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Green Synthesis of Zinc Oxide Nanoparticles Using Fresh Stem of *Cissus quadrangularis* Extract and its Various *in vitro* Studies

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The green synthesis of metal and metal-oxide nanoparticles is a growing research area due to the potential applications in the growth of novel technologies. The present work reports low-cost, green synthesis of zinc oxide nanoparticles using *Cissus quadrangularis* stems extract. The biosynthesized nanoparticles were characterized by UV, FTIR, XRD and SEM. The synthesized ZnO nanoparticles were pure, predominantly spherical in shape with the size ranging from 23 to 64 nm. In the present study the biosynthesized ZnO nanoparticles have been used for various *in vitro* activities such as antihelmintic, antibacterial, antiarthritic and antioxidant activities. Zinc oxide nanoparticles demonstrated antioxidant activity by scavenging 36 % hydrogen peroxide at 100 µg/mL and revealed excellent antihelmintic effect by show casing the death of the worm at all the concentrations at different times. The antibacterial study was done by agar-well diffusion method and the maximum inhibition zones around the ZnO nanoparticles were observed in *E. coli* followed by *S. aureus*, *Listeria* sp, *Salmonella* sp and *Klebsiella* sp. The percentage stabilization of aqueous extract was found to be 93 % inhibition on bovine serum albumin method and 91 % inhibition on egg albumin denaturation method which confirms the antiarthritic activity. Therefore, the study reveals an eco-friendly, efficient and simple method for the green synthesis of multifunctional ZnO nanoparticles using green synthetic approach.

Keywords: Earthworms, Egg albumin denaturation method, Hydrogen per oxide, SEM.

INTRODUCTION

The development of the processes for the synthesis of nanoparticles is evolving into a vital branch of engineering [1]. Green synthesis of nanoparticles is an eco-friendly approach which could pave the method for researchers across the world to explore the potential of various herbs so as to synthesize nanoparticles [2]. In recent years, a fast development of nano-technology has opened up a world of recent potentialities for fabricating nanomaterials of desired particle size, shapes suitable for uses in biomedicine, trade and agriculture field [3]. Zinc oxide may be a distinctive material that exhibits conductive, electricity and pyro-electric properties and has versatile applications in, transparent electronics, ultraviolet (UV) light emitters, electricity devices, chemical sensors, spin electronics, personal care products, coating and paints [4]. The ZnO nanoparticles have been prepared by both physical and chemical methods [5]. Recently, biological methods for the synthesis of ZnO nanoparticles using microorganisms, enzymes and plant extracts have been suggested as possible eco-friendly alternatives to chemical and physical methods [6]. Plants and their extracts provide a biological synthesis route of several metallic and metal oxide nanoparticles which are more eco-friendly and allows a controlled synthesis with well-defined size and shape [4]. Among the metal oxide nanoparticles, ZnO nanoparticles are considered more and used because of their chemical stability and strong adsorption ability [7]. Many researchers reported the biosynthesis of ZnO nanoparticles using plant extracts such as *Camellia sinesis* [8], *Olea europea* [6], *Trifolium pretense* [9], *Solanum nigrum* [10], *Couroupita guianensis* [11], *Limonia acidissima* L [12].

Cissus quadrangularis (Family: Vitaceae) also known as *Vitis quadrangularis* is a vining plant native to India and Africa that has been used medicinally for centuries. It is a common perennial climber and is distributed throughout India, particularly in tropical regions. It has employed in Ayurveda as an alternate antihelminthic, dyspeptic, digestive, tonic, analgesic in eye and ear diseases and within the treatment of irregular menses and respiratory disease. The whole plant is employed in oral rehydration; while the leaf, stem and root extracts of this plant are vital within the management of assorted ailments [13]. The plant has been prescribed in Ayurveda as an alternative to treat diseases such as digestive, tonic, analgesic in ear and eye diseases and also in the treatment of irregular

menstruation and asthma. Some other reports on *Cissus quadrangularis* justifies its effectiveness in management of obesity and complications associated with metabolic disorders. The aim of the present study is to develop a green process for the production of ZnO nanoparticles using aqueous stem extract of *Cissus quadrangularis* and to test its various biological applications.

EXPERIMENTAL

Zinc nitrate was obtained from Hi-media and distilled water was used throughout the experiments.

