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Molluscicidal activities of green-synthesized *Alstonia congensis* silver nanoparticles

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ABSTRACT

Objective: To evaluate the efficacy of *Alstonia congensis* green-synthesized nanoparticles as a molluscicide against snail hosts of trematodes.

Methods: The ethanolic leaf extract of *Alstonia congensis* was used to synthesize silver nanoparticles. The formulation was characterized by Fourier transform infrared spectroscopy, X-ray powder diffraction, and scanning electron microscope/energy-dispersed X-ray. The ovicidal and molluscicidal activities of the *Alstonia congensis* extract and its nanoparticles were tested against *Physa acuta* and *Bulinus forskalii* at different concentrations.

Results: The green-synthesized nanoparticles inhibited embryonic development within the egg masses of the two snails in all the tested concentrations. *Alstonia congensis* extract did not show molluscicidal properties against adult *Physa acuta* but showed a very weak activity against *Bulinus forskalii*. Moreover, the synthesized nanoparticles showed significantly high molluscicidal activity against adult snails within 5-40 min of exposure in a concentration-dependent manner ($P<0.05$).

Conclusions: The *Alstonia congensis*-based nanoparticles show molluscicidal activities against adults and embryos of *Physa acuta* and *Bulinus forskalii*, and can be further explored as a potent molluscicide for the control of intermediate host of trematode parasites.

KEYWORDS: *Alstonia congensis*; *Physa acuta*; *Bulinus forskalii*; Nanoparticles; Embryo inhibition; Molluscicide

1. Introduction

Molluscicide development has become an important endeavor in human life in view of the economic and public health implications of certain freshwater snails[1-3]. *Physa acuta* (*P. acuta*) (Draparnaud, 1805), later transferred to the genus *Haitai*[4], has been reported as an intermediate host for several trematodes including *Choanocotyle* and *Echinostoma*[5,6]. In a similar vein, *Bulinus forskalii* (*B. forskalii*) is an implicated intermediate host of the trematode *Schistosoma haematobium* in several Sub-Saharan African countries[1,7].

Medicinal plants have become important sources of bioactive agents for freshwater snails' control[8]. The justification for the advocacy of their use as molluscicides is due to their eco-friendly nature[9]. Nevertheless, reports have shown that the molluscicidal activities of extracts from some plants are weak[10] and optimizing the molluscicidal efficacies of these plants could necessitate the application of admixture of two or more different plants[1]. Other recent methods that have been adopted to improve the delivery of bioactive agents in plants to freshwater snails' tissues are nanotechnological-based[11,12].

Significance

To date, no research has been done on the application of *Alstonia congensis*-based nanoparticles as molluscicides. This study demonstrates that *Alstonia congensis*-based nanoparticles had efficacy against the eggs and adults of snail hosts of trematodes compared with the *Alstonia congensis* extract. This suggests that the *Alstonia congensis*-based nanoparticles could be a promising candidate for further development as a molluscicide.

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Nanotechnology is an emerging technology that has been widely applied in diverse fields such as agriculture, food processing, material science, and health amongst others[13]. Particle sizes between 1-100 nm can be developed physically, chemically, and biologically[14]. The formulation of nanoparticles in the first two methods often involves the use of hazardous chemical reagents, unique and large facilities, and controlled conditions of pressure, temperature, and pH[13]. Moreover, the physical and chemical synthesis of nanoparticles is expensive and often produces by-products that are toxic to the environment[15]. The biologically-mediated synthesis of nanoparticles, in contrast to the physical and chemical approaches, is cheap, easy to adopt, and produces a formulation that is safe for the environment[16]. As a result, studies have explored the biological or green synthesis approaches in the production of nanosized materials using biological-based components derived from algae, plants, bacteria, fungi, *etc*[11,13,17–19].

Studies on plant-based nanoparticles against different stages of the intermediate snail hosts of trematodes are currently evolving and have shown promising potential as molluscicide agents[8,11,20,21]. *Alstonia congensis* (*A. congensis*) is a 10-15 m tall tree with creamy white heartwood and sapwood that is not distinctly demarcated. It belongs to the Apocynaceae family and is native to several African countries[22]. The plant possesses several alkaloids which could be responsible for its numerous medicinal activities. Particularly, various preparations from the different parts of the plants have been reported in the folkloric management of rheumatic pain, gonorrhea, intestinal problems, diarrhea, scabies, leucorrhea, headache, ulcers, yaws, and malaria[22,23].

