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In vitro scolicidal activity of synthesised silver nanoparticles from aqueous plant extract against *Echinococcus granulosus*

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ABSTRACT

At present, biosynthesis of AgNPs is a very effective method to produce less toxic nanoparticles. The vision of this research is to use three different plant extracts derived from leaves of *Piper nigrum*, *Ziziphus Spina-Christi* and *Eucalyptus globulus* for rapid biosynthesis of AgNPs. This is in addition to investigating the scolicidal activity against *Echinococcus granulosus*. The methods of UV–vis spectroscopy, X-ray diffraction (XRD), scanning electron microscopy (SEM), and energy-dispersive X-ray analysis (EDX) were employed to characterise the nanoparticles. UV spectra disclosed a maximum absorption at 437 nm for the biosynthesised AgNPs using EUCGLO extract. The XRD patterns revealed the (fcc) structure of the AgNPs with slightly shifted characteristic peaks at 2θ degree of 37.3° and 43.4°, respectively. The scolicidal activity against *E. granulosus* revealed that the AgNPs, which were synthesised using *Eucalyptus globulus*, have powered scolicidal of 47.8 % after 45 min. which is comparable to the treatment by Albendazole.

1. Introduction

Echinococcus granulosus is a type of parasitic worms (*Cestoda*), which is considered as the main causative agent of the Hydatidosis. This is due to the migration and development of larvae in different human organs [1]. In particular, it develops in the liver with no symptoms for several years before forming cysts inside the liver. Hydatidosis is reported as a worldwide harmful epidemic disease [2]. Iraq (where the research was carried out), is considered one of those countries that are affected by this endemic disease. Generally, the most effective treatment of that disease includes surgery which is quite difficult for some cases when cysts have spread out into many organs or formed in risky locations [3]. Chemotherapeutic treatment is also used for healing. However, the only available licensed medication in the market is the Benzimidazole. Furthermore, their use is limited due to the lower solubility in water in addition to the risk of side effects [1,4,5].

Nanotechnology and nanoscience are important for the prosperity of many fields and sciences. The nanoscale size of the materials makes it applicable to the different potential applications. The usage of nanomaterials has been developed in many fields such as semiconductors [6], thermoelectric materials [7], catalysts [8], carbon capture [9], biomaterials [10], drug delivery [11], supporting materials [12,13], porous materials [14], cancer treatment [15,16], medical treatment [17] and other applications. Recently, the researchers are putting in a great effort in investing the nanomaterials in biomedical and pharmaceutical applications. Despite the traditional methods to produce nanoparticles, plant extracts represent an eco-friendly biological synthesis of the nanoparticles [18]. The phytochemicals in the plant extracts act as a reducing agent to the metal ions to produce metal nanoparticles [19].

Over the recent years, tremendous researches investigated the biosynthesis and utilization of silver nanoparticles in germination [20], in medicine as antibacterial agent [21,22], and as an anticancer agent [23]. Silver nanoparticles also employed as alternative scolicidal agents of hydatid cyst. For example, the scolicidal activity of the selenium nanoparticles, which are derived from marine bacterial strain (Bacillus sp.), was investigated against *E. granulosus* [24]. Aqueous aerial extract of Penicillium aculeatum is reported in the synthesis of silver nanoparticles (AgNPs) for the scolicidal activity [2]. Most recently, a published paper compared the scolicidal activity of some nanoparticles (AgNPs, FeNPs, CuNPs, SiNPs, and ZnNPs) against hydatid cyst protoscolices [3].

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The objective of the current work is to explore the *in vitro* scolicidal activity of silver nanoparticles (AgNPs) against hydatid cyst protoscolices. The AgNPs were derived from different concentrations of plant extracts of *Piper nigrum* (PN), *Ziziphus Spina-Christi* (ZSC) and *Eucalyptus globulus* (EUCGLO). A comparative study was conducted to figure out the optimum plant extract for the biosynthesis of AgNPs and the optimum exposure time for treatment of the hydatid cyst.