Preparation of *C. quadrangularis* plant extract: Fresh green young stems of *Cissus quadrangularis* (Commonly called as Pirandai) were collected from Sevoor (Vellore district, Tamil Nadu, India) and used for the preparation of aqueous extracts. The surface of the plants was cleaned with running tap water, followed by double distilled water to remove the impurities. 25 g of young stem was cut into fine pieces and boiled with 100 mL of sterile distilled water at 60 °C for 1 h. Then, the extract was filtered through Whatmann No. 1 filter paper and used for further experiments.

Qualitative phytochemical screening: The aqueous extracts of *C. quadrangularis* were screened for different phytochemical constituents' *viz.*, carbohydrates, phenol, alkaloid, tannin, flavonoid and saponin. Phytochemical screening of the extracts was carried out by the standard methods [14].

Synthesis of zinc oxide nanoparticles: For the synthesis of nanoparticles, zinc nitrate (5 mM) was mixed with 100 mL of double distilled water. 75 mL of zinc nitrate solution was mixed with 25 mL of plant extract. Then, the suspension was kept under stirring for 10 h at room temperature.

Characterization of synthesized zinc oxide nanoparticles: The synthesized ZnO nanoparticles were characterized by UV-visible spectroscopy, scanning electron microscope (SEM), X-ray diffraction (XRD) and Fourier transform infrared spectroscopy (FTIR) analysis [15]. Eco-friendly synthesized ZnO nanoparticles were monitored by UV-visible spectrometry (Schimadzu UV-visible spectrophotometer, model UV-1800). FT-IR spectrum analyzed for functional group identification using (Jasco-6600). The XRD Patterns were recorded in an X-ray diffractometer (Seifert Jso-Debyerex-2002). The morphology and size distribution were characterized using SEM [10].

Antibacterial activity: The antimicrobial activity of ZnO nanoparticles was evaluated against bacterial pathogens such as *Escherichia coli, Staphylococcus aureus, Salmonella* sp, *Listeria* sp and *Klebsiella pneumonia* by disc diffusion method [14]. Sterilized discs were soaked in aqueous plant extract and synthesized ZnO nanoparticles. The sterile, dried antimicrobial discs impregnated with crude plant extracts and ZnO nanoparticles were carefully dispensed with uniform distances placed on Muller-Hinton agar plates and incubated for 18-24 h at 37 °C. The zone of inhibition was measured from the centre of the disc to the clear zone in millimeter and the results were recorded.

Antihelminthic activity: Indian adult earthworms (*Pheretima posthuma*) were used for the evaluation of *in vitro* antihelmintic activity. Earthworms were collected from moist soil (VIT University, Vellore) and washed with normal saline to remove the external matter. Prepared *C. quadrangularis* extract and ZnO nanoparticles were used for antihelmintic study. Solution of standard antihelmintic drug (Albendazole, 15 mg/mL) was also prepared in distilled water. Normal saline is used as a control. Five groups of the approximately equal size of earthworms, consisting of three in number in each group, were released into each petri-dish. Observations were made for the time taken for paralysis and death of individual worms. Time for the death of worms was recorded when ascertaining that the worms neither moved when shaken vigorously nor when dipped in warm water at 500 °C. The antihelmintic activity was evaluated by adopting the quality technique [16].

Antioxidant activity

Hydrogen peroxide scavenging activity: The ability of *C. quadrangularis* extracts to scavenge hydrogen peroxide was determined according to the method [17]. A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). Extracts and ZnO nanoparticles (100 µg/mL) were added to a hydrogen peroxide solution. The absorbance of hydrogen peroxide at 230 nm was determined after 10 min against a blank solution containing the phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenging of both *C. quadrangularis* extracts and ZnO nanoparticles with standard compounds (α-tocopherol) were calculated:

$$\text{Scavenged } [H_2O_2]\ (\%) = \frac{(A_C - A_S)}{A_C} \times 100$$

where A_C is the absorbance of the control and A_S is the absorbance in the presence of the sample of *C. quadrangularis* extracts and ZnO nanoparticles or standards [18].

Antiarthritic activity: The *in vitro* antiarthritic activity was studied using bovine serum protein denaturation method and egg albumin denaturation method.