Silver nanoparticles (AgNPs) are desired as a better candidate for several applications due to their size and shape-dependent optical, chemical, and electronic properties[24]. The chemical reduction process that results in the formation of bulk colloidal AgNPs is simple, fast, and capable of producing AgNPs of different sizes[24]. Reduction of silver ions using plant materials or other biological agents as reducing and capping agents has been proven as a better alternative to commercial reducing/capping agents[25,26]. This study aimed to synthesize AgNPs using leaf extracts from *A. congensis* as a capping/reducing agent and test the formulation against potential snail intermediate hosts of trematodes.

2. Materials and methods

2.1. Collection of plant and identification

The fresh leaves of *A. congensis* were obtained from Igba area in Ondo city, Nigeria. A taxonomist, Professor Emmanuel Izaka Aigbokhan from the Department of Plant Biology and Biotechnology at the University of Benin, Benin City, Edo State, identified the plant and allocated a voucher number (UBH-555). The plant was then deposited at the department's Herbarium. The leaves collected were cleaned and dried at room temperature (25 ℃) for three weeks. After drying, the leaves were ground and used for extraction. The plant collection and usage were in accordance with all relevant national guidelines.

2.2. Preparation of extracts

The extraction of plant materials was carried out using a method that was previously reported[18]. To extract the plant materials, 400 g of ground plant leaves were soaked in 2 L of absolute ethanol (95%) for 7 d while shaking manually. The mixture was filtered in a series using a muslin cloth and cotton wool. The resulting leaf extract was concentrated with a rotary evaporator and kept at 25 ℃ until needed.

2.3. Formulation of green–synthesized A. congensis–based AgNPs

The method of synthesizing AgNPs was based on the method described by Okeke *et al*[18]. Initially, 60 mg of extract from *A. congensis* leaf was suspended and dissolved in 30 mL of distilled water. The solution was filtered thrice, resulting in a clear filtrate. Then, 10 mL of 10 mM $AgNO_3$ was added to 4 mL of the *A. congensis* leaf extract filtrate. The solution was kept stirred at 70-80 ℃ for 1 h, and a color change from colorless to dark brown indicated the reduction of Ag^+ to Ag^0. The formation of AgNPs was confirmed using UV-Vis spectroscopy. The synthesized AgNP suspension was then centrifuged at 10 000 rpm for 15 min. Pellets containing AgNPs were washed thrice with distilled water to remove silver ions and leaf extract residues. Finally, the precipitated AgNPs were lyophilized.

2.4. Fourier transform infrared analysis (FTIR)

To detect the functional groups present in the lyophilized AgNPs and leaf extracts, FTIR analyses were conducted. Before analysis, each sample tablet was prepared in a 1∶100 ratio of sample to potassium bromide (KBr). An FT-IR spectrometer (Infrared spectrometer Varian 660 MidIR Dual MCT/DTGS Bundle with ATR) was used to record the spectra in the frequency range of 4 000 cm^{-1} to 500 cm^{-1}, with 200 scans per sample and a detector resolution of 4 cm^{-1}[18].

2.5. X–ray diffraction (XRD)

To determine the crystalline structure of *A. congensis*-AgNPs, the prepared samples were analyzed using X-ray diffraction on a Shimadzu XDS 2400H diffractometer with a Cu anode control. The

samples were placed in a Lucite holder on the goniometer of the instrument, which was outfitted with a PW3064 spinner stage and a divergence slit. XRD pattern analysis was carried out on each sample with a step size of 0.017° and a counting time of 14 s per step[18].

2.6. Scanning electron microscopy (SEM) and energy dispersive X-ray (SEM-EDX) analysis

An SEM (Hitachi SU 3500 scanning microscope, Tokyo, Japan) was utilized to examine the shape of the *A. congensis*-AgNPs and analyze their element compositions through SEM-EDX analysis[18].