2. Materials and methods

2.1. Collection and preparation of the aqueous extracts

The PN, ZSC, and EUCGLO plant leaves were collected somewhere in western Iraq during January, February and March 2019. The plants were washed thoroughly with distilled water to remove the soil and the impurities. The leaves were kept inside the oven on a non-adhesive plate at 25 ° C for drying. The dried leaves were ground using electrical miller then the powder was sieved using 15 mesh size sieve. The final plant powders were kept in well-sealed nylon bags for further treatments.

2.2. Preparation of aqueous extraction

In a typical synthesis, 10 g of the dried leaves powder was mixed with 100 mL of distilled water in a 250 mL size beaker. The mixture then magnetically stirred for 2 h at 50 ° C. The supernatant then filtered using a paper filter size 0.4 μm. The supernatant then dried in the oven at 60 ° C to obtain a fine powder of plant leaves extract. Finally, 10 mg of the powder extract was suspended in a 100 mL distilled water to obtain 100 ppm of the aqueous plant extract.

2.3. Biosynthesis of silver nanoparticles

Typically, 1 mL (100 ppm) of aqueous extract was mixed with 10 mL (0.001 M) silver nitrate in a test tube. The mixture was shaken for 5 min then kept for 1 h at room temperature in a dark place. A change from colourless to brown colour was a primary indicator of the AgNPs existence. Another two sets of AgNPs were synthesised using 2 mL and 3 mL of the extracts. X-ray diffractometer (XRD, type PANalytical X'Pert PRO, Almelo, Netherlands) was utilized to evaluate the crystalline structure of the samples. The test was performed with Cu-Kα radiation (λ = 1.54178 Å), at a power of 40 kV and 40 mA over 2θ range from 10 ° to 80 °. The morphology and the particle size of the synthesised AgNPs were investigated using scanning electron microscopy (SEM; FEG-SEM MIRA3 TESCAN, Czech Republic). The energy-dispersive X-ray analysis was carried out using the same SEM. Finally, the surface plasmon resonance (SPR) of the silver nanoparticles was characterised using a UV–vis spectrum, Shimadzu UV-1800/Visible spectrophotometer.

2.4. Hydatid cysts collection

Hydatid cysts of the infected sheep livers were collected from the local slaughterhouse in Ramadi city – the capital of the Anbar province west of Iraq. The infected livers were taken directly to the lab. The hydatid cysts were firstly sterilised twice with 1% alcoholic iodine using medical cotton. To reduce the internal pressure of the cysts, 10 mL of hydatid fluid was sucked using a G21 syringe needle. After which, using a sterile medical scalpel, the cysts were carefully opened and the hydatid fluid was drained using Pasteur pipette. Then the protoscolices transferred into tubes and washed three times. The first wash was carried out using PBS solution then centrifuged for 15 min. at 3000 rpm. The second wash was with PBS containing penicillin (20 IU/mL) and streptomycin (1 mg/mL). The final wash was carried out with PBS. The supernatant was removed from the liquid and a fresh PBS was added into the hydatid cysts precipitate.

2.5. Viability of protoscolices

The viability test was performed according to a method reported by Smyth and Barrett [25]. Briefly, 20 micrometres of suspended protoscolices in the PBS was mixed with the same volume of 0.1 % aqueous eosin dye. The samples were dropped on glass substrates and monitored under the microscope with a magnification of 40 ×. After five minutes, the bright green protoscolices, which did not absorb the dye, were considered as viable, while the reddish ones were considered dead protoscolices. The percentage of protoscolices at time zero was calculated before conducting the treatments with AgNPs according to the following equation [26]

$$\text{Viability percentage} = \frac{\text{No. of viable protoscolices}}{\text{Total no. of protoscolices calculated in the sample}} \times 100\%$$

The process was repeated five times before taking the rate of the viable ratio. The percentage of protoscolices was calculated after each exposure time. For accuracy, the operations were performed on the day of collecting the hydatid cysts.

