Biosynthesis, characterization and antivenom activities of *Moringa oleifera* silver nanoparticles: an experimental approach

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**Abstract**

*Moringa oleifera* has been previously established to possess neutralizing potentials against *Echis ocellatus* venom. This study however, investigated the bioeficacy of silver nanoparticles biosynthesized from *M. oleifera* leaf extract aimed at improving its bioactivity against *E. ocellatus* venom-induced toxicities using *in vivo* and *in vitro* methods. The intrinsic characteristics of the produced *M. oleifera*-Silver nanoparticles (MO-AgNPs) were carried out using energy dispersive X-ray, X-ray diffraction, scanning electron microscopy, transmission electron microscopy and Fourier-transform infrared spectroscopy. Twenty-five male Wistar rats divided randomly into five groups (n=5) were used for the antivenom study. Group 1 received saline while groups 2 to 5 were envenomed intraperitoneally with 0.22mg/kg (LD$_{50}$) of *E. ocellatus* venom. Group 2 was left untreated while groups 3 to 5 were treated with 0.2ml of antivenom, 5 and 10mg/kg MO-AgNPs post-envenomation, respectively. Blood and tissue of treated rats were analyzed for haematological parameters and histopathology, respectively. The MO-AgNPs formation was confirmed with a colour change from light brown to yellowish-brown with maximum SPR band at 420nm from UV-Vis analysis, indicating a reflection of the bio-reduction of Ag$^+$ to Ag$^0$. The Transmission electron micrographs showed well dispersed spherical AgNPs with average particle size of 15.7nm. Treatment with MO-AgNPs caused a significant improvement of acute anemia, leucopenia and thrombocytopenia induced by the venom in the envenomed treated rats. Also, MO-AgNPs inhibited the haemorrhagic, haemolytic and anticoagulant activities of the venom. Tissue lesions observed in heart of envenomed untreated rats were attenuated after treatment with MO-AgNPs. The biosynthesized MO-AgNPs exhibited potent neutralizing potentials than *M. oleifera* crude extract against *E. ocellatus* venom toxicities.

**Introduction**

Snakebite envenoming poses a typical ravaging occupational and environmental menace, particularly in rustic communities of tropical developing countries (WHO 2015). *Echis ocellatus*, also known as carpet viper is in the Viperidae family, a species liable for numerous clinical complexities of extreme systemic and local pathology (Chippaux 2011). It has been reported that *E. ocellatus* accounts for 60% of fatalities resulting from 90% of bites in sub-Saharan Africa (Yusuf et al 2015). This species is commonly found in different parts of Nigeria and its envenoming has reportedly caused numerous deaths, particularly in the North-Eastern region (Harrison et al 2003). Envenomination by *E. ocellatus* results in pathophysiological conditions like swelling, blistering, coagulopathy, necrosis, haemotoxicity and haemorrhages (Chippaux 2011) as a result of the presence of toxic enzymes majorly snake venom metalloproteinases (SVMPs) (Harrison et al 2003).

For some years now, studies on the treatment of snake envenoming using other remedies of natural or synthetic origins have gained more popularity due to drawbacks in the use of conventional antivenom (de Silva et al 2016; Adeyi et al 2021a, 2023 and Ajisebiola et al 2022, 2023). Also, research interest gaining scientific importance and attention is focus on the utilization of silver nanoparticles (AgNPs) synthesized via the green route, as therapeutic agents most especially the bio-based process, using synthesis with plant extracts (Irvani et al 2014; Lee and Jun 2019). AgNPs plant extracts synthesis is generally known as “green synthesis” and its processes are deemed non-toxic, less expensive and environmentally friendly when compared to conventional AgNPs chemical synthesis (Srikar et al 2016). The greener approach towards the synthesis of AgNPs is a novel, promising area of study with robust potential and serve as one of the many bases of potent drugs with excellent prospects to combat many diseases such as cancer, diabetes, inflammation and antimicrobial effects (Ge et al 2014). Plant-derived AgNPs have been recognized in the field of nano-medicine owning to their


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promising potential in a wide sphere of bio-medical applications and many researchers have demonstrated therapeutics potentials of green synthesized AgNPs as antimicrobial, antibacterial, antifungal and anticancer (Khorrami et al 2018; Oves et al 2018). The significant potentials and fewer safety concerns of plant-derived AgNPs have prompted diverse research towards harnessing the full potential of AgNPs for treatment in the biomedical field (Wei et al. 2015). Also, the synthesis of nano-particles utilizing plants gives large-scale options for the production of nanoparticles (Sharma et al 2009).

