Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2019; 8(6): 2116-2121
Received: 06-09-2019
Accepted: 10-10-2019

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In-vitro anthelmintic evaluation of synthesized silver nanoparticles of *Moringa oleifera* seeds against strongyle nematode of small ruminants

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Abstract
Moringa oleifera is one of the most commonly used medicinal plants for various conditions in India. The present experiment was much focused to explore the *in-vitro* anthelmintic activity of aqueous extract of *Moringa oleifera* seeds and its synthesised silver nanoparticles against strongyle nematodes of small ruminants. The synthesised silver nanoparticles were characterized by UV spectrophotometer, TEM, SEM and FTIR analysis. The *in-vitro* anthelmintic activity was carried out by egg hatch assay using various concentrations of aqueous extract and its synthesised silver nanoparticles and compared with the standard anthelmintic drug, thiabendazole.

The qualitative phytochemical analysis of the aqueous seed extract revealed the presence of flavonoids, tannins, terpenoids and sterols. On characterization, the UV- spectrophotometer analysis showed a peak at 460.8 nm. The Scanning and Transmission Electron Microscopy analysis showed particles with the size of 10-30 nm and 30 nm respectively. The Fourier transform infrared analysis exhibited peaks at various absorbance, indicating the presence of alkynes, amines, ketones, aldehydes and carboxylic acids.

The results of the egg hatch assay indicated a significant difference in the percentage of inhibition of hatching at different doses. The aqueous extracts and silver nanoparticles of *Moringa oleifera* seeds showed 95% and 81% inhibition on egg hatching at the concentration of 50 mg/ml and 8 mg/ml respectively.

This study demonstrated an ovicidal activity by both aqueous and synthesised silver nanoparticles of *Moringa oleifera* seed extract and paved the way for further research with their bioactive metabolites as nano preparation responsible for this activity.

Keywords: *Moringa oleifera,* silver nanoparticles, egg hatch assay, ovicidal effect

Introduction
Livestock is one of the fastest growing agricultural subsectors in our country. This fast pace of growth in this sector could probably be triggered by increasing demands for livestock products due to ever growing population, unchecked urbanization and increase per capita income. In addition, as the crop failures due to vagaries of monsoon have been increasing year after year, the small ruminant rearing has become a vital component of rural economy and livelihood for the marginal, landless and poor farmers (Misra *et al.,* 2006) [9]. However, further growth in the small ruminant production mainly lies on proper disease control programme.

Among the various diseases affecting the small ruminants, gastrointestinal nematode parasitism continues to be one of the most important constraints for its production in tropical and sub-tropical countries, causing retarded growth, weight loss and impaired fertility (Cavalcante *et al*., 2009) [4].

Currently, the use of synthetic anthelmintics is the mainstay for the control of gastrointestinal nematode infections. However, frequent and improper drenching of anthelmintics in animals over the years has not only resulted in development of resistant helminthic population, but also contamination of meat and milk with residues of synthetic anthelmintics and environmental pollution (Janina demeler *et al*., 2013) [8].

The emergence of anthelmintic resistance coupled with consumer awareness about residue free animal products has prompted the scientists in recent years to look into alternative novel approaches for sustainable control of gastrointestinal nematode infections in small ruminants. One such novel approach is using medicinal plants as green dewormer, which is easily available, biodegradable, eco-friendly and nontoxic to the host (Akhtar and Malik, 2000) [1].

Considering the advantages of plant based anthelmintics, the plant *Moringa oleifera* was selected for this study to evaluate their anthelmintic properties against nematodes of small ruminants and to evaluate their anthelmintic activity of synthesized silver nano particle using extracts of *Moringa oleifera* seeds.

Materials and Methods

Collection of *Moringa oleifera* seeds and preparation of its aqueous extract

The dry seeds of *Moringa oleifera* were collected from the agricultural section of Veterinary College and Research Institute, Namakkal, Tamil Nadu. The collected seeds were cleaned with distilled water and allowed to dry at room temperature and then pulverized with domestic mixer. To 10 grams of *Moringa oleifera* seed powder, 100 ml of Millipore water was added and heated at 60˚C for 30 minutes. Finally, the extract was collected by filtering process using Whatman no.1 filter paper.