Bovine serum protein denaturation method: Various concentrations (25, 50, 75, 100 µg/mL) of test dugs (plant extract and ZnO nanoparticles) and standard drug diclofenac sodium (100 µg/mL) were taken respectively and 0.5 % w/V BSA is mixed. The samples were incubated at 37 °C for 20 min and the temperature was increased to 57 °C for 3 min. After cooling, 2.5 mL of phosphate buffer was added to the above solutions. The absorbance was measured using UV-visible spectrophotometer at 255 nm. The control represents 100 % protein denaturation. The results were compared with diclofenac sodium. The percentage inhibition of protein denaturation can be calculated as follows.

$$\text{Inhibition } (\%) = \left(100 - \frac{OD_{control} - OD_{test}}{OD_{control}}\right) \times 100$$

Egg albumin denaturation method: The reaction mixture (5 mL) consist of 0.2 mL of egg albumin (from fresh hen's egg), 2.8 mL of phosphate-buffered saline (PBS, pH 6.4) and 2 mL of varying concentrations (25, 50, 75, 100 µg/mL) of plant extract and ZnO nanoparticles. A similar volume of double-distilled water served as the control. Next, the mixtures were incubated at 37 ± 2 °C in a BOD incubator for 15 min and then heated at 70 °C for 5 min. After cooling, their absorbance was measured at 660 nm.

Diclofenac sodium was used as the reference drug and treated similarly for the determination of absorbance. The percentage inhibition of protein denaturation wascalculated by using the following formula:

$$\text{Inhibition } (\%) = 100 \times \left(\frac{V_t}{V_c} - 1 \right)$$

where, V_t = absorbance of the test sample, V_c = absorbance of control.

RESULTS AND DISCUSSION

Qualitative phytochemical analysis: Preliminary phytochemical screening of *Cissus quadrangularis* has shown the presence of alkaloids, tannins, flavonoids, saponins, steroids, triterpenoids, proteins, carbohydrates and glycosides.

UV-visible spectroscopy: The optical absorption spectra of ZnO dispersed in water were recorded using UV-visible spectrophotometer. UV-spectra were measured at room temperature in a quartz cuvette with the path length of 1 cm. It is known that UV-visible spectroscopy is the most widely used technique for the structural characterization of nanoparticles. Also, the absorbance of the reaction mixture was monitored after 24 h of reaction. Fig. 1 shows the UV-visible absorption spectrum of ZnO nanoparticles sample at different times. Typical absorption at 310 nm was observed at room temperature. This reveals that *Cissus quadrangularis* exhibits the stable synthesis of ZnO nanoparticles.

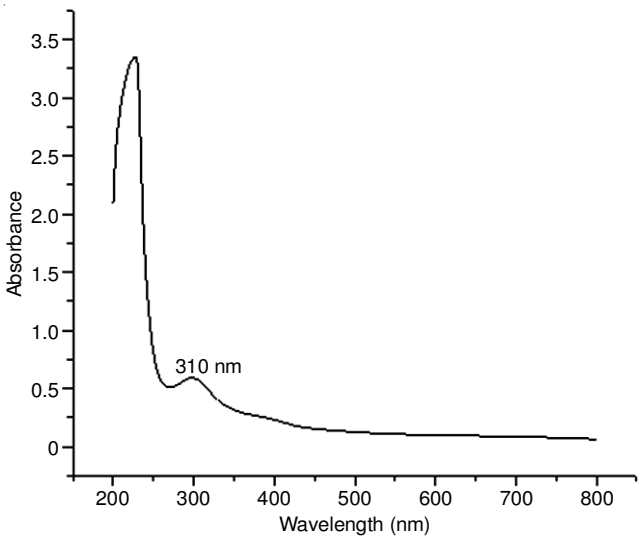

Fig. 1. UV-visible spectrum of ZnO nanoparticles

FTIR: The results were further reinforced by FT-IR analysis, which showed the shifts and difference in areas of the peaks. Fig. 2 shows the IR spectrum of ZnO nanoparticles. The FTIR bands of biosynthesized ZnO nanoparticles using *Cissus quadrangularis* were indicated at 3334.92 (Bond: O-H stretch), 2920.23 (Bond: C-H Stretch), 2850.79 (Bond: O-H stretch), 1726.29 (Bond: C=O stretch) and 1631.78 (Bond: C=C stretch). This present study revealed that the FTIR band proved the appearance of alcohol, alkyl, carboxylic acids, amides, ketones, aldehydes, esters, alkene, aromatic ring.

XRD: Fig. 3 shows XRD patterns of ZnO nanoparticles synthesized. The characteristic patterns are corresponding to

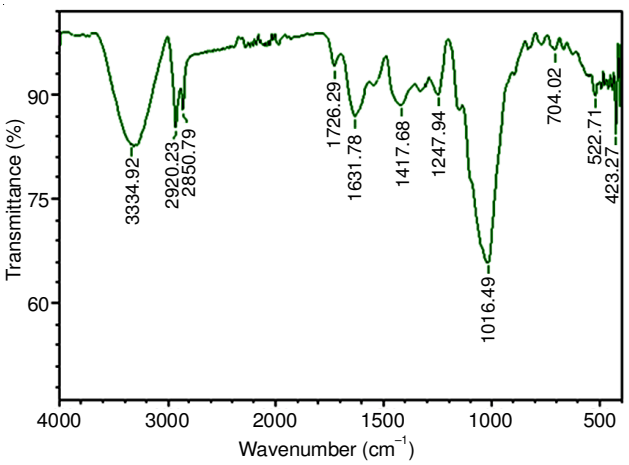

Fig. 2. FTIR spectrum of ZnO nanoparticles

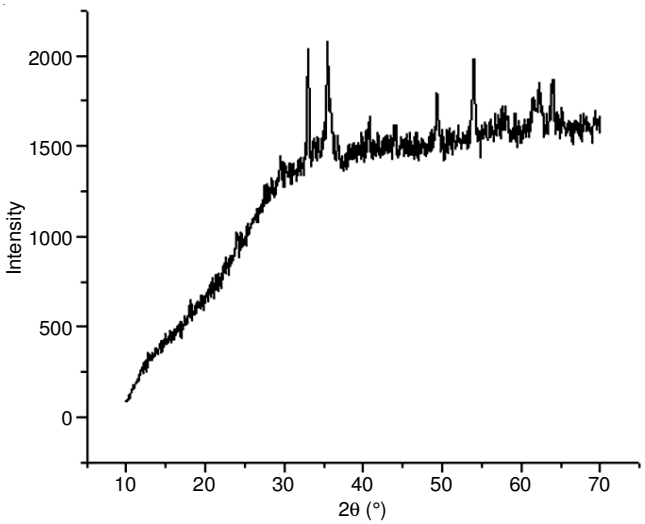

Fig. 3. XRD spectrum of ZnO nanoparticles

the diffraction patterns of hexagonal wurtzite phase of ZnO. No other peaks appeared in the patterns. The cell constants were observed to be a = 3.25 Å and c = 5.21 Å and all the peaks were well matched with the JCPDS card No. 89-7123. The XRD peaks were identified as (100), (002), (101), (110), (112) and (202) reflections, respectively. Apparently, the introduction of different plant extracts favour relatively small-size ZnO nanoparticles, since the average size of ZnO (82 nm) is larger compared to ZnO samples prepared with plant extracts in the size range 23-64 nm. This suggested that the use of plant extract restricts the growth of crystallites.

SEM: The pure ZnO nanoparticles formed was agglomerated with a hexagonal and cubical structures and a particle size ranging from 76 to 97 nm with some deviations. This agglomeration is due to polarity and electrostatic attraction of ZnO nanoparticles.

Antibacterial activity: The synthesized ZnO nanoparticles and aqueous plant extract *C. quadrangularis* had tested for its ability to inhibit the growth of *Escherichia coli, Staphylococcus aureus, Salmonella* sp, *Listeria* sp, *Klebsiella* sp. Among the tested bacterial isolates, the nanoparticles showed maximum inhibition against *E.coli* followed by *S. aureus, Listeria* sp, *Salmonella* sp, *Klebsiella* sp. The aqueous plant extract *C. quadrangularis* showed maximum inhibition against *E. coli*

followed by *Listeria sp, S.aureus, Salmonella* sp, *Klebsiella* sp. The zones are presented in Table-1. This result suggests that the ZnO nanoparticles and the aqueous plant extract *C. quadrangularis* have the potential against several bacteria species. Similar quite results stating that the ZnO nanoparticles produces a maximum zone of inhibition against *Pseudomonas aeruginosa* followed by *Proteus mirabilis*, *Bacillus cereus*, *Escherichia coli* and *Staphylococcus aureus*. The author recommended that the green synthesized ZnO nanoparticles are often used as an alternate to existing antimicrobial agents [8].

TABLE-1
ANTIBACTERIAL ACTIVITY

Microorganism	ZnO nanoparticles (mm)	Plant extract (mm)
E. coli	21 ± 0.23	11 ± 0.62
S. aureus	15 ± 0.77	8 ± 0.36
Listeria	10 ± 0.58	9 ± 0.45
Salmonella	8 ± 0.46	7 ± 0.18
Klebsiella	6 ± 0.13	3 ± 0.91

Antihelmintic activity analysis: Zinc oxide nanoparticles using