2.7. Snail collection and identification

Adult *P. acuta* and *B. forskalii* were collected from freshwater ponds and a canal that constantly receives water from nearby ponds during the peak of raining season. An expert malacologist from the Department of Biological Sciences, University of Medical Sciences, Ondo, Nigeria, identified the snails. As freshwater snails do not require approval in Nigerian institutions, we were granted a waiver.

2.8. Ovicidal activities of plant extracts and A. congensis-based AgNPs

The snails' egg masses were laid on a clear polythene bag, which lined the container in which the snails were being cultured. The attached egg masses were then carefully removed from the bag and placed in distilled water in a beaker, with their surface facing the water interface to promote embryonic development and prevent dehydration. To investigate the effect of ethanolic extracts of *A. congensis* and its nanoparticle derivative on embryonic development, 2-3 egg masses containing 12-15 embryos in *B. forskalii* and 2 egg masses containing 14-18 embryos in *P. acuta* were exposed to varying concentrations of the extracts and nanoparticles for 6 d. Photomicrographic evaluation of the embryo development was carried out after the 6-day exposure at ×40 magnification.

2.9. Molluscicidal testing

A modified method described by the World Health Organization (WHO) was utilized to evaluate the molluscicidal activity of the extracts and AgNPs against adult snails[27]. After collection, the adult snails were fed with dried blanched water lettuce and placed in a bowl of distilled water lined with a transparent polythene bag. A minimum of 24 hours was allowed for the snails to adjust to the laboratory conditions prior to experimentation. The concentrations of the plant extract and the green synthesized nanoparticles were as previously prepared for ovicidal activity *i.e.* 0.5, 1, 2, 4 mg/mL and 0.013, 0.06, 0.13, and 0.25 mg/mL, respectively. A total number of 5 adult snails were exposed to 5 mL of each at varying concentrations. Mortality was determined after 24 h in ethanolic extract but between 5 and 40 min in *A. congensis*-based AgNPs. The experiment was performed in duplicate and distilled water was used as the negative control. The lethal concentrations (LC_{50}) were determined. Mortality was determined by lack of motility after a recovery period in distilled water.

2.10. Statistical analysis

The recorded snail mortality data were analyzed using SPSS statistical software version 23.0 (IBM Corp., Armonk, N.Y., USA). The accuracy of the data was verified before conducting the analysis. Two-way analysis of variance (ANOVA) was performed to assess significant differences in snail mortality at different concentrations and exposure periods. The LC_{50} of the extracts and AgNPs was determined using probit analysis. A *P*-value of less than 0.05 was considered statistically significant.

3. Results

3.1. FTIR results

The FTIR spectrum of *A. congensis* leaf extract revealed absorption peaks at 3 450.17, 1 725.63, 1 649.38, 1 548.36, 1 225.55, 1 000.65, 849.32, 775.09, and 710.52 cm^{-1}. The peak at 3 450.17 cm^{-1} indicated the presence of hydroxyl and phenols, as it represented the O-H stretch. Additionally, the N-H bending vibration of amines was represented by the peak at 1 725.63 cm^{-1}, while the band at 1 649.38 cm^{-1} represented C-C stretching vibrations of alkenes. The band observed at 1 548.36 cm^{-1} revealed the C-O stretching vibrations of esters, ethers, alcohols, or carboxylic acid. Aromatic C-C stretching vibrations in alkanes were indicated by the peak at 1 225.55 cm^{-1}. The presence of C-O stretching vibrations of the alkoxy group was revealed by the band at 1 000.65 cm^{-1}. Furthermore, the mono-substituted aromatic C-H bond of alkenes was shown by the band observed at 849.32 cm^{-1}, and the band at 775.09 cm^{-1} demonstrated the alkyne C-H bending vibration. Finally, the C-Cl, C-Br, and C-I stretching vibrations of alkyl halide were visible in the band at 710.52 cm^{-1}, as depicted in Figure 1.

