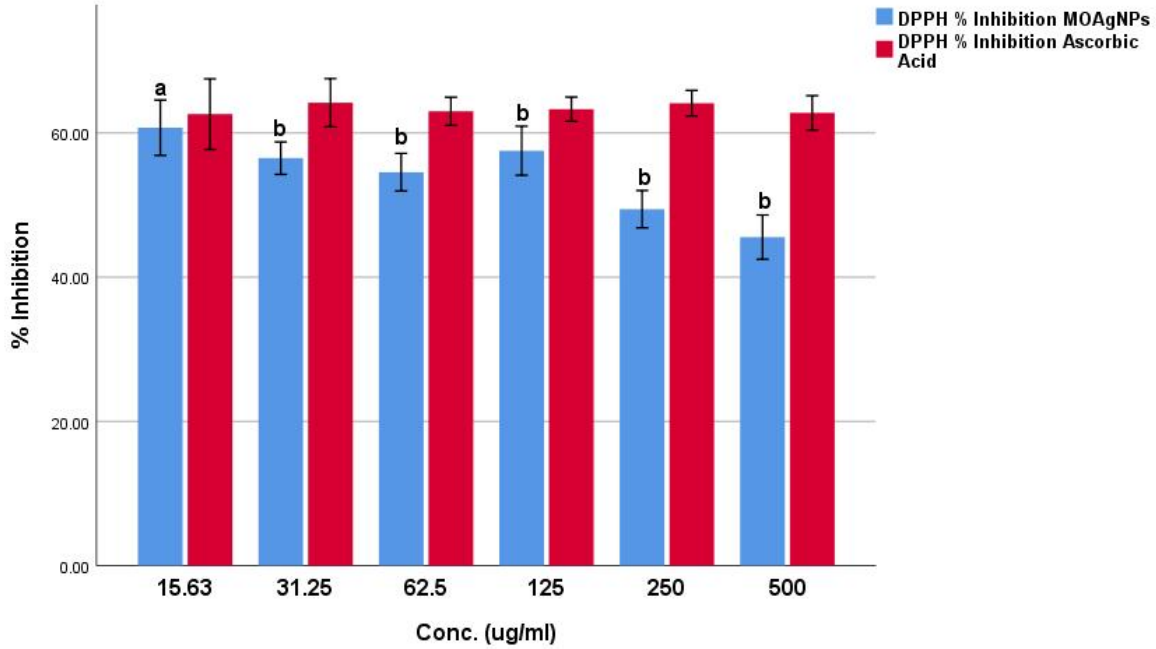


Figure 3. DPPH radical scavenging activity. Bars are mean ± SEM of 3 determinations [n=3]. [a]:($p<0.05$) vs ascorbic acid, [b]:($p>0.05$) vs ascorbic acid.

3.2.2 Nitric Oxide Radical Scavenging Activity of Mo-AgNPs

Figure 4 shows the NO radical scavenging activity of Mo-AgNPs. Results showed no significant difference in percentage inhibition of Mo-AgNPs on NO against the standard at the highest concentration of 500 µg/ml. However, at lower concentrations of 31.23, 62.50, 125, and 250 µg/ml, the percentage inhibitions of Mo-AgNPs were significantly different from the standard.