2.6. Preparing the protoscolices samples for examination

The scolicidal activity of the biosynthesised AgNPs and the negative control (plant extracts) was carried out at different times of inhibition. The experiments were designed in five replicates. Initially, 1 mL of the protoscolices solution (each 1 mL contains 500 protoscolices) was poured in a sterile glass tube. The same volume of the aqueous colloidal of AgNPs was mixed with the PBS solution of the protoscolices. The same process was repeated with all controls. The samples were kept in a water bath at 37 °C before investigating the viability of the protoscolices at times of (15, 30, 45 min.). Besides, a 10 μg/ mL of Albendazole (Haryana, India) was used as a useful and reliable positive control for validating the experimental procedure.

2.7. Preparing the samples for the optical microscopy

The slides of the optical microscopy were prepared by dropping 20 μL of each sample. The samples were investigated before and after adding the same volume of solution contains eosin dye (0.1 %). The number of live protoscolices was counted. The stained protoscolices with a green colour were considered to be vital. The plasma membrane of the live protoscolices has a permeable property which prevented the eosin dye to penetrate. While the red protoscolices were considered dead cells due to the penetration of the eosin stain through the plasma membrane [27].

2.8. Statistical analysis

The mean and the standard deviation were calculated for the data. In addition, variance analysis and Dunkin test were performed using SPSS® Statisticsv23.0 software.

3. Result and discussion

3.1. Characterisation of AgNPs

The characteristic properties of the silver nanoparticles were monitored using a double beam UV–vis spectroscopy, XRD

spectroscopy, SEM imaging and EDX. Further to the colour changing confirmation, the UV–vis spectra were performed to validate the formation of the AgNPs. The surface plasmon resonance (SPR) was recorded for the maximum absorbance. SPR of the AgNPs was distinctly observed at 437 nm for the synthesised AgNPs using EUCGLO extract (Fig. 1). Fig. 2 illustrates the X-ray patterns of the dried AgNPs derived from leaves extracts of PN, ZSC, and EUCGLO. The patterns demonstrate the presence of two characteristic peaks of AgNPs corresponding to the crystalline planes of (111) and (200). According to the standard JCPDS file No. 04-0783, the Bragg's angles of the fcc structure of AgNPs are reflecting at 2 θ of 38° and 44°. However, the characteristic peaks slightly shifted than the standard at 2 θ of 37.3° and 43.4°, respectively. Scherrer equation was utilised to calculate the crystalline particle size. Sample E3 exhibited a smaller average crystalline particle size (17 ± 1 nm) among other samples. The average crystalline particle size of the biosynthesised AgNPs decreased with increase in the concentration of the plant extract. This can be seen in Table 1.

The particle size of the AgNPs is distributed in the range of 8−35 nm. Some agglomerated nanoparticles have been distinguished in the images might be occurred during preparing samples for the SEM analysis. In parallel to the XRD technique, the purity of the AgNPs, which was verified by XRD analysis, was confirmed by the presence of the signals of Ag atoms in the spectra of the energy-dispersive X-ray analysis. The elemental analysis EDX was performed to determine the composition of the biosynthesised AgNPs. Fig. 4 illustrates the EDX spectrum of the AgNPs. The spectrum includes additional peaks denoted the X-ray emission of C, O and Cl atoms, which might be emitted from the biomolecules of the plant extracts.