Phytochemical analysis

The phytochemical analysis of the aqueous extracts of *M. oleifera* seeds (Varthini *et al.*, 2018) [6] was done at Chromopark, Namakkal to identify the phytochemical chemical composition.

Synthesis of silver nanoparticle of *Moringa oleifera* seeds

Aqueous solution of silver nitrate was prepared by mixing 1 milli Mole of silver nitrate with 100 ml of Millipore water. Then 1 ml of aqueous seed extract of *Moringa oleifera* was added into 100 ml of aqueous solution of 1mM silver nitrate and kept at room temperature for 24 hours. After 24 hours, the solution turned to brown colour from yellowish, indicated the synthesize of herbal silver nano particles (Varthini *et al.*, 2018) [6].

Characterization of silver nanoparticles

The green synthesized silver nanoparticles of *Moringa oleifera* seed were characterized by UV spectrophotometer, Transmission Electron Microscopy (TEM), Scanning Electron Microscopy (SEM) and Fourier Transformed Infrared Analysis (FTIR).

UV- vis- Spectrophotometric analysis

The UV-vis-spectrophotometric measurements of synthesized silver nanoparticles were carried out on Double Beam UV-vis-Spectrophotometer-2202 (Systronics) at Department of Meat Science and Technology, Veterinary College and Research Institute, Namakkal. A cuvette contained 5 ml of hydrosol was scanned from 250-650 nm optical regions to obtain absorption spectrum of the formed silver nanoparticles of herbal extracts.

Transmission electron microscopy

The synthesized silver nanoparticles were submitted to determine the surface morphology and particle size distribution using JEOL JEM2100 high resolution transmission electron microscope. The sample was prepared by air drying drops diluted solutions of preparations on carbon films supported by copper grids.

Scanning electron microscopy

The scanning electron microscopic analysis was carried out to determine the size and shape of the particles using Hitachi S-4500 SEM machine. Thin films of the samples were prepared on a carbon coated copper grid by just dropping a very small amount of sample on the grid, extra solution was removed using a blotting paper and then the film on the SEM grid were allowed to dry by putting a mercury lamp for 5 min.

Fourier transformed infrared analysis

The synthesized silver nanoparticles were also subjected to FTIR analysis for identification of the functional groups. In order to remove, any free biomass residue or compound that is not the capping ligand of the nanoparticles, the residual solution of 100 ml after reaction was centrifuged at 5000 rpm for 10 min. The supernatant was again centrifuged at 10000 rpm for 60 min to obtain pellet, followed by re-dispersion of the pellet of Ag – NPs into 1 ml of de-ionized water. Thereafter, the purified suspension was freeze dried to obtain dried powder. Finally, the dried nanoparticles were analysed by FTIR Nicolet Avatar 660 (Nicolet, USA).

In-vitro evaluation of anthelmintic activities of synthesized silver nanoparticles by Egg hatch assay

Mass harvesting of Strongyle eggs

Approximately 100gm of pooled faecal samples were collected from a suspected small ruminant flock located in Namakkal, Tamil Nadu in an air tight container and brought to the laboratory. Then the faecal samples were used for harvesting the Strongyle eggs (Ponnudurai *et al.*, 2018) [12].

Preparation of working concentration of silver synthesized plant extracts

One ml of stock solution of silver synthesized nanoparticles containing 10 mg of plant powder was prepared. This stock solution was diluted with distilled water so as to obtain 0.5, 1.0, 2.0, 4.0 and 8.0 mg per ml and used as working concentrations to carry out Egg Hatch Assay.

Preparation of working concentration of thiabendazole

Hundred mg of pure thiabendazole powder (Sigma Aldrich, USA) was added with 40 ml of dimethyl sulfoxide (DMSO) in two installments of equal volume in a flask. After complete dissolution, the total volume was made up to 100 ml with distilled water to get the stock solution of 1000 ppm. Finally, the working solution (80 ppm) was prepared from the stock solution (1000 ppm), by adding 0.8 ml of stock solution to 9.2 ml of distilled water (Ponnudurai *et al.*, 2018) [12].