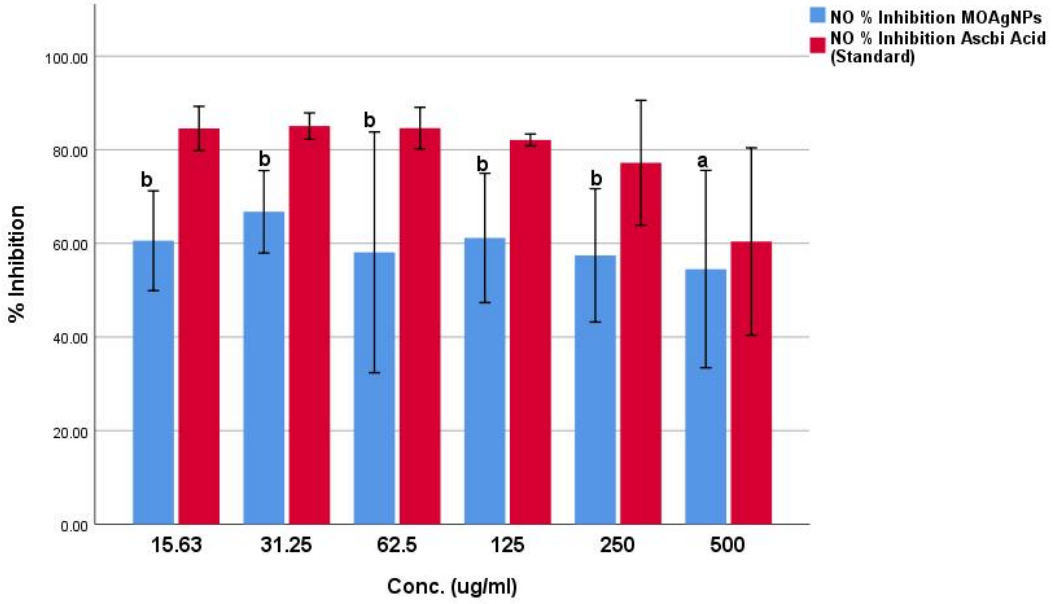


Figure 4. NOradical scavenging activity. Bars are mean ± SEM of determinations [n=3]. [a]:($p<0.05$) vs ascorbic acid, [b]:($p>0.05$) vs ascorbic acid.

3.2.3 Total Antioxidant Capacity of Mo-AgNPs

Figure 5 shows the TAC of Mo-AgNPs. It was above 100% in AAEat the lowest concentrations of 15.63 µg/ml. TAC of Mo-AgNPs was also ≥ 90 AAE at concentrations of 31.23, 250, and 500 µg/ml, while at concentrations of 62.5 and 126 µg/ml TAC values of Mo-AgNPs were above 70% in AAE.

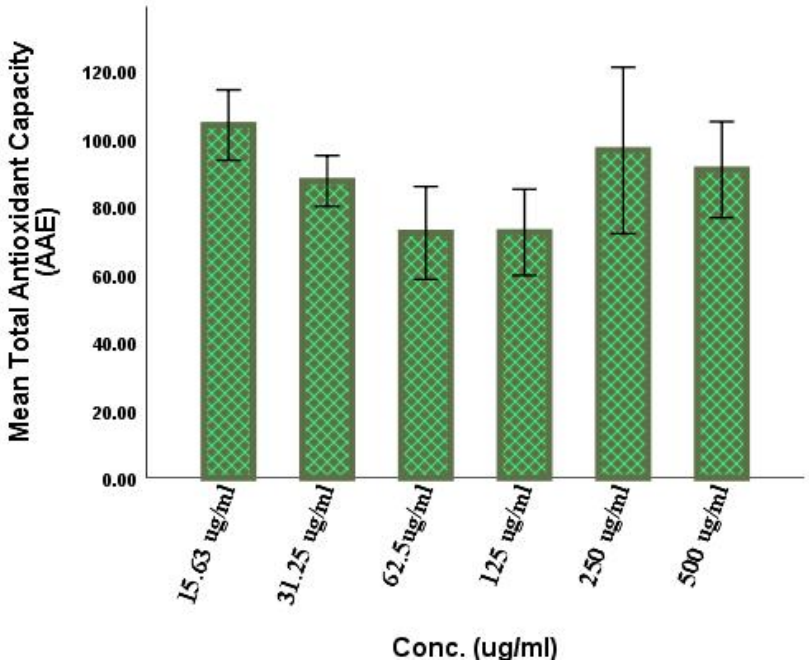


Figure 5. TAC of Mo-AgNPs in AAE. Bars are mean ± SEM of 3 determinations (n=3).

Table 1 shows no significant difference in Hb before induction (Hb1). After induction of amenia (Hb2) Hb decreased ($p<0.05$) in group 3, 4, 5 and group 6 when compared to the control. After treatment, Hb of untreated group 3 decreased ($p<0.05$) against group 1. Hb increased ($p<0.05$) significantly in treatment groups 5 and 6 against untreated group 3.

Table 1. Haemoglobin (Hb) level of PHZ-induced anaemic rats.