*Moringa oleifera* (Moringaceae) is a vital medicinal plant cultivated in the tropics and subtropical areas of the world (Irfan et al 2021). A lot of pharmacological potentials of *M. oleifera* crude extract have been recorded as anti-inflammatory, anti-diabetic, antihypertensive anti-ulcer, antitumor, antispasmodic, antipyretic, antioxidant (Irfan et al 2021) and antivenom (Adeyi et al 2021b). Also, the potential therapeutic action of synthesized AgNPs from *M. oleifera* leaf extract has been studied and reported as anticancer (Khorrami et al 2020), antibacterial (Irfan et al 2021) and also in insect control (Idowu et al 2021). Previous research by Adeyi et al (2021) has documented the antivenom efficacy of *M. oleifera* crude extract against *E. ocellatus* envenoming. However, there is dearth of information on the antivenom application of biosynthesized AgNPs using *Moringa oleifera* leaf extract. This current study aimed at providing insights into the potential increase or otherwise in the antivenom efficacy of biosynthesized AgNPs using aqueous extract of *M. oleifera* leaves against *E. ocellatus* venom induced toxicity.

### Materials and methods

#### Procurement of venom and anti-venom

Lyophilized *E. ocellatus* venom was obtained from the Department of Veterinary Pharmacology and Toxicology, Ahmadu Bello University, Zaria, Nigeria and preserved at 4°C. EchiTab-Plus (ICP) polyclonal antivenom made by Instiudo Clodomiro Picado in Costa Rica was utilized as drug control for this research.

#### Collection and preparation of plant extract

Freshly harvested *M. oleifera* plants were identified at the Herbarium of Department of Botany, University of Ibadan, Nigeria with the coupon identification number, UIH-22442. Thereafter, fresh leaves were carefully detached from the plant stalk and rinsed completely and cleaned using double distilled water (DDW). Exactly 10g of the rinsed and air-dried leaves were weighed and put into a 500ml beaker having 300ml DDW and then heated at 100°C for 10 minutes. The fresh aqueous leaf extract was filtered using Whatman filter paper (185m), generating a pale amber-coloured filtrate. The extract was preserved in the laboratory at temperatures range of 25-28°C for further use.

#### Green synthesis of *M. oleifera* AgNPs (MO-AgNPs)

MO-AgNPs were synthesized according to methods by Labulo et al (2016). A measure of 10ml of the leaf extract of *M. oleifera* was added to 40ml of 1mM AgNO₃ at room temperature. The mixture was stored in the dark cupboard for 24 hours and then isolated by centrifuging at 6000rpm for 30 minutes. The clear fluid obtained was disposed and the residue (i.e. nanoparticles) was rinsed twice using DDW and oven dried at 50°C for 45 minutes. Obtained nanoparticles were kept in a 5ml vial tube and labelled MO-AgNPs for further use.

### Characterization of MO-AgNPs

Biochrom Libra PCB 1500 Ultra Violet-Vis spectrophotometer was utilised for Surface Plasmon Resonance (SPR) of synthesized MO-AgNPs. AgNPs absorbance evaluation was done by dispersing it in a quartz cuvette with a one-centimetre optical path. This was measured by collecting a small aliquot from the mixture of the reaction and performing a wavelength scan after 24 hours. The inherent characteristics of produced AgNPs were studied using Energy Dispersive X-ray (EDX), x-ray Power Diffraction (XRD), Fourier Transformed Infrared (FTIR), Scanning Electron Microscope (SEM), Transmission Electron Microscope (TEM), and Thermogravimetric Analysis (TGA). FTIR analysis was carried out with a SHIMADZU FTIR machine with model number IR8400s spectrophotometer, this gave a record for present functional groups and biomolecules found available on the outer layer of MO-AgNPs in the process of synthesis. The surface structures or morphologies and

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Plate 1. Synthesis of MO-AgNPs

Characterization of MO-AgNPs

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the elemental constituents of MO-AgNPs were recorded employing EDX (TESCAN Vega TS 5136LM), SEM normally was at 20kV at a working stretch of 20nm. The shapes plus sizes of the MO-AgNPs were examined using Transmission Electron Microscope by plunging an aqueous mixture having the AgNPs onto the carbon-smudged grids with an infrared lamp and for drying. Micrographs were gotten by employing a Philips Morgagni (M-268) working at 80kV. XRD analysis was carried out employing model D8 ADVANCE equipment (Bruker, USA).