3.2. Scolicidal activity of AgNPs

Up to date surgery is still considered the appropriate treatment choice for the problematic cases of the hydatidosis. Today, many researchers investigated the antiparasitic and inhibitory effects of several nanoparticles on protoscolices. For example, H. Barabadi and his group investigated the effect of different concentrations and inhibition times of AgNPs on the scolicidal activity against protoscolices of CHD [28]. Also, Roghayeh recommended Ag, Fe, Cu, Si and Zn nanoparticles, as scolicidal agents against hydatid cyst protoscolices [3]. However, for the best of our knowledge, nobody carried out a comparative study on the socolicidal activity of the nanoparticles concerning and medicine (Albendazole). In addition,

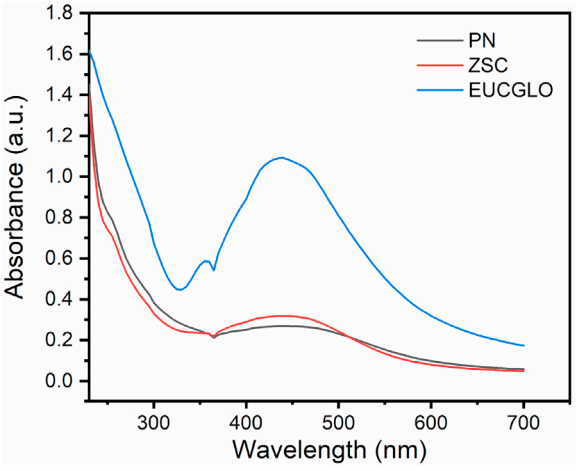

Fig. 1. UV–vis spectrum of AgNPs synthesised using PN, ZSC, and EUCGLO plant extracts.

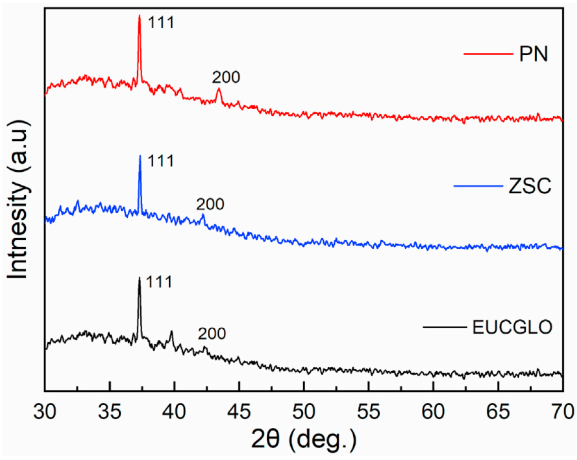

Fig. 2. X-ray patterns of AgNPs synthesised using PN, ZSC, EUCGLO plant extracts.

The morphology and the topography of the AgNPs were studied using a scanning electron microscope (SEM). The spherical shape of the AgNPs is distinctly observed from the SEM images (Fig. 3).

the majority of the research does not consider the maximum concentration of nanoparticle of the dose to avoid the toxic effect. Finally, the impact of particle size of the nanoparticles has not been taken into considerations. In the present research, the emphasis was placed on one-step green synthesis of silver nanoparticles from different plant extracts of different concentrations. The plant extracts are alternative reducing and stabilising agents to the toxically chemical reducing agents. The natural compounds in the plant extract grant the direct use of the aqueous colloidal of AgNPs for the treatment of the protoscolices after synthesis. The validity of AgNPs as a scolicidal agent was assessed by the Albendazole (positive control). The treatment of hydatid protoscoleces by Albendazole was reported a long time ago using different concentrations [29]. Fig. 5 shows the protoscolices of *E. granulosus* before stained with eosin dye (image-a) and after stained with eosin dye (images- b and c). The number of dead protoscolices in one millilitre of the solution has been calculated during the exposure time before and after the treatment with the AgNPs. Before investigating the scolicidal activity of AgNPs, the mortality of the protoscolices has been recorded and eliminated from the real percentage of the scolicidal activity of AgNPs as illustrated in Fig. 6.