Group	Hb1 (g/dL)	Hb2 (g/dL)	Hb (g/dL)
1	11.37±0.24	11.16±0.17	16.11±0.19
2	11.45±0.32	11.67±0.19	15.27±0.58[b]
3	11.47±0.29	6.91±0.12[a]	9.24±0.23[a]
4	11.14±0.16	6.91±0.16[a]	14.73±0.38[ab]
5	11.67±0.19	6.82±0.07[a]	14.46±0.37[ab]
6	11.06±0.13	6.99±0.15[a]	14.87±0.25[ab]

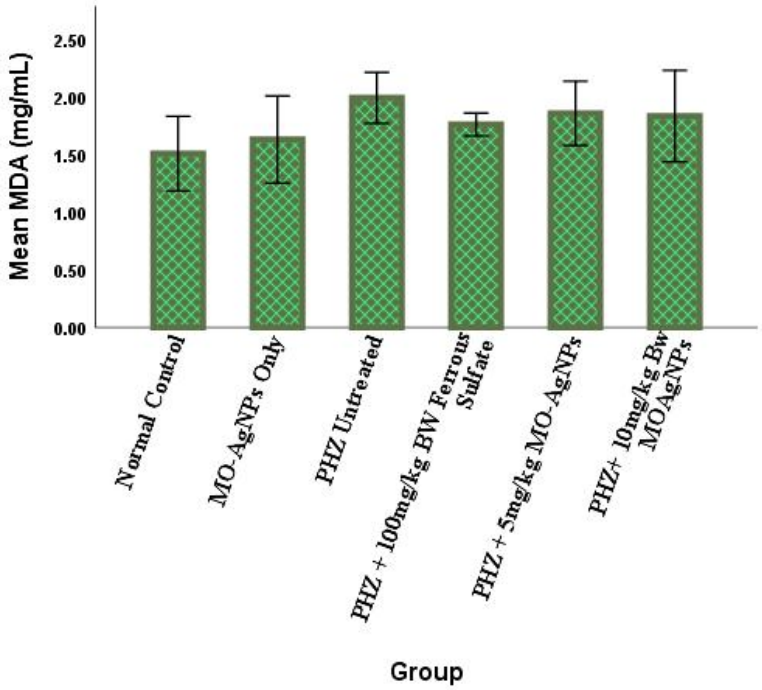
Values are Mean ± SEM of 5 determination (n=5). [a]:($p<0.05$) vs group 1, [b]:($p<0.05$) vs group 3.

Group 1: Normal control, Group 2: MoAgNPs only, Group 3: PHZ untreated, Group 4: PHZ + 100mg/kg Bwt Ferrous Sulfate, Group 5: PHZ + 5mg/kg Bwt MoAgNPs, Group 6: PHZ+ 10mg/kg Bwt MoAgNPs. Hb1: Hemoglobin before induction, Hb2: Hemoglobin after induction, Hb: Hemoglobin after treatment.

3.3 Effect of Mo-AgNPs on Oxidative Stress Markers

3.3.1 Malondialdehyde Level

Figure 6 shows the MDA level of PHZ-induced anemic rats.MDA level increased significantly ($P<0.05$) in group 3 against the normal control (group 1). A non-significant decrease ($P>0.05$) in MDA level was obtained in groups 4, 5, and 6 when compared with group 3. However, groups 2, 4, 5, and 6 demostrated a non-significant ($P > 0.05$) increase in MDA level againstgroup 1.