The absorption peaks in the FTIR spectrum of *A. congensis*-based AgNPs were at 3 000.35, 1 725.63, 1 552.83, 1 504.27, 1 301.41, 1 228.08, 1 150.49, 948.73, 900.35, 803.47, and 700.83 cm^{-1}. The peak at 3 000.35 cm^{-1} indicated the presence of O-H stretching vibration in carboxyl and amino groups. The band at 1 725.63 cm^{-1} revealed the stretching vibrations of the aliphatic C=C group of alkenes. The stretching vibrations of the C=C group of alkenes, the N-H group of amides, and amine salts were represented by the band

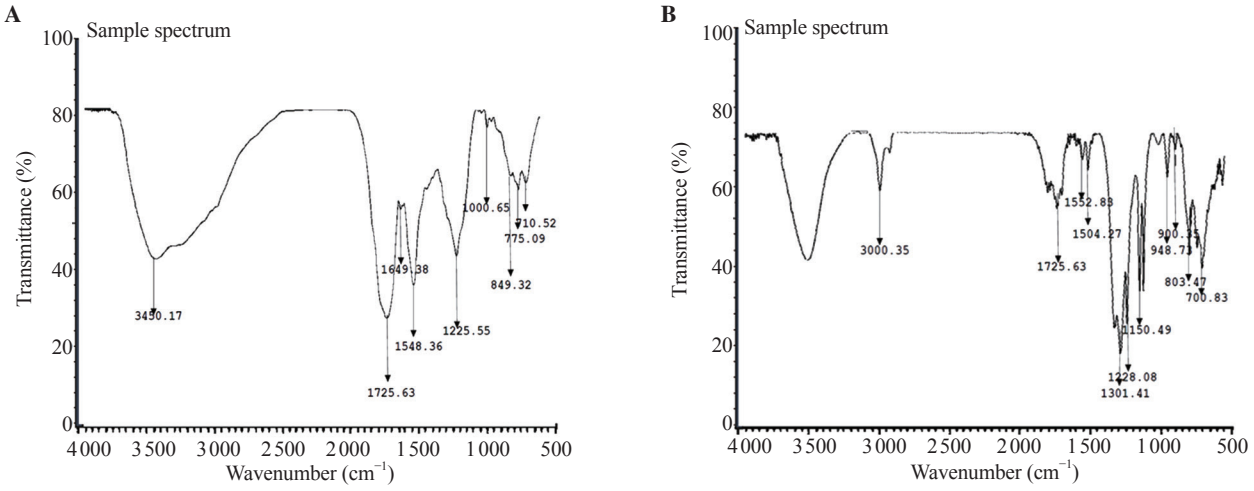

Figure 1. FTIR spectra of (A) *Alstonia congensis* leaf extract and (B) its silver nanoparticles.