Animals and ethical consideration
Twenty-five male albino wistar rats with weight range of 140-160g were acquired from the Department of Zoology, University of Ibadan, Nigeria. They were categorized into five groups each in a well-airy transparent plastic cage at average room temperature. The rats were well fed with regular rat pelletized feed and water ad libitum. Animal experiments of this study were approved by the University of Ibadan-Animal Care and Research Ethics Committee (UI-ACUREC), with Assigned number: UI-ACUREC: 18/0108. The processes as stipulated by the ethical board were precisely complied with for proper handling of the animals. National Research Council’s guidelines on animal experiments and care were strictly adhered to (National Research Council 2011).

Experimental design
Rats for experimentation were divided into five groups each with five rats (n=5). The first group was administered with normal saline (Normal control), while the second was envenomed and not treated (control venom). Rats in the third group were envenomed and treated with polyvalent antivenom. Also, rats in the fourth and fifth groups were envenomed and treated with 5 and 10mg/kg of MO-AgNPs.

Envenoming and treatment procedures
Rats in the envenomed groups were injected intraperitoneally with 0.2ml of 0.22mg/kg (LD50) of E. ocellatus venom dissolved in 1ml of saline (Adeyi et al., 2021). A solution of each dose (5 and 10mg/kg) of MO-AgNPs was prepared in 1ml of saline and given orally to envenomed MO-AgNPs-treated rats while antivenom was administered intravenously to the rats treated with antivenom. The treatment of the animals was done one hour post envenomation for seven consecutive days. Clinical signs of toxicity and mortality were recorded.

Organ and blood sample collection
After seven days of therapy, blood was obtained from the experimental rats through retro-orbital sinus punctation and then collected in bottles containing Ethylene diamine tetaacetic acid (EDTA) for haematological examination. Afterwards, the animals were euthanized by cervical dislocation and dissected according to guidelines by Rowett (1997). The hearts of the experimental rats were collected and stored in 10% formalin for histological examination.

Measurement of haematological indices
Blood samples obtained from the experimental rats were analysed for White Blood Cell (WBC), Packed Cell Volume (PCV), Red Blood Cell (RBC), Haemoglobin (Hb) level, white blood differential counts, and platelet (Baker and Silverton 1985).

In vitro antivenom activity of M. oleifera-silver nanoparticles (MO-AgNPs)
Anti-haemorrhagic assay
The procedure reported by Theakston and Reid (1983) was followed for the anti-haemorrhagic assay with slight modifications. Fifteen (15) male wistar rats were randomly divided into 5 groups (n=5). The first group of rats was injected with 0.2ml of saline solution, which was used as control. In the second group, rats were injected with 0.2ml of E. ocellatus venom and LD50 solution of 1ml of saline (venom control). In separate tubes, 0.2ml of antivenom and 0.2ml of 5 and 10mg/kg of MO-AgNPs dissolved in 1ml of saline were added to 0.2ml of LD50 venom. The mixtures were incubated at 37°C for an hour. Thereafter, 0.2ml of the prepared solution was administered intradermally into rats in the third, fourth and fifth groups, respectively. The haemorrhagic lesion was observed, measured and recorded after 3 hours.

Anti-haemolytic assay
In the anti-haemolytic assay, 20ml of citrated bovine blood was used, following the method described by Gomes and Pallabi (1999). The erythrocytes were cleaned with 5ml of saline solution (0.9%) and centrifugation at 2400rpm for 10 minutes. This was repeated ten times to obtain free packed cells. After several rinsing with saline, a 10% cell suspension was prepared in normal saline. In separate tubes, 0.2ml of E. ocellatus venom LD50 was made to a solution employing 1ml of saline which was combined with 2ml of 10 per cent cell suspension, as well as 0.2ml of 5 and 10mg/ml of MO-AgNPs and polyvalent antivenom (0.2ml) respectively. The control tube for the venom contained 0.2ml of venom and 10% cell suspension. The combinations were prepared in triplicates and incubated at 37°C for 60 minutes. Haemolysis was halted by adding 3mL of cooled phosphate buffer saline (PBS pH 7.2). Solutions in the tubes were centrifuged at 2400rpm for 10 minutes and the absorbance of each supernatant was read at 540nm.