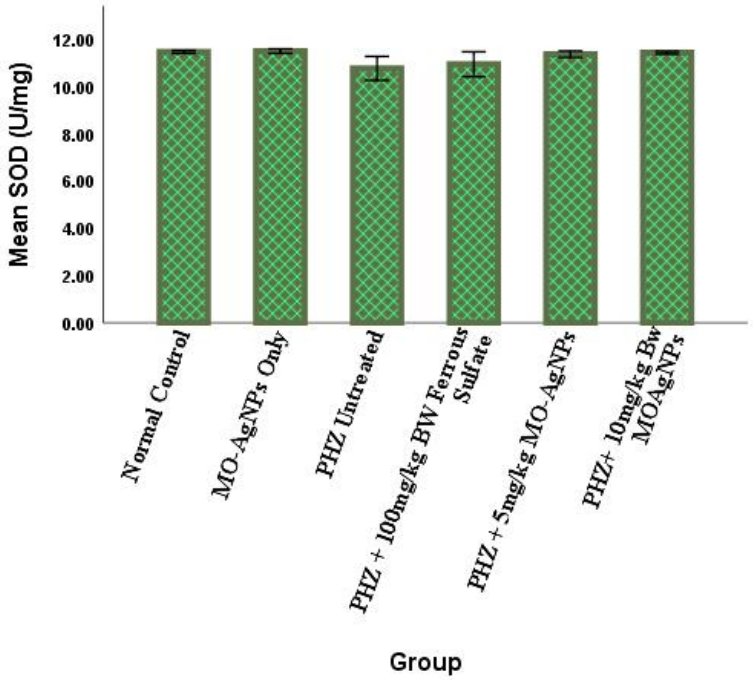


Group 1: Normal control
Group 2: MoAgNPs only
Group 3: PHZ untreated
Group 4: PHZ + 100mg/kg Bwt Ferrous Sulfate
Group 5: PHZ + 5mg/kg Bwt MoAgNPs
Group 6: PHZ+ 10mg/kg Bwt MoAgNPs

Figure 6. MDA level of PHZ-induced anemic rats. Bars are mean ± SEM of 5 determinations (n=5).

3.3.2 Superoxide Dismutase Activity

Superoxde dismutase activities in PHZ-induced anemic rats are shown in Figure 7. SOD activity in group 3 significantly ($P<0.05$) decreased compared to the control (group 1). There was significant ($P<0.05$) increased in SOD activity in groups 5, and 6 against group 3.

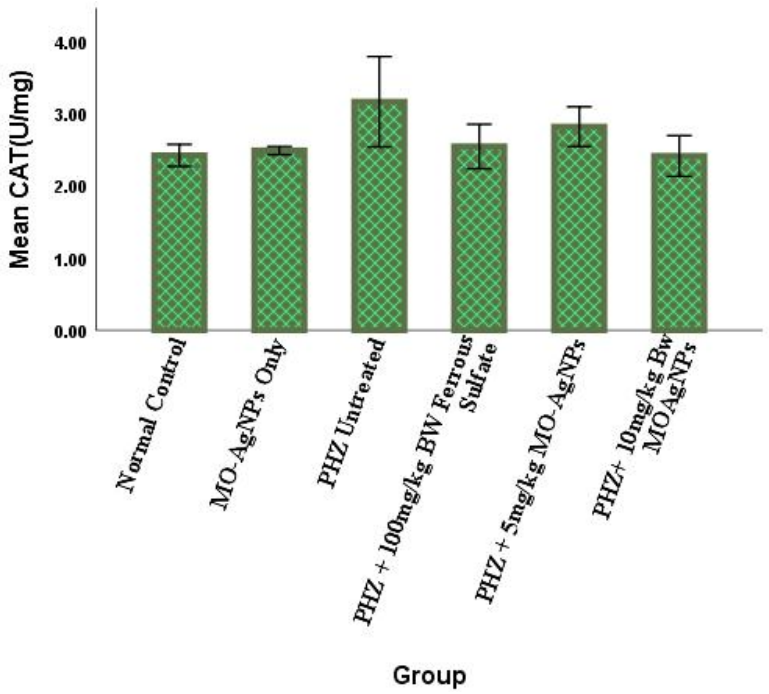


Group 1: Normal control
Group 2: MoAgNPs only
Group 3: PHZ untreated
Group 4: PHZ + 100mg/kg Bwt Ferrous Sulfate
Group 5: PHZ + 5mg/kg Bwt MoAgNPs
Group 6: PHZ+ 10mg/kg Bwt MoAgNPs

Figure 7. SOD activity of PHZ-induced anemic rats. Bars are mean ± SEM of 5 determinations(n=5).

3.3.3 Catalase Activity

CATactivity in PHZ-induced anemic rats as shown in Figure 8 indicates that CAT activity increased in group 3 and was significant ($P<0.05$) relative to group1.Subsequently, significant ($P<0.05$) increased in CAT activity was observed in groups 4, and 5 against group 1.