Test procedure

In a 24 well plate, 100 µl of egg suspension containing approximately 100 eggs was added into each well of a 24 well plate. After that, 1ml of each working concentration of silver nanoparticles (0.5, 1.0, 2.0, 4.0, and 8.0 mg) was added into 3 wells in each row, while the same quantity of water was added into three wells in the last column, which were maintained as negative control. Ten microliter of thiabendazole having 80 ppm solution was added to the 4th well of each row and which served as positive control. Then, the distilled water was added to each well to make a total volume of 2 ml. The plate was then incubated at 26 ^{o}C for 48 hr. After 48 hr, a drop of helminthological iodine was added to each well. The larvae and unhatched eggs were counted under inverted tissue culture microscope and the percentage of hatch was calculated as follows:

$$\text{Percentage of hatch} = \frac{\text{No of larvae hatched out}}{\text{No of eggs + No of larvae}} \times 100$$

Statistical analysis

The data obtained from egg hatch assay was subjected to one-way analysis of variance. The means were compared by the Duncan test with 5% and 1% significant level using the SPSS 20.0 program (Tayo *et al*., 2014) [15].

Results

Phytochemical analysis

The phytochemical analysis of aqueous extracts of *Moringa oleifera* seeds results revealed the presence of flavonoids, tannin and terpenoids (Table 1).

Table 1: Phytochemicals identified in Aqueous extracts *Moringa oleifera* seeds

S. No	Phytochemicals	Aqueous extracts *Moringa oleifera* seeds
1	Alkaloids	-
2	Carbohydrates	-
3	Flavonoids	+
4	Phenols	-
5	Saponins	-
6	Tannins	+
7	Terpenoids	+
8	Quinone	-
9	Sterols	+
10	Proteins	-

Characterization of *Moringa oleifera* silver nanoparticles

The UV spectrophotometer analysis of was *Moringa oleifera* seed silver nanoparticles showed a peak at 460.8 nm with absorbance 4 (Fig.1). The SEM analysis study showed face centred cubic form and a large distribution of particles sized ranging from 10-30 nm with no visible agglomeration between the nanoparticles (Fig.2).

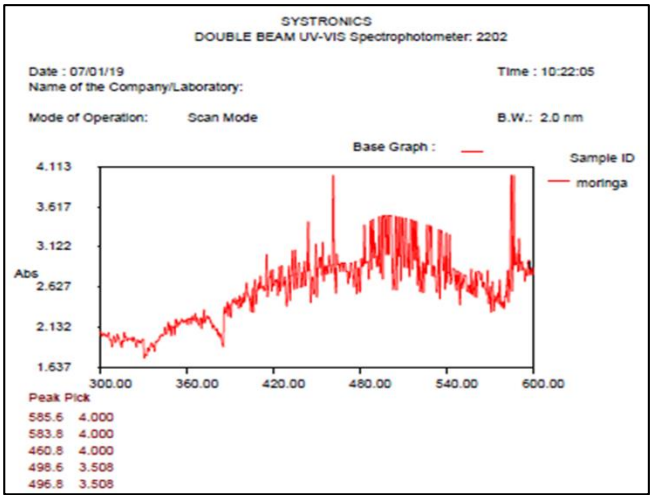
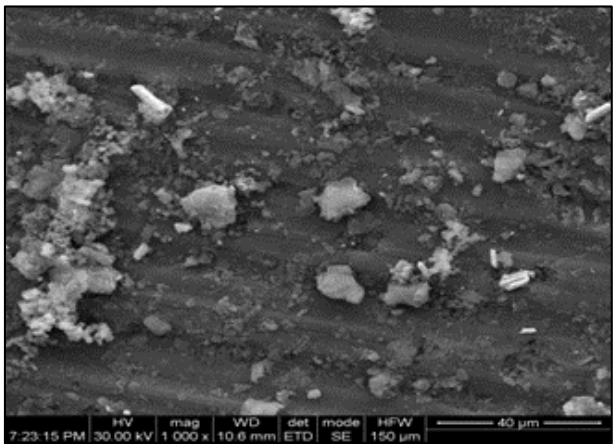


Fig 1: UV-vis-Spectrophotometric analysis of silver nanoparticles of *Moringa oleifera* seeds