Fig. 7 illustrates the scolicidal activity of the synthesised AgNPs derived from different concentration of the PN, ZSC extracts, and EUCGLO extracts against the protoscolices of *E. granulosus*. In addition, the figure includes the treatment of the *E. granulosus* protoscolices with the Albendazole as positive control and plants extracts as a negative control. According to the obtained results, generally, the death percentage of the protoscolices increased with the exposure time for the different samples. Furthermore, concerning the concentration of the plant extracts, the highest scolicidal activity increased as the higher concentration of different plant extract is used to synthesise AgNPs. It can be seen from Fig. 7 that the mortality rate of the AgNPs derived from EUCGLO was at high rates compared to those derived from PN and ZSC extracts. The highest mortality rate of the protoscolices increased from 10.4 % after 15 min. to 47.8 % after 45 min. of exposing the protoscolices to the nanoparticles. Considering the low concentration of the AgNPs, the scolicidal activity of this biosynthesised Ag nanoparticles demonstrated a reasonable efficiency when compared with treatment by Albendazole (68.15 %) after 45 min. While the highest scolicidal activity of the AgNPs derived from PN extracts increased from 6.8 % after 15 min. to 22.6 % after 45 min.. In general, the treatment with

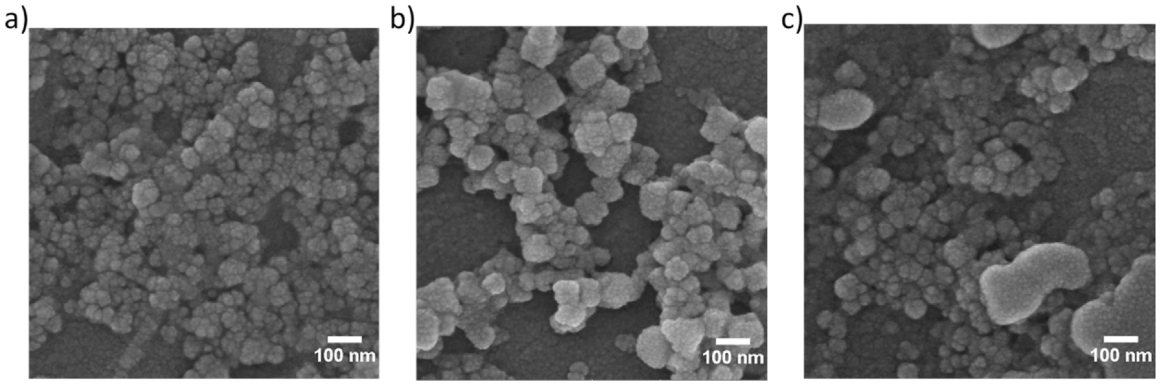

Fig. 3. SEM images of AgNPs synthesised using : (a) PN extract, (b) ZSC extract, and (c) EUCGLO extract.

Table 1
The XRD particle size of AgNPs derived from different concentrations of PN, ZSC and EUCGLO extracts.

	Scilicidal agent	Concentration	XRD particle size (±1 nm)
Piper nigrum (PN)	P1	1:10	22
	P2	2:10	21
	P3	3:10	19
Ziziphus Spina-Christi (ZSC)	Z1	1:10	21
	Z2	2:10	21
	Z3	3:10	19
Eucalyptus globulus (EUCGLO)	E1	1:10	21
	E2	2:10	20
	E3	3:10	17

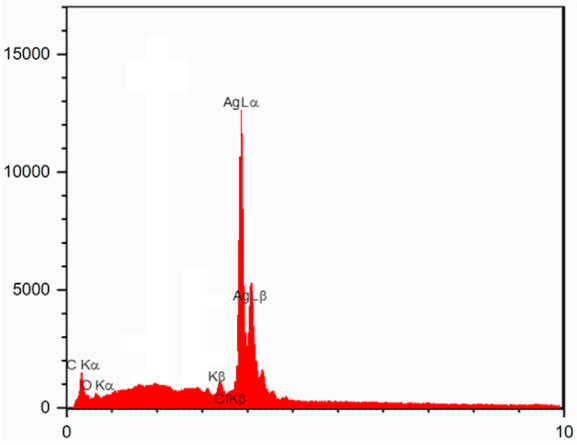

Fig. 4. EDX analysis of AgNPs derived from EUCGLO (E3).