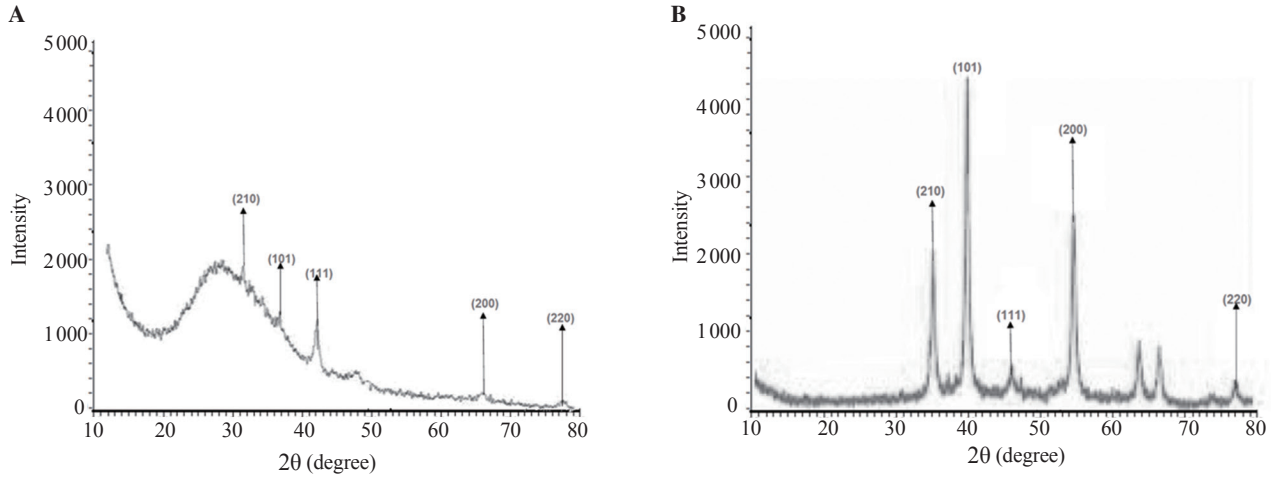

Figure 2. X-ray diffraction analysis of (A) *Alstonia congensis* leaf extract and (B) its silver nanoparticles.

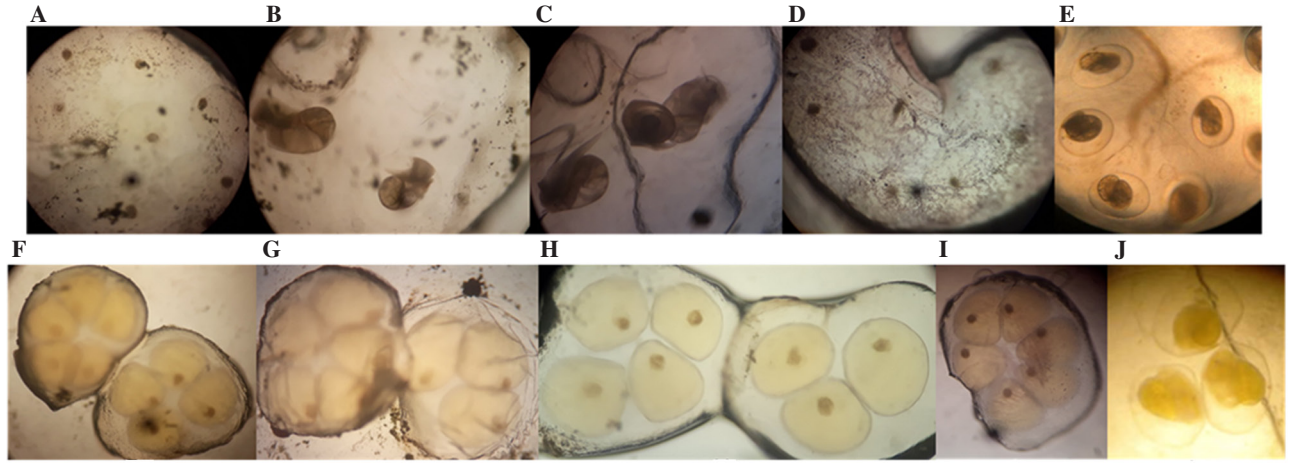

Figure 3. Ovicidal activities of *Alstonia congensis* leaf extract and its green-synthesized nanoparticles (ACNPs) against *Physa acuta* (A-E) and *Bulinus forskalii* (F-J) eggs (Magnification: ×40; scale bar: 21 µm). A and F: *Alstonia congensis* leaf extract 2 mg/mL; B and G: *Alstonia congensis* leaf extract 1 mg/mL; C and H: *Alstonia congensis* leaf extract 0.5 mg/mL; D: ACNPs 0.06 mg/mL; E: Negative control for *Physa acuta*; I: ACNPs 0.013 mg/mL; J: Negative control for *Bulinus forskalii*.

at 1 552.83 cm^{-1}. The band at 1 504.27 cm^{-1} demonstrated C=O stretching of the amide group, which is responsible for Ag^{+} reduction to Ag^{0}. Moreover, the peak at 1 301.41 cm^{-1} revealed the presence of C-O-C stretching vibrations of carboxyl. The band at 1 228.08 cm^{-1} denoted the presence of carboxylic acid C-H bending vibrations. The stretching vibrations of C-O groups of anhydrides, esters, ethers, alcohols, and carbonyl phenols were indicated by the band at 1 150.49 cm^{-1}. The band observed at 948.73 cm^{-1} showed C-O-C, C-O-P, and O-H stretching vibrations of carboxyl polysaccharides. Additionally, the 900.35 cm^{-1} band represented an aliphatic C-N stretching vibration of carboxyl groups and amines. The band at 700.83 cm^{-1} revealed C-H bending vibrations of alkynes, while the band at 803.47 cm^{-1} denoted C-O stretching vibrations of alkoxyl groups, as shown in Figure 1.