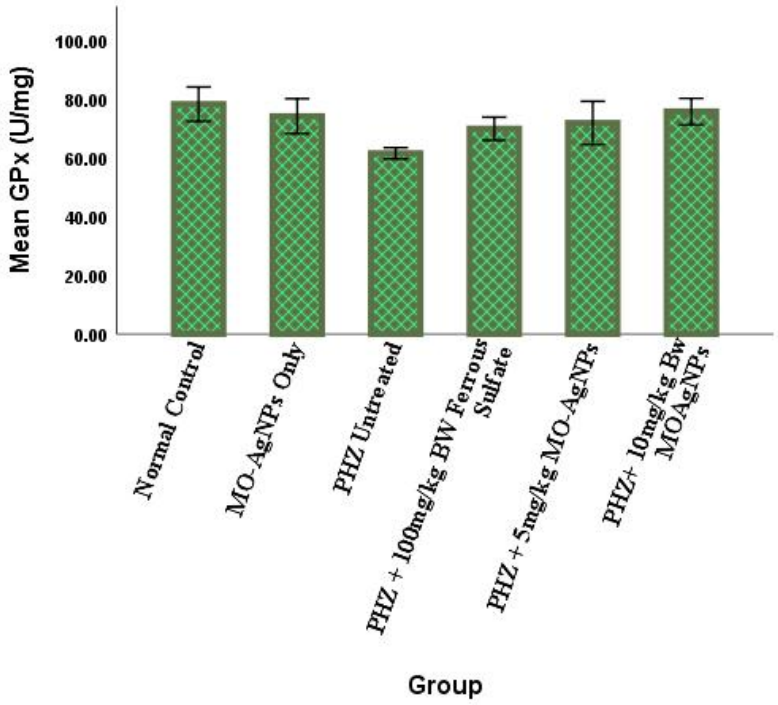


Group 1: Normal control
Group 2: MoAgNPs only
Group 3: PHZ untreated
Group 4: PHZ + 100mg/kg Bwt Ferrous Sulfate
Group 5: PHZ + 5mg/kg Bwt MoAgNPs
Group 6: PHZ+ 10mg/kg Bwt MoAgNPs

Figure 8. CAT activity in serum of PHZ-induced anemic rats. Bars are mean ± SEM of 5 determinations (n=5).

3.3.4 Glutathione Peroxidase Activity

Figure 9 shows GPx activity of phenyl hydrazine-induced anemic rats. GPx activity decreased in groups 3 and 4 and was reduced significantly (P≤0.05) relative to the control. No significant difference in GPX activities of the Mo-AgNPs-treated groups 5 and 6 against the control. However, GPX activity increased in groups 4, 5, and 6 against untreated group 3.



Group 1: Normal control
Group 2: MoAgNPs only
Group 3: PHZ untreated
Group 4: PHZ + 100mg/kg Bwt Ferrous Sulfate
Group 5: PHZ + 5mg/kg Bwt MoAgNPs
Group 6: PHZ+ 10mg/kg Bwt MoAgNPs

Figure 9. GPx activity of PHZ-induced anemic rats. Bars are mean ± SEM of 5 determinations.

3.4 Effect of Mo-AgNPs on Lipid Profile, Albumin, and Total Protein in PHZ-Induced Anemic Rats

Table 2 presents the lipid profile, albumin, and total protein of rats with phenylhydrazine-induced anaemia. High-density lipoprotein levels reduced significantly (P<0.05) in group 3 compared to the normal control. Total protein levels significantly reduced (P<0.05) in all treatment groups relative to the normal control. Low-density lipoprotein (LDL) levels dropped in group 2 against group 3. Triacylglycerol levels considerably (P<0.05) diminished in group 2 relative

to groups 3, 4, and 1, but cholesterol, triacylglycerol, low-density lipoprotein, and albumin levels in groups 3, 4, 5, and 6 exhibited non-significant decreases (P>0.05) compared to the control group.

Table 2. Lipid profile, albumin and total protein in serum of PHZ-induced anemic rats.

Group	CHOL [mmol/L]	TAG [mmol/L]	HDL[mmol/L]	LDL[mmol/L]	ALB [g/dL]	TP [g/dL]
1	4.74±0.24	1.56±0.05	2.14±0.15	2.24±0.36	4.12±0.14	5.62±0.11
2	4.62±0.21	1.34±0.02[abc]	1.94±0.09	1.96±0.29[b]	4.28±0.19	5.00±0.11[a]
3	4.94±0.14	1.64±0.02	1.82±0.04[a]	2.68±0.07	3.96±0.21	4.98±0.08[a]
4	4.80±0.24	1.60±0.04	1.98±0.07	2.36±0.23	4.10±0.20	5.14±0.09[a]
5	4.86±0.16	1.62±0.06	1.96±0.07	2.61±0.14	4.04±0.13	5.04±0.05[a]
6	4.86±0.11	1.58±0.04	2.02±0.08	2.57±0.16	4.06±0.13	5.30±0.25[a]

Values are Mean ± SEM of 5 determination [n=5]. [a]:(P<0.05) vs group 1, [b]:(P<0.05) vs group 3, [c]:(P<0.05) vs group 4.