AgNPs derived from PN extract does not prove to have high scolicidal activity comparing to Albendazole.

Furthermore, the mortality rate of the AgNPs derived from ZSC extracts was slightly higher than that recorded for the AgNPs derived from PN extracts. The highest mortality rate of the AgNPs was 23.8 % after 45 min. of the treatment. The similarity between the results, which were obtained from the treatment by both types of biosynthesised AgNPs, might be related to the close particle size of both types of AgNPs.

The scolicidal activity of the biosynthesised AgNPs derived from EUCGLO extracts was approximately 2 folds potent than that of the AgNPs derived from PN and ZSC extracts. With regards to the results of treatment with 10 μg/ mL of Albendazole, the treatment with AgNPs derived from EUCGLO extract produced reasonably and comparable results.

The cytotoxicity of the Ag nanoparticles evolved from the released Ag+. Many mechanisms described the cells death by interacting with the Ag+. Ag ions, which are capable of changing

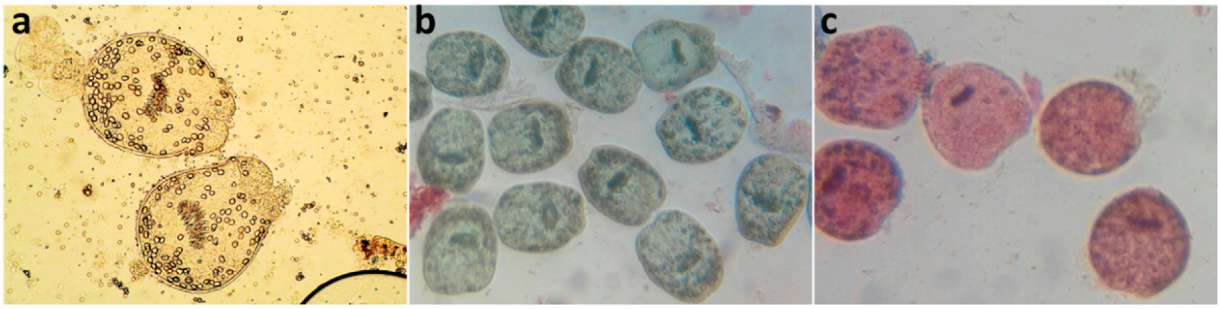

Fig. 5. The live and dead protoscolices. (a) The live protoscolices before staining with 0.1 % eosin. (b) the live protoscolices (green colour), (c) the dead protoscolices (red colour), after staining with 0.1 % eosin dye (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

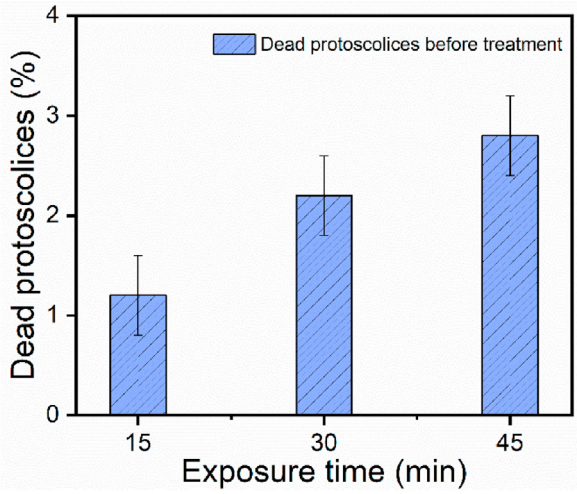

Fig. 6. The number of mortality of E. granulosus parasite protoscolices before treatment with the AgNPs.