Coagulant assay
For the anticoagulant assay, citrated bovine plasma was used according to the method described by Gomes and Pallabi (1999). Five (5) test tubes were numbered 1-5 each in triplicate. For the control setup, 0.2ml of normal saline was measured into test tube 1. Test tubes 2 to 5 contained an exact dose of 0.2ml LD50 of the venom and 0.2ml of bovine-citrate plasma. Setup in tube 2 was used as the venom control. To test tubes 3 to 5, 0.2ml of 5 and 10mg MO-AgNPs solution in 1ml normal saline and 0.2ml of polyvalent anti-venom were added, respectively. Subsequently, 0.2ml of CaCl2 was added to all test tubes, and the combination was incubated at
37°C. The clotting time was monitored and recorded for each test tube.

Histopathological evaluation
The heart tissues from the experimental rats were processed routinely for histological examination and stained with hematoxylin-eosin stain according to Drury and Wallington (1980).

Statistical analysis
The data analysis was done using the Statistical software (SPSS version 20). The figures were presented as average ± standard error (S.E). The relationship between varying groups was analysed using one-way Analysis of Variance (ANOVA), followed by the Duncan Multiple Range Test (DMRT) at p< 0.05.

Results
UV-Vis for MO-AgNPs
The formation of the MO-AgNPs was confirmed by a colour transition from light-brown to yellowish-brown as a result of the reaction of M. oleifera leaf extract and AgNO₃ solution. This is as a result of the bio-reduction from Ag⁺ to Ag⁰, which was observed from the UV-vis spectra (Figure 1). The maximum SPR band was recorded at 420 nm.

Transmission Electron Microscopy and particle size distribution
The TEM micrographs showed aggregated and dispersed spherical AgNPs while some are irregular in shape (Figures 2a and 2b). The average particle size of MO-AgNPs was arithmetically estimated using Debye-Scherrer’s equation. Figure 2b indicates an average particle size of the dispersed AgNPs of 15.7 nm with a polydispersity index (PDI) of 0.186 indicating a stable and colloidal dispersion.

EDX and SEM analyses
The EDX spectra showed a high intensity for Ag confirming the formation of AgNPs (Figure 3). As a result of the SPR, strong count for metallic nanosilver (61.9%) for other elements (i.e., 0-23.0%, N - 6.6%, S - 4.3%, Fe - 1.3%, Si - 0.9%, Cu - 0.7%, P - 0.6%, Zn - 0.3%, Al - 0.2%, Cr - 0.1%) were recorded. Metallic silver is the highest constituent compared to Si, Fe, Zn and Al. These other elements may come from the biomolecules of M. oleifera that are attached to the surface of MO-AgNPs thereby confirming the participation of biomolecules from the plant as stabilizers of the nanostructure (Figure 4).

FTIR analysis
The FTIR spectrum of MO-AgNPs showed peaks at 3303, 2917, 2137, 1619, and 1021 cm⁻¹, showing −OH group, C-H vibrations, −C≡C−, N-H, and C-O-C functional groups (Figure 5). These peaks suggest that the silver nanoparticles may be surrounded by hydroxyl groups and aliphatic or carboxylic compounds. In addition, the FTIR spectra of the aqueous leave excerpts of M. oleifera revealed absorption peaks at 3235 cm⁻¹ for O-H stretching combined alcohols, 2095.76 cm⁻¹ for −C ≡C− of the alkyne group, and 1644 cm⁻¹ for amine groups present in the proteins. The absorption band at 1644 cm⁻¹ in the spectra of extract of M. oleifera was moved to a lesser frequency of 1619 cm⁻¹ for MO-AgNPs, indicating amine class are primarily active in the lowering and stabilization of silver ions to AgNPs and prevent agglomeration.

XRD analysis
The crystallinity of MO-AgNPs was affirmed by XRD characterization. Figure 6 indicated the XRD result of the MO-AgNPs at 2(θ) = 26.3, 38.6, 44.2, and 64.5. The
calculated average particle size using full-width at half maximum (FWHM) of the (111) and (200) peaks were 19.3 and 22.5 nm, respectively.