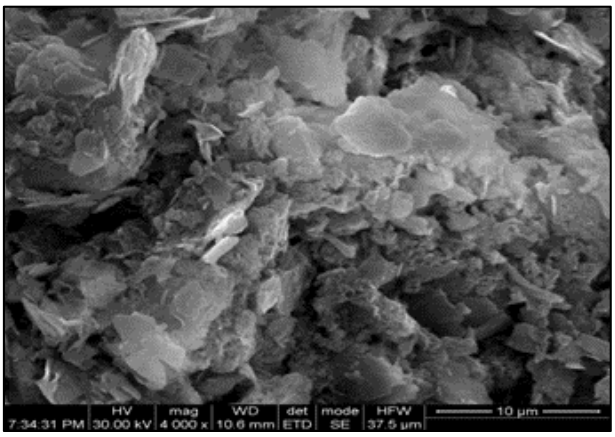


Fig 2: Scanning electron microscopy analysis of silver nanoparticles of *Moringa oleifera* seeds showing distribution of nano particles

The TEM analysis confirmed the presence of silver nanoparticles with detailed size and shape. The data obtained from the micrograph showed distinct shape and size of polydisperse nanoparticles. Mostly particles were spherical, but some were ellipsoidal in shape with an average size of 30 nm without significant agglomeration (Fig. 3).

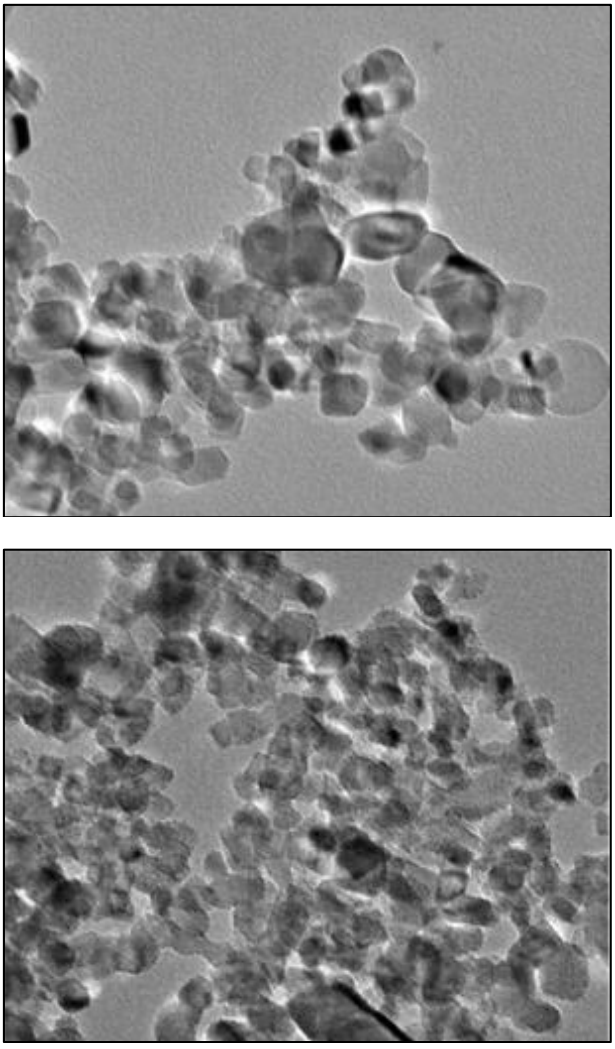
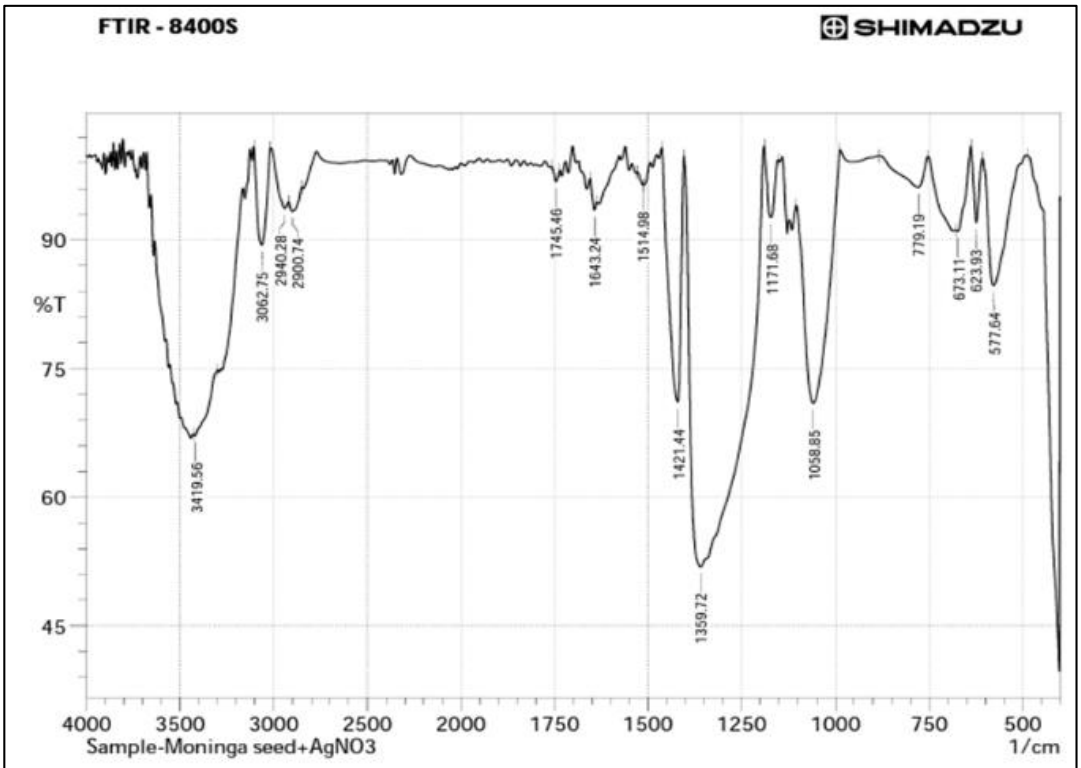