Key: Group 1: Normal control, Group 2: Mo-AgNPs only, Group 3: PHZ untreated, Group 4: PHZ + 100mg/kg Bwt Ferrous Sulfate, Group 5: PHZ + 5mg/kg BwtMo-AgNPs, Group 6: PHZ+ 10mg/kg BwtMo-AgNPs.

Table 3. Antibacterial activity of MO-AgNPs.

Sample	Zones of Inhibition (mm)	
	E. Coli	***S. Aureus***
MO-AgNPs 100% v/v	15.57±0.15*	13.67±0.15*
MO-AgNPs 50% v/v	11.70±0.12*	9.37±0.09*
MO-AgNPs 25% v/v	6.23±0.15*	5.00±0.06*
Moringa (aq) extract 100% v/v	4.33±0.09	4.03±0.07
$AgNO_3$ 0.001M	4.10±0.06	3.80±0.12
Water	0.0 ± 0.0	0.0 ± 0.0

Values are mean ± SEM of triplicates determination. Values with asterisk are significant.

4. Discussion

Chemicals and metabolites derived from plants with therapeutic potential present challenges, including their inability to reach target locations due to their insolubility and high molecular sizes, which significantly diminishes the therapeutic efficacy of the plant. Researchers have utilised nanotechnology to improve drug characteristics, safeguard them during circulation, and facilitate targeted delivery. Encapsulating phytochemicals within nanoparticles has benefits such as enhanced stability, solubility, and targeted distribution, hence augmenting the therapeutic efficacy of plant extracts [6,7,8,16]. This study examined the impact of Mo-AgNPs on PHZ-induced anaemia in rats. The *in vitro* and *in vivo* antioxidant properties of Mo-AgNPs were examined, encompassing lipid profile, albumin, and total protein.

A SEM was used to examine the surface morphology and uniformity of the Mo-AgNPs. The SEM micrograph (Figure 1) at 200nm magnification revealed that the particles were predominantly spherical in shape. The size distribution of the Mo-AgNPs (Figure 2) ranged from 10-28 nm. The results indicate that the prepared silver nanoparticles solution was stable with no observable particle agglomeration. This is comparable with work by Shousha et al. [19], who characterized *M. oleifera* leaf extract silver nanoparticles (Ag-NPs) using transmission electron microscope (TEM) and reported particles size ranged between 5 and 10 nm.

The antioxidant activity of Mo-AgNPs exhibits assay-dependent variability, suggesting distinct mechanisms of action. In DPPH assays, Mo-AgNPs demonstrate potent electron-donating capacity at low concentrations (15.63 µg/ml), attributed to electron-donating phytoconstituents capping the nanoparticles. The decreased efficacy at higher concentrations shows decrease antioxidant efficacy of Mo-AgNPs, this could arise from pro-oxidant behavior of the capping phytochemicals at elevated doses, altered redox kinetics in the methanol medium, pH-dependent alterations in redox potential, surface-site saturation or steric hindrance at high nanoparticle density. Phytochemicals on the nanoparticle surface might get saturated at higher concentrations, limiting further increase in antioxidant activity [23-24]. Some phytochemicals or AgNPs themselves might as well exhibit pro-oxidant behavior at higher concentrations, contributing to the non-linear dose-response. This implies that optimal concentration ranges for therapeutic use of Mo-AgNPs might be narrow.In contrast, Mo-AgNPs effectively scavenged NO at 500 µg/mL in aqueous buffer (pH 7.4). This suggests assay-specific mechanisms. NO scavenging likely involves functional groups (e.g., catechol moieties) on the capping layer that selectively interact with NO radicals or inhibit its generation, and these groups may require a threshold concentration to be effective. The differences between DPPH (organic solvent, electron-transfer based) and NO (aqueous, nitrosylation/reaction based) assays highlight that the antioxidant action of Mo-AgNPs depends on radical type, solvent system, nanoparticle dose, and the accessibility of specific phytochemical groups on the particle

surface. These findings suggest that Mo-AgNPs act as effective *in vitro* antioxidants, but their activity is modulated by radical type, assay conditions, and nanoparticle dose.