Observed mortalities from the envenomed animals during treatment with MO-AgNPs
No mortality was recorded in the control rats whereas 20% mortality occurred in the untreated envenomed group. The envenomed group that was treated with 5 mg/kg of MO-AgNPs and antivenom had 10% mortality each, respectively. However, no mortality was recorded in envenomed rats treated with 10 mg/kg of MO-AgNPs. Also, clinical symptoms of toxicity shown by the envenomed rats included sluggishness, mild bleeding at the site of venom injection, and reduced physical activities.

Figure 3: EDX Spectra of MO-AgNPs

Figure 4: SEM images of MO-AgNPs

In vivo antivenom activity of MO-AgNPs against E. ocellatus venom
Haematological parameters of rats envenomed with E. ocellatus venom after treatment with MO-AgNPs
The levels of RBC, haemoglobin concentration (Hb), PCV, and platelet counts in the untreated group (venom control) were significantly lower than in the control. Conversely, envenomed rats treated with antivenom and MO-AgNPs showed a substantial increase in PCV, platelet counts, RBC, and Hb, and compared to the venom control. However, dose dependent increase was observed in RBC, PCV counts and Hb concentration of envenomed groups treated with varying concentrations of MO-AgNPs. It is also noteworthy that PCV, RBC, platelet counts and Hb concentration of the envenomed group treated with 10 mg/kg of MO-AgNPs were higher when compared with group treated with antivenom. (Table 1).

WBC and Differentials of the Envenomed treated with MO-AgNPs
The WBC and lymphocytes counts in the envenomed group were significantly lower compared to the control. However, these parameters substantially increased in the envenomed rats treated with antivenom and MO-AgNPs in comparison to the venom control group. Similarly, the values obtained for neutrophils (NEUT), monocytes
and eosinophils (EO) were significantly higher in envenomed treated rats compared to the control (Table 2).

Figure 5. FTIR of Synthesized MO-AgNPs

Antihemorrhagic action of MO-AgNPs

Intradermal injection of *E. ocellatus* venom in rats showed a visible spot with 100% haemorrhage recorded in the venom control but no lesion was observed in the control rats injected with saline. However, the group injected with the venom and a high dose of MO-AgNPs showed a highly significant inhibition of haemorrhage induced by the venom when compared to groups treated with a low dose of MO-AgNPs and antivenom (Table 3).

Table 1: Haematological parameters of *E. ocellatus* envenomed rats after treatment with MO-AgNPs

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group 1 (normal control)</th>
<th>Group 2 (venom control)</th>
<th>Group 3 (venom/antivenom)</th>
<th>Group 4 (venom/5mg MO-AgNPs)</th>
<th>Group 5 (venom/10mg MO-AgNPs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCV (%)</td>
<td>48.33±1.33&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>39.45±1.55&lt;sup&gt;c&lt;/sup&gt;</td>
<td>42.23±2.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>44.5±1.56&lt;sup&gt;a&lt;/sup&gt;</td>
<td>47.33±1.76&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hb (g/dl)</td>
<td>16.62±0.45&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>12.60±0.65&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>14.64±0.63&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15.20±1.90&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.32±0.55&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>RBC (cell/L)</td>
<td>8.43±0.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.32±0.23&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.75±0.46&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.78±0.45&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.10±0.28&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Platelet (&lt;10&lt;sup&gt;3&lt;/sup&gt; cell/l)</td>
<td>109.33±3.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>100.62±2.88&lt;sup&gt;c&lt;/sup&gt;</td>
<td>104.95±1.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>106.5±1.52&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>108.66±2.67&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>MCH (pg/cell)</td>
<td>209.08±1.92&lt;sup&gt;b&lt;/sup&gt;</td>
<td>199.36±1.29&lt;sup&gt;a&lt;/sup&gt;</td>
<td>201.54±4.22&lt;sup&gt;b&lt;/sup&gt;</td>
<td>206.24±2.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>208.23±1.76&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>MCHC (g/dl)</td>
<td>34.85±0.36&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30.3±1.67&lt;sup&gt;b&lt;/sup&gt;</td>
<td>32.5±1.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>32.64±0.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>33.58±0.21&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>629.38±4.79&lt;sup&gt;a&lt;/sup&gt;</td>
<td>617.08±3.25&lt;sup&gt;b&lt;/sup&gt;</td>
<td>618.05±5.72&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>620.76±4.45&lt;sup&gt;a&lt;/sup&gt;</td>
<td>626.91±3.30&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data demonstrated as average ± Standard error means (SEM), (n=3). Figures in similar columns carrying varying superscripts are taken substantial (p<0.05). HGB: Hemoglobin, MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration

Table 2: Differentials of *E. ocellatus* and WBC envenomed rats post-treatment with MO-AgNPs

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group 1 (normal control)</th>
<th>Group 2 (venom control)</th>
<th>Group 3 (venom/antivenom)</th>
<th>Group 4 (venom/5mg MO-AgNPs)</th>
<th>Group 5 (venom/10mg MO-AgNPs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC (10&lt;sup&gt;3&lt;/sup&gt; cell/l)</td>
<td>2.41±0.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.6±0.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.5±0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.42±0.37&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.25±0.07&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>79.34±0.57&lt;sup&gt;a&lt;/sup&gt;</td>
<td>68.12±2.32&lt;sup&gt;b&lt;/sup&gt;</td>
<td>76.51±3.50&lt;sup&gt;a&lt;/sup&gt;</td>
<td>76.43±1.32&lt;sup&gt;a&lt;/sup&gt;</td>
<td>79.21±1.15&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Neutrophils (10&lt;sup&gt;3&lt;/sup&gt; cm&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>22.33±0.88&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.24±1.74&lt;sup&gt;b&lt;/sup&gt;</td>
<td>19.23±3.60&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20.51±4.54&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.66±1.20&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Monocytes (10&lt;sup&gt;3&lt;/sup&gt; µl)</td>
<td>2.66±0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.12±1.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.53±0.52&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.46±1.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.33±0.33&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Eosinophils (10&lt;sup&gt;3&lt;/sup&gt; µl)</td>
<td>2.45±0.57&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.32±0.45&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.61±0.36&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.53±0.52&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.45±0.57&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
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</table>

Data demonstrated as average ± Standard error means (SEM), (n=3). Figures in similar columns with varying superscripts are seen as substantial (p<0.05)

Anthaemolytic activity of MO-AgNPs

The control setup had 100% haemolysis as the venom exhibited conventional hemolysis of bovine red blood cells. However, haemolysis induced by the venom was strongly inhibited by the high dose of MO-AgNPs and antivenom (Table 4).

Coagulation activity of MO-AgNPs

The control sample clotted within 50s, whereas the venom prolonged the clotting time to 292s in venom...
control setup. However, the setup treated with high dose of MO-AgNPs and antivenom substantially (p<0.05) reduced the clotting time to 68 and 60 s, respectively (Figure 7).

Histopathological observation of the heart of the envenomated treated rats
Heart of control rats indicated regular formation of cardiomyocytes without observable defects while slides prepared from the heart tissues of venom control indicates moderate foci of interstitial cell hyperplasia, haemorrhage, and hypertrophy of myofibres. The observed morphological alterations were extenuated in groups envenomed and those treated with antivenom and MO-AgNPs (Plate 2).

Table 3. Inhibition of haemorrhagic action by MO-AgNPs

<table>
<thead>
<tr>
<th>Therapy</th>
<th>Percentage inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (Saline)</td>
<td>0 (100% haemorrhage)</td>
</tr>
<tr>
<td>Group 2 (Venom only)</td>
<td>62.00±2.42\textsuperscript{a}</td>
</tr>
<tr>
<td>Group 3 (Venom/antivenom)</td>
<td>64.00±2.68\textsuperscript{a}</td>
</tr>
<tr>
<td>Group 4 (Venom/5mg/kg MO-AgNPs)</td>
<td>82.00±3.21\textsuperscript{b}</td>
</tr>
<tr>
<td>Data demonstrated as average ± Standard error means (SEM), (n=3). Figures in similar columns with varying superscripts are considered substantial (p&lt;0.05)</td>
<td></td>
</tr>
</tbody>
</table>