Fig 3: Transmission electron microscopy analysis of silver nanoparticles of *Moringa oleifera* seeds showing distinct shape and size of polydisperse nanoparticles

The FTIR spectra of *Moringa oleifera silver nano particles* exhibited the prominent peak with different values as 3419.56, 3062.75, 2940.28, 2900.74, 1745.46, 1643.24, 1514.93, 1421.44, 1352.72, 1171.68, 1058.85, 779.19, 671.11, 621.93 and 577.64 cm^{-1}. The spectra showed a sharp and strong absorption peak at 3419.56 cm^{-1} which assigned to the stretching vibration of O-H groups, H bonded alcohols and Phenols (3500-3200), 3300-2500-OH stretch carboxylic acids,3000-2850(C-H stretch alkanes), 1600-1585 (C-C stretch (in ring) aromatics), 1390-1350(C-H rock alkanes), 1360-1290(N-O symmetric stretch nitro compounds), 1320-1000(C-O stretch esters, ethers), 1250-1020(C-N stretch aliphatic amines), 910-665 (N-H wag 1,2 amines), 900-675(C-H "oop" aromatics), 690-515 (C-Br stretch alkyl halides) etc. (Fig. 4).



No.	Peak	Intensity	Corr. Intensity	Base (H)	Base (L)	Area	Corr. Area
1	577.64	84.68	14.95	606.57	487.96	3.86	3.72
2	623.93	92.03	8.25	638.39	606.57	0.53	0.56
3	673.11	91.01	1.73	679.86	638.39	1.06	0.31
4	779.19	96.13	3.62	883.34	752.19	1.26	1.13
5	1058.85	70.97	25.57	1104.17	988.45	10.37	8.94
6	1171.68	92.63	7.51	1190.96	1153.35	0.69	0.72
7	1359.72	51.88	48.16	1404.08	1190.96	40.68	41.04
8	1421.44	71.07	29.02	1461.94	1404.08	4.94	5.01
9	1514.98	96.39	0.21	1528.48	1513.05	0.2	0
10	1643.24	93.45	1.98	1654.81	1636.49	0.44	0.09
11	1745.46	96.78	1.52	1757.99	1735.81	0.23	0.07
12	2900.74	93.33	1.55	2920.03	2848.67	1.86	0.36
13	2940.28	93.66	2.09	3015.5	2920.03	1.62	0.57
14	3116.75	89.39	11.4	3107.11	3015.5	2.54	2.85
15	3419.56	67.14	0.85	3431.13	3299.98	20.24	0.72