The TAC of Mo-AgNPs was evaluated. Mo-AgNPs exhibit strong antioxidant capacity, especially at low concentrations (15.63 µg/ml >100 AAE), suggesting efficient phytochemical-AgNP interactions. TAC ≥ 90 AAE at multiple concentrations indicates robust antioxidant activity, potentially useful for therapeutic applications. These findings corroborate the assertions of Azmi *et al*. [9] and Watanabe *et al*. [10] about the possible health-enhancing attributes of the Moringa plant. The concealed efficacy of biomedically active nanoparticles is contingent upon the plant part extracts employed in their manufacture [25-27]. The bioactive phytochemical composition of *M. oleifera* includes quinine, saponins, flavonoids, tannins, steroids, glycosides, niazinins, niaziminins, among others, [28-31], which contribute to the potent antioxidant activity.

The elevated MDA levels in the untreated group signify heightened lipid peroxidation, serving as an indicator of oxidative stress [32]. Increased MDA levels indicate oxidative damage to lipids, potentially resulting in tissue degradation and dysfunction of biological processes. The diminished activities of SOD, CAT, and GPx in untreated group suggest the presence of oxidative stress, which promotes free radicals and overwhelms the antioxidant defence mechanism. Decreased SOD activity can hinder the transformation of superoxide anion into hydrogen peroxide, resulting in heightened oxidative stress that undermines the functionality of biological molecules. Elevated CAT activity may serve as a compensatory mechanism to mitigate heightened oxidative stress by detoxifying hydrogen peroxide. CAT is essential for antioxidant defence, and heightened activity may signify an effort to alleviate oxidative stress [4]. Moreover, diminished GPx activity in the untreated anaemic cohort may arise from compromised antioxidant defence, resulting in heightened oxidative stress. GPx is essential for the neutralization of the effect of hydrogen peroxide and lipid hydroperoxides, and its reduced activity may lead to oxidative damage. The alterations in oxidative stress indicators offer significant insights into the mechanisms of Mo-AgNPs actions and indicate possible therapeutic advantages. Our findings corroborated those of Udeozor et al. [33], indicating that therapy enhanced protection against oxidative damage in PHZ-induced anaemic rats. Our data (Table 1) showing reduced Hb in untreated anemic rats point to hemolyticanemia driven by PHZ-induced oxidative stress. PHZ acts as an oxidant, generating ROS and PHZ-derived radicals that damage RBC membranes/proteins and promote hemolysis [33]. Since oxidative stress accelerates erythrocyte loss and eryptosis, the antioxidant potential of MO-AgNPs could mitigate these effects by neutralizing ROS and protecting membrane components, explaining the improved Hb in treated groups.

The modification of lipid profile, albumin, and total protein in PHZ-induced anaemic rats signifies oxidative damage to biomolecules. Oxidants produced by PHZ may have targeted the electron-rich molecules, leading to their degradation. The consequence is that the antioxidant defence system has been surpassed by the oxidants. The reduction in HDL levels in the untreated anaemic group relative to the normal control indicated the extent of oxidative damage to biomolecules. Total cholesterol, triglycerides, and LDL levels were influenced. Anaemia substantially influences lipid metabolism, generally resulting in diminished levels of total cholesterol, LDL, and triglycerides, with the decrease correlating to the severity of anaemia [34]. This phenomenon is frequently noted in both human and animal research and is postulated to entail enhanced cholesterol utilisation by proliferating cells and/or diminished hepatic oxygenation resulting in lower cholesterol production in the context of anaemia [35]. The reduced HDL in the untreated anaemic cohort may result from inflammation and oxidative stress, which can adversely affect HDL function and concentrations. Inflammation and oxidative stress caused by anaemia may hinder reverse cholesterol transfer, resulting in reduced HDL levels [36-37]. Niesor et al. [38] showed that elevated HDL may also be a reaction to oxidative stress, which can also occur in anaemia. The current study implies an indirect link between anaemia and lipid metabolism. The absence of discernible alterations in the lipid profile indicates that anaemia may not substantially influence lipid metabolism. The lipid profile results in this study contradict those of Ojimelukwe et al. [39], who documented a notable elevation in LDL, total cholesterol, and TAG levels in untreated anaemic rats.