Table 4. Inhibition of hemolytic action by MO-AgNPs

<table>
<thead>
<tr>
<th>Therapy</th>
<th>Percentage inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (Normal control)</td>
<td>0 (100% haemolysis)</td>
</tr>
<tr>
<td>Group 2 (Venom control)</td>
<td>26.00±1.45\textsuperscript{a}</td>
</tr>
<tr>
<td>Group 3 (Venom/antivenom)</td>
<td>82.00±2.24\textsuperscript{b}</td>
</tr>
<tr>
<td>Group 4 (Venom/5mg/kg MO-AgNPs)</td>
<td>68.00±2.00\textsuperscript{a}</td>
</tr>
<tr>
<td>Group 5 (Venom/10mg/kg MO-AgNPs)</td>
<td>78.00±2.86\textsuperscript{b}</td>
</tr>
<tr>
<td>Data demonstrated as average ± Standard error means (SEM), (n=3). Figures in similar columns with varying superscripts are considered substantial (p&lt;0.05)</td>
<td></td>
</tr>
</tbody>
</table>

Discussion

The field of nanotechnology has turned into an important domain in medical sciences due to its great potentials and utilization for treatment of many chronic diseases by researchers most especially the use of phytoconstituents as valuable candidates in synthesizing green silver nanoparticles (AgNPs) (Jain et al 2021). Reports abound that metal combined with entire-plant extracts would better potentiate the activities of the phytoconstituents (Chaudhary and Singh 2010; Gomes et al 2016; Hingane et al 2018). Recently, the application of metal nanoparticles and nano-based bio-products in the sphere of biomedicine is gaining more research attention and has paved way for capable drug molecules (Gomes et al 2016; Hingane et al 2018).

The MO-AgNPs revealed diagnostic peak characteristics of present functionalities that caused a decline of Ag\textsuperscript{+} to afford the Ag\textsuperscript{0}. Also, aliphatics amine, carbonyl of amide, and hydroxyl are characteristic functionalities that were noticed and the manifestation of the outer layer functionality examination means functional groups presence can reduce the Ag\textsuperscript{+}, and also cause the stabilization of MO-AgNPs which is in tandem with report of Idowu et al (2021). The corresponding single peak absorbance spectra of MO-AgNPs could be attributed to feature vibrations due to disparity in the electronic energy levels of AgNPs. The maximum SPR band was localized around 420nm, the same as the wavelength absorption band of a single metallic Ag\textsuperscript{0} (Idowu et al 2021; Patil and Ashok 2021). The average particle size distribution of the MO-AgNPs was found to be 15.7nm, which is similar to findings from other works using aqueous leave extract of M. oleifera (Awawd et al 2012; Jayshree and Nallamuthus 2016; Shousha et al 2019).

Snake venoms most especially viper venoms contain hydrolytic proteins and enzymes that manifest toxic actions resulting in clinical complications (Hamza et al 2010). These proteins may cause haematological disturbances, damage blood capillaries and induce haemorrhage through their associated proteins and changed the primary hemostasis by disrupting platelet adhesion ( Cherifi and Laraba 2013). As observed in this study, E. ocellatus venom altered the haematological indices by inducing acute anaemia and thrombocytopenia. Finding also showed induced leucopenia in the venom control rats as levels of WBC and differentials significantly decreased and these are vital components of the blood. Leucopenia induced by the venom may be a result of bone marrow failure, tumour and collagen vascular diseases (Al-Sadoon and Fahim 2012) which could diminish the ability of the body cells to fight body foreign invaders that can cause infections.
Furthermore, *E. ocellatus* venom induced various histopathological alterations in heart of the envenomed untreated rats as moderate foci of interstitial cell hyperplasia, haemorrhage, and hypertrophy of myofibres were noticed. Cardiac lesions is one of the pathological effects associated with snakebite envenoming as a result of cardiotoxin(s) and PLA2 leading to cytotoxicity and cellular necrosis (Asad et al 2014). Additionally, SVMPs present in viper venom are considered liable for regular haemorrhage, reducing the constituent of the vascular basement membrane, and leading to dislodgement of the vascular structure (Silveira et al 2004; Fox and Serrano 2005).

![Plate 2. Histopathology of the heart tissues of envenomed treated rats.](image)

**Group 1 (Control):** Regular cardiomyocytes with no observable defect, **Group 2 (Venom control):** There are foci of moderate interstitial cell hyperplasia, haemorrhage, and hypertrophy of myofibres, **Group 3 (Venom/antivenom):** Moderate interstitial cell hyperplasia and hypertrophy of myofibres, **Group 4 (Venom/5mg/kg MO-AgNPs):** Mild hypertrophy of myofibres, **Group 5 (Venom/10mg/kg MO-AgNPs):** Mild hypertrophy of myofibres.