Fig 4: FTIR analysis of silver nanoparticles of *Moringa oleifera* seeds

Evaluation of anthelmintic activity by *in-vitro* egg hatch assay

The results of egg hatch assay carried out in this study using aqueous extracts of *Moringa oleifera* seeds (Tab. 2) indicated a significant difference in the percentage of inhibition of hatching among the different doses of each extracts.

A significant difference in the ovicidal effect was noticed between the aqueous extracts of *Moringa oleifera* seeds and its silver nanoparticles. The aqueous extract of the *Moringa oleifera* seeds showed 95.41 ± 1.08 percent inhibition on egg hatching at 50 mg/ml concentration and showed no significant difference with the standard anthelmintic drug thiabendazole.

Similarly, the AgNPs of *Moringa oleifera* seeds (Tab. 3) produced a maximum of 80.59 ± 5.65 per cent inhibition of egg hatching at 8 mg/ml concentration and showed no significant difference with the standard anthelmintic drug thiabendazole.

Table 2: Effect of aqueous extracts of *Moringa oleifera* seeds on egg hatching (Mean ± SE) at different concentrations

Conc. (mg/ml)	Mean No. of eggs hatched	Mean No. of eggs unhatched	% Inhibition of hatching
1.5	16.67 ± 0.88^{a}	28.67 ± 2.60^{ab}	63.08 ± 1.46^{bcd}
3.125	13.00 ± 2.24^{a}	91.33 ± 26.98^{bcd}	79.64 ± 6.79^{bcde}
6.25	11.50 ± 1.3^{a}	94.33 ± 26.40^{cd}	81.22 ± 6.64^{bcde}
12.5	11.00 ± 2.21^{a}	101.17 ± 28.19^{d}	82.96 ± 5.45^{cde}
25	11.67 ± 1.80^{a}	101.50 ± 32.94^{d}	83.81 ± 5.71^{e}
50	08.67 ± 2.03^{a}	180.00 ± 3.21^{e}	95.41 ± 1.09^{e}
Thiabendazole	01.14 ± 0.30^{a}	77.34 ± 9.40^{abcd}	97.63 ± 0.63^{e}
Control	107.35 ± 18.01^{b}	15.70 ± 3.59^{a}	12.01 ± 2.28^{a}

Table 3: Effect of silver nanoparticle of *Moringa oleifera* seeds on egg hatching (Mean ± SE) at different concentrations

Conc (mg/ml)	Mean No. of eggs hatched	Mean No. of eggs unhatched	% inhibition of hatching
0.5	39.06 ± 12.41^{a}	45.22 ± 6.02^{abcd}	58.36 ± 7.73^{b}
1	41.07 ± 8.26^{a}	49.64 ± 8.35^{abcd}	59.92 ± 4.38^{bc}
2	37.00 ± 8.52^{a}	48.75 ± 8.65^{abcd}	63.34 ± 9.31^{bcd}
3	23.25 ± 8.56^{a}	28.00 ± 2.45^{ab}	63.61 ± 4.65^{bcd}
4	31.46 ± 8.04^{a}	49.11 ± 8.74^{abcd}	64.94 ± 5.69^{bcd}
6	17.00 ± 3.49^{a}	31.25 ± 6.26^{abc}	67.24 ± 4.79^{bcd}
8	21.42 ± 7.29^{a}	58.29 ± 11.99^{abcd}	79.59 ± 4.68^{bcde}
10	34.50 ± 6.02^{a}	51.83 ± 10.67^{abcd}	59.19 ± 1.74^{b}
Thiabendazole	01.14 ± 0.30^{a}	77.34 ± 9.40^{abcd}	97.63 ± 0.63^{e}
Control	107.35 ± 18.01^{b}	15.70 ± 3.59^{a}	12.01 ± 2.28^{a}

Discussion

Phytochemical analysis of aqueous extracts

The aqueous extracts of *M. oleifera* in this study revealed the presence of flavonoids, tannin and terpenoids. This observation confirms the findings of Ishwar Chandra Giri *et al*. (2010) recorded the presence of saponins, carbohydrates, alkaloids, tannins, proteins, flavonoids in methanolic extracts of *Moringa oleifera* seed extract.