3.2. XRD analysis

Figure 2 displays the XRD pattern obtained from the leaf extract of *A. congensis*, revealing major peaks at (2θ) 32.00, 37.00, 42.05, 66.00, and 77.05, which corresponded to the (210), (101), (111), (200), and (220) planes, respectively. Similarly, the XRD pattern obtained from the AgNPs using *A. congensis* is presented in Figure 2, which demonstrates the primary peaks at (2θ) 35.00, 40.00, 46.00, 54.06, and 77.00, corresponding to the (210), (101), (111), (200), and (220) planes, respectively.

3.3. SEM/EDX analysis

The supplementary figure displays the SEM image of the AgNPs, which revealed that the surface morphology of the green-synthesized nanoparticles contained clusters of irregularly shaped nanoparticles. The nanoparticles had a surface area of 23.068 m^2/g, a pore volume of 0.089 cm^3/g, a pore diameter of 18.97 nm, and a size of 39.72 nm. The EDX spectrum showed a typically optical absorption peak at approximately 4 eV and indicated that the nanoparticles were composed primarily of silver (Ag) at 70.48%, with oxygen (O) at 17.58%, nitrogen (N) at 4.57%, iron (Fe) at 2.15%, copper (Cu) at 2.04%, and chloride (Cl) at 2.01%.

3.4. Ovicidal activities of A. congensis extract and green-synthesized nanoparticles

The plant extract inhibited embryonic development within the egg masses of *B. forskalii* in all tested concentrations (0.5-4 mg/mL), but the ovicidal activity was only observed in 4 mg/mL and 2 mg/mL of *A. congensis* extract against *P. acuta*. No development of embryos of *B. forskalii* and *P. acuta* beyond the blastula stage was observed in all the tested concentrations of AgNPs. The ovicidal activities of the plant extract and the AgNPs in selected concentrations are shown in Figure 3.

3.5. Molluscicidal activity against adult snails

The *A. congensis* extract showed no molluscicidal efficacy against adult *P. acuta* at all concentrations tested, but a very weak molluscicidal activity against *B. forskalii* was recorded after 24 h of exposure (Figure 4). The molluscicidal activities of *A. congensis*-based AgNPs against adult *P. acuta* are presented in Figure 5. The molluscicidal activities were very strong at higher concentrations of 0.13 and 0.25 mg/mL as all snails were observed dead within 5 min of exposure. Only 2 (40%) and 4.5 (90%) of the snails were observed dead at lower concentrations of 0.013 and 0.06 mg/mL after 40 min of exposure (Figure 5). The snail mortality was both concentration and time-dependent ($P<0.05$).

The mortality of adult *B. forskalii* exposed to *A. congensis*-based AgNPs was concentration-dependent ($P<0.05$) but not time-dependent ($P>0.05$). The mortalities of *B. forskalii* exposed to the AgNPs after 5 and 10 min were significantly higher than the snails' mortalities recorded in the *A. congensis* extract after 24 h of exposure ($P<0.05$). All adult *B. forskalii* snails were observed dead within 5 min of exposure to 0.13 mg/mL of the nanoparticles (Figure 6).

The LC_{50} of AgNPs against adult *P. acuta* were 0.078, 0.078, 0.075, 0.061, and 0.052 mg/mL at 5, 10, 20, 30, and 40 min, respectively, while the LC_{50} against adult *B. forskalii* were 0.072 and 0.056 mg/mL at 5 and 10 min, respectively. A higher LC_{50} of 0.49 mg/mL was recorded for the *A. congensis* extract after 24-h exposure of *B. forskalii*.