The reduction in total protein (TP) in the untreated anaemic cohort signifies inflammation and oxidative stress, potentially resulting in heightened protein breakdown and reduced protein synthesis [21]. Anaemia may be linked to nutritional inadequacies, which can lead to reduced total protein levels. The present investigation demonstrated no significant change in albumin concentration over the entire cohort. This indicates that ALB levels were sustained within a limited range despite fluctuations in total protein levels, reinforcing the notion that albumin regulation is meticulously governed [40]. Compensatory mechanisms may exist to sustain albumin levels despite the presence of anaemia.

The agar well diffusion assay showed dose-dependent antibacterial activity of MO-AgNPs against the test organisms (Table 3). At 100% v/v, MO-AgNPs produced zones of inhibition of 15.57±0.15 mm and 13.67±0.15 mm against *E. coli* and *S. aureus*, respectively. Activity decreased with dilution. Moringa aqueous extract alone showed moderate activity, while $AgNO_3$ showed minimal activity, affirming the efficacy of *M. oleifera*-synthesized nanoparticles. The findings are consistent with previous reports. Ali et al. [41] reported zones of inhibition of 12-16 mm for green-synthesised Mo-AgNPs against similar Gram-negative and Gram-positive bacteria using the agar well diffusion method. Similar findings were reported by Bamigboye and Ajiboye [42], who also reported that the ethanol extract of moringa leaves and its nanoparticles showed higher antibacterial and antifungal activities compared to its aqueous extract. The comparable zone sizes confirm that phytochemicals in moringa act as effective reducing and capping agents, leading to its effectiveness.

5. Conclusion

The antioxidant activity of Mo-AgNPs exhibits assay-dependent variability, suggesting distinct mechanisms of action. Mo-AgNPs show promise as potent antioxidants, particularly at lower concentration suggesting potential for therapeutic/nutraceutical applications. Mo-AgNPs possess multifaceted antioxidant properties which is the basis of its activity. The Moringa plant's phytochemicals are responsible for the protective effect against oxidative insult and enhancement of haematological recovery. Incorporating herbal extracts into NPs improves its therapeutic potential by enhancing its antioxidant properties, eco-friendliness, and permeability.

Recommendation

Further studies are recommended for complete elucidation of the bioactive components in Mo-AgNPs responsible for the pharmacological effects and to investigate deeply the various mechanisms by which the Mo-AgNPs protect against anemia. There is also a need to investigate the use of Mo-AgNPs to improve other organ functions, such as kidney, liver, and cardiac functions, in rats and other higher animals, including humans.

The Limitations of the Study

The biogenic nanoparticles are favored for their biocompatibility, low toxicity but often exhibit lower stability, inconstant size distribution, and lower production yields. PHZ creates excessive reactive oxygen species (ROS), which not only causes hemolysis but also leads to severe lipid peroxidation, making it difficult to isolate the exact therapeutic effect of the nanoparticle from the body's innate recovery mechanisms. High activity shown *in vitro* antioxidant study failed to correlate directly to *in vivo* results possibly because of digestion, metabolism, and physiological barriers in rats.

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Ethics Statement

The experiment's protocols received approval (MOUAU/BCH/EC/2025/01) from the Animal Ethics Committee at the College of Natural Science, University of Agriculture, in accordance with the Guide for the Care and Use of Laboratory Animals.

Data Availability Statement

All data are included in the paper. The authors confirm that the figures are original.

Author Contributions

Emmanuel N. Uhuo: Methodology, Investigation, Writing-original draft, Writing-review and editing. Godwin Kingsley Okechuku: Supervision, Methodology, Formal analysis and Data curation. Ajah Obinna: Investigation, Project administration, Formal and Data curation. Parker E.Joshua: Conceptualization, Supervision, review and editing.

Conflict of Interest

The authors assert that they possess no conflicts of interest.

Generative AI Statement

The authors declare that no Gen AI was used in the creation of this manuscript.

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