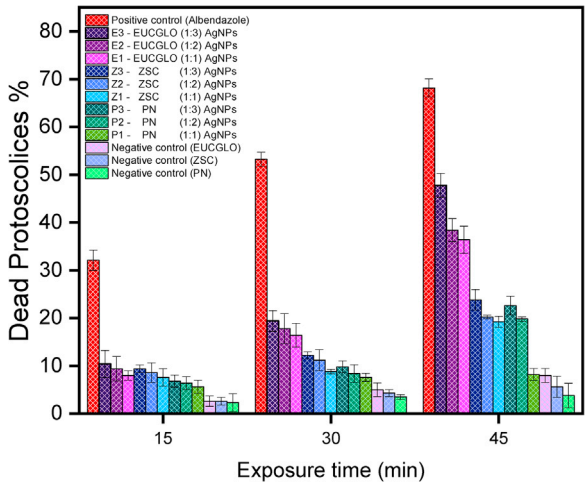

Fig. 7. The scolicidal activity of AgNPs synthesised using PN extracts, ZSC extracts, and EUCGLO extracts against protoscolices of E. granulosus with exposure times.

the three-dimensional structure of the microorganism, can interact with the disulphide bonds of the proteins. That interaction prevents the functionality of the microorganisms [30]. Other mechanisms include proton motive force, forming complexes with DNA and RNA, and breaking the mitochondrial membranes of the cells [31]. In general, for biological systems, as the complexity increases, it is assumed that the toxicity of the nanomaterials decreases [32]. Therefore, the cells of the higher organisms such as plants and animals are less sensitive to the toxic effects of the Ag nanoparticles. It is thought that the complex biological systems own several defence mechanisms that enable them to resist the toxicity of high concentrations of AgNPs [33]. The concentration of AgNPs, in the current research, is less than 100 μg/mL. The reason is to reduce the toxicity effect on the human cellular system.

The high scolicidal activity of the biosynthesised AgNPs derived from EUCGLO extracts might be due to the smaller particle size of the nanoparticles and the optimum synthesis of the nanoparticles. The optimum synthesis of AgNPs using EUCGLO extract compared to the PN and ZSC extracts are distinctly observed from the UV–vis spectra (Fig. 1) and the particle size results (Table 1). This might be due to the superior role of the EUCGLO extracts as reducing and capping agents than the PN and ZSC extracts. The morphology and the particle size of the metallic nanoparticles relay on the percentage of the metallic ions in the solution to the capping and stabilising agents [34]. The greater amount of the capping and stabilising agents present in the higher concentration of plant extract, the increased the amount of the Ag nanoparticles. The scolicidal activity of the Eucalyptus against protoscolices of hydatid cyst is reported somewhere in the literature [35]. This foundation is in agreement with the conclusion of the current research.

4. Conclusion

In the current work, the rapid successful synthesis of AgNPs has been reported using PN, ZSC and EUCGLO plant extracts. The characterisation revealed the crystalline fcc nature of the AgNPs. The findings of the current work denoted that the EUCGLO plant extract displayed a powerful role as reducing and capping agents than the PN and ZSC extracts. Moreover, the biosynthesised AgNPs using EUCGLO exhibit a significant scolicidal activity against *E. granulosus* of 47.8 % after 45 min.. Although a good scolicidal agent should be able to kill more than 90 % of protoscolices in minimum time. The comparable lower scolicidal activity of the AgNPs (47.8 % after 45 min.) is due to implementing a low concentration of Ag nanoparticle to avoid the toxicity into the humane textures. As regards, the AgNPs exhibited reasonably comparable results to that results which were obtained from treatment the protoscolices with 10 μg/ mL of Albendazole.

Author agreement

All authors declare that descriptions are accurate and agreed to submit the final version of the manuscript.

CRediT authorship contribution statement

Thaer Abdulqader Salih: Methodology, Writing - original draft. **Khalil T Hassan:** Supervision, Validation, Visualization, Writing - review & editing. **Sattar Rajab Majeed:** Methodology. **Ibraheem J. Ibraheem:** Investigation. **Omar. M. Hassan:** Software, Data curation. **A.S. Obaid:** Formal analysis, Writing - original draft.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.btre.2020.e00545.

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