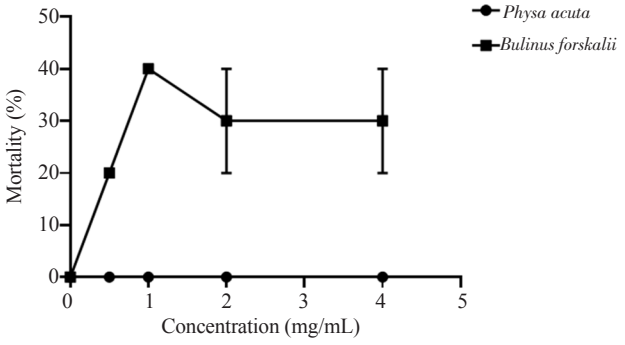

Figure 4. Molluscicidal activities of ethanolic extract of *Alstonia congensis* against *Physa acuta* and *Bulinus forskalii* after 24 hours of exposure.

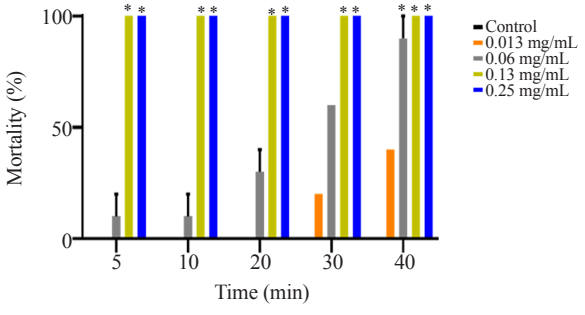

Figure 5. Effect of different concentrations of *Alstonia congensis*-based silver nanoparticles against *Physa acuta*. *denotes a significant difference at $P < 0.05$ compared with the control.

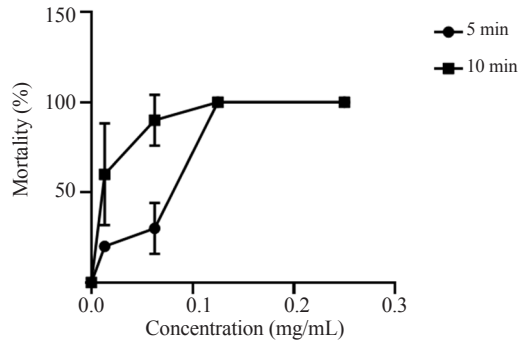

Figure 6. Molluscicidal activity of the green-synthesized nanoparticles against *Bulinus forskalii*.

4. Discussion

The use of naturally derived molluscicides has been widely advocated in recent times. While plant molluscicides have recorded remarkable activities against freshwater snail intermediate hosts of trematodes[8,28], the fabrication of plants into green-synthesized nanoparticles has further enhanced their molluscicidal potential[11,21]. This approach is however just emerging and it needs to be more explored owning to the acclaimed potentials reported in the few available studies.

The formation of *A. congensis*-based AgNPs can be confirmed by the color change of the solution, which transforms from colorless to dark brown due to the interaction of electromagnetic radiation with conduction band electrons, a phenomenon known as surface plasmon resonance[29]. UV-Vis spectroscopy was proved to be a valuable tool for the characterization of AgNPs, as it allowed for easy identification of the surface plasmon resonance absorption peak[24]. The absorption wavelength of the formulation at 425 nm was found to be comparable to the value of 426 nm observed for silver nanoparticles synthesized using *Alstonia scholaris*, which were employed to improve the catalytic decomposition of methylene blue in water[24]. The major phytochemicals in *A. congensis* are alkaloids, saponins, tannins, and flavonoids[22]. The alkaloidal and carbonyl functionalities present in the plant extract used for green synthesis are responsible for the sharp absorption bands observed in the formulation, which represent the N-H bending, C-N stretching, and C-O stretching vibrations[24]. The small band at 700.83 cm^{-1} is attributed to the bending vibrations of –C-H groups[30], hence, the FTIR spectrum of the *A. congensis*-based AgNPs suggested the involvement of phytochemicals of *A. congensis* in the reduction of Ag^{+} ions and protection of the green-synthesized nanoparticles[24]. The sharp XRD peaks depict the crystallinity properties of the nanoparticles and a similar observation was reported for green-synthesized *Alstonia scholaris* nanoparticles[24]. Other elements such as Cl, O, Cu, and Fe observed in the formulation could be natural phytoconstituents present in the plant used for green synthesis. For example, Cl, Cu, and Fe are important plant micronutrients. The aggregation properties of nanoparticles as revealed by SEM can be associated with the structural and electronic properties of phytoconstituents[31].

Several plants have been reported to elicit molluscicidal and ovicidal activities[10,27,32]. The present study showed the ovicidal activities of the plant extract (except against *P. acuta*) and the *A. congensis*-based AgNPs, howbeit, the latter was more efficacious against *P. acuta* at more than 30-fold decreased concentration compared with the plant extract. The least concentration in AgNPs (0.013 mg/mL) was able to inhibit *P. acuta* embryonic development with the embryos not developing beyond the blastula stage. Only 2 and 4 mg/mL concentrations of *A. congensis* extract showed impressive ovicidal activities similar to what was observed in AgNPs. The efficacy of a very low dosage of green-synthesized nanoparticles against the parent plant extract to achieve total elimination of developing snail embryos within the egg masses is important in the sustenance of a green environment and the practicability of the approach. Our earlier study also showed superior ovicidal activity of plant-based green synthesis over a crude extract from the parent plant[18]. The superior potential of our green-synthesis formulation to inhibit embryonic development could be a result of the increased bioavailability of the plant bioactive ingredients to the developing embryos within the gelatinous egg masses[11]. The fabrication of the plant material into a nanosized form resulting in an alteration or an increase in composition or abundance of certain functional groups could influence the lipophilicity of the formulation which may in turn facilitate the egg-penetrating ability of the nanoparticle[11].

This study further showed the superior efficacy of green-synthesized nanoparticles over the parent plant extract against adult *P. acuta* and *B. forskalii*. The relationship between the concentration of nanosized *A. congensis* and its effectiveness against adult snails differed from a previous study on polymeric entrapped nanoparticles containing curcumin-nisin[11], but was consistent with other research that used plants as molluscicides[27,33]. While no death was recorded in *P. acuta* exposed to the plant extract after 24 h, a weak molluscicidal activity was observed against *B. forskalii*. In the latter, there was hypersecretion of mucus and complete retraction into the shell among the snails that died[34]. Others, however, attempted to escape the extract treatment. The impressive and rapid molluscicidal activities of the green-synthesized nanoparticles against the two snail species in this study were probably due to an acute toxic effect of the nanoparticles, and this is desirable as it reduces the possibility of escape behavior observed in plant extract at sub-lethal doses[32,35].

The differential responses of the two snails to the plant extract and the nanoparticles could be associated with marked differences in the stress tolerance level in the two pulmonates. The *P. acuta* capacity to survive in plant extract at sub-lethal concentrations has been

attributed to the invasive behavior of the snail, which is associated with its tolerance to high levels of pollutants[35]. On the other hand, *B. forskalii* has been reported to be one of the most sensitive snails to toxicants[36], therefore, it is susceptible to the highly efficacious green-synthesized nanoparticles.

However, this study has some limitations. We did not compare the molluscicidal activities of the green-synthesized nanoparticles with niclosamide, a standard molluscicide agent. Additionally, although the formulation showed impressive molluscicidal activities, we did not investigate the mechanisms behind the molluscicidal activity of the nanoparticle against freshwater snails. Furthermore, we did not assess the toxicity of the nanoparticle to non-targeted organisms.

In conclusion, this study showed the high efficacy of *A. congensis*-based nanoparticles against the developing embryos within the gelatinous egg masses and the adult snails at a very low concentration. It can be further explored for possible development of molluscicides. Although green-synthesized nanoparticles are generally acclaimed as safe, subjecting the formulation to *in vitro* toxicity assessments against non-targeted organisms, and *in vivo* testing in the murine model will provide more data to support its potential adoption as a future molluscicide. It is also recommended that mechanistic studies be carried out on the molluscicide to gain insight into its activities' pathways.

Conflict of interest statement

The authors declare no competing interests.

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Authors' contributions

OTO conceived, designed, supervised the work, and wrote the paper. OTO, BMB, TCA, PAA and ITO conducted the experiments. Both OTO and ITO analyzed the data. All authors approved the final manuscript.

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