Characterization of silver nanoparticles

UV- vis –Spectrophotometer analysis

In the present study, the UV- vis –spectrophotometer analysis of synthesized nanoparticles of *M. oleifera* showed a peak at 460.8 nm with 4.000 absorbance spectrum at 250 – 650 range at the concentration of 1 mM. And this observation is in consonance with the findings of Moodley *et al*. (2018) [10] who also observed a peak at 450 nm and 440 nm while analysing the extracts of fresh and freeze dried leaves of *M. oleifera*.

SEM and TEM analysis

Scanning electron microscopy analysis of synthesized silver nanoparticles of *Moringa oleifera* in the present study showed face centred cubic form particles with the size of 10-30 nm. The TEM analysis of *Moringa oleifera* silver nanoparticles showed the particles as mostly spherical, but some as ellipsoidal in shape with an average size 30 nm. This observation is in close agreement with the results of Anandalakshmi *et al*. (2015) [2] who reported that an average particle size of *Pedalium murex* showed in TEM analysis was 50 nm, though there is slight difference owing to variation in plant species.

Fourier transform infrared analysis

The Fourier Transform Infrared Analysis of *Moringa oleifera* extracts exhibited peaks at various absorbances like from 577.64 cm^{-1} to the maximum of 3419.56 cm-1, indicating the presence of alkines, and amines and this result is in accordance with the findings of Varthini *et al.*, 2018 [6].

In vitro evaluation of anthelmintic activity by egg hatch assay

In the present study, the aqueous extracts and nano synthesized particles of *Moringa oleifera* seeds produced a significant percentage of inhibition on egg hatching. The results of this study are akin to the findings of Tayo *et al* (2014) [15] who observed that aqueous and ethanolic extract of *Moringa oleifera* exhibited ovicidal and larvicidal activity against *Haemonchus contortus.* On comparison, the silver nanoparticles of *Moringa oleifera* seed extract showed significant inhibition on egg hatching than aqueous extract even in low doses. Nanoparticles normally improve the target selectivity, drug delivery at specific target site and effectiveness of the drug (Ansari *et al.*, 2012) [3] could be the reasons for this effect. Also, they were utilized to increase the solubility of herbal drugs and also favouring the localization of phytomolecules in a specific site (Sharma, 2014) [14].

Phytochemicals in herbs are responsible for their pharmacotherapeutic potentials (Nagwa *et al.*, 2014). The qualitative phytochemical analysis of *Moringa oleifera* proved an enriched phytocomponents responsible for their anthelmintic property. The observation of the present study also in compliance with findings of Salles *et al*. (2014) [13] who observed that the crude aqueous extract of *Moringa oleifera* seeds produced more than 90 per cent inhibition on egg hatching against *Haemonchus contortus.*

Conclusion

The present study was undertaken to evaluate the *in-vitro* anthelmintic activity of aqueous extract and silver synthesized nanoparticles of *Moringa oleifera* seeds against Strongyle nematode of small ruminants by egg hatch assay. In this study, the aqueous extract of *M. oleifera* exhibited a significant inhibition on egg hatching. Similarly, the silver nanoparticles of *Moringa oleifera* showed a significant inhibitory effect. On comparison, the silver nanoparticles based preparation showed better results than the other. Enriched phytochemicals and advantages of nanoparticles utilised herbal preparation could be the reason for the inhibition of hatching of Strongyle eggs effectively even in low doses. Further investigations on identification of specific phytomolecule responsible for this anthelmintic property and molecular mechanism of phytochemicals with specific target site will bring a novel anthelmintic preparation for nematodes of small ruminants in future.

Acknowledgement

The authors acknowledge the financial assistance and facilities provided by Tamil Nadu Veterinary and Animal Sciences University, Chennai-51 to carry out the research.

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