The manifestations of pathophysiology as observed in the envenomed untreated rats are evidence of the toxic actions exhibited by viper venom toxins as earlier reported in envenomed animals (Al-Sadoon and Fahim 2012; Adeyi et al 2021; Ajisebiola et al 2021) and in viper envenomed victims (Netto et al 2004). However, treatment with a low and high dose of MO-AgNPs significantly improved the haematological indices dose-dependently and ameliorated the pathological defects noticed in the heart of envenomed treated rats. The observed improvement could be attributed to the neutralizing potentials of MO-AgNPs after treatment as studies have reported the neutralizing potency of silver nanoparticles-based plant extract against venoms of snake species (Singh et al 2020; Ghosh et al 2021).

Phospholipase A2 (PLA2) and SVMPs are widespread proteins that constitute Viperidae venom. The latter is known to cause hemostasis disturbance by hydrolyzing fibrinogen or degrading the fibrin clot and wall of blood capillaries thereby enhancing the haemorrhagic effect resulting in spontaneous pathological bleeding (Al-Sadoon and Fahim 2012) while the former hydrolyzes cell membrane and blood cells resulting in tissue necrosis and haemolysis (Lomonte et al 2003). The results showed that, *E. ocellatus* venom displayed complete haemorrhagic action in the venom control rats as expected of viper venom which was in tandem with earlier study (Adeyi et al 2021). Also, the venom-induced direct haemolysis on citrated bovine erythrocytes and prolonged blood coagulation could be attributed to the action of SVMPs and PLA2 enzymes respectively (Adeyi et al 2021; Ajisebiola et al 2021). In this study, MO-AgNPs effectively countered the haemorrhagic and blood-lytic activities of the venom and restored coagulation by shortening the clotting time.
As earlier stated, our previous study has reported the antivenom effects of crude extract of *M. oleifera* at a high dose of 600 mg/kg as to *E. ocellatus* venom toxicities *in vitro* and *in vivo* (Adeyi et al 2021) whereas, findings from this study demonstrated successful neutralization of the venom *in vitro* and *in vivo* by MO-AgNPs at a high dose of 10 mg/kg thereby, confirming the antivenom potentials of MO-AgNPs and indicating that biosynthesized silver nano-particles with plant extract possess more potent antivenom activity than a whole-plant extract, and this observation corroborated with Singh et al (2020).

The mechanism of MO-AgNPs inhibitory action against the venom toxic activities is fully unknown yet but it should be noted that successful use of plant biosynthesized silver nanoparticles using *M. oleifera* extract against some diseases has been reported (Idowu et al 2021; Irfan et al 2021; Khorrami et al 2020). The possible mechanism of AgNPs successes in the treatment of diseases may be due to the unique electrical and magnetic properties including their capability to merge biomolecules, high layer area to volume ratio, significant upper layer reactivity, ease of synthesis and characterization, low cytotoxicity, and potential to enhance gene expression for redox processes (Luis et al 2020).

Report showed that phenolic, alkaloids, enzymes, coenzymes, proteins sugars and terpenoids are the main phytochemicals that are liable for the bioreduction of Ag salts (Roy et al 2019). *M. oleifera* leaf is a great source of tannins, flavonoids, phenolic compounds, alkaloids and terpenoids (Vergara-Jimenez et al 2017) and successful bioreduction of silver and transformation of silver ions to nanoparticles were achieved as relevant contributions of these functional groups (Singh et al 2019). Another possible mechanism of the antivenom effects exhibited by green synthesis MO-AgNPs could be attributed to the synergistic effect between the MO-AgNPs and the biological coating of the plant excerpts on the outer layer of the nanoparticle (Khorrami et al 2018).

**Conclusion**

This study reported the successful bio-synthesis of Ag nanoparticles employing aqueous extracts of *M. oleifera* and its antivenom bioactivity against *E. ocellatus* venom toxicities *in vivo* and *in vitro*. This study affirmed that venom MO-AgNPs have great potential as an antidote against snake venom toxicities when compared to the *M. oleifera* crude extract and could be used as part of an integrated approach towards effective treatment of snakebite envenomation. However, further study is required in understanding the mechanism of MO-AgNPs in countering venom's toxic effects.

**Acknowledgements**

Not applicable

**Conflict of Interest statement**

All authors declare no conflict of interest.

References


Moringa oleifera, and nanoparticles. Inhibition of crude viper venom action by silver and metallic nanoparticles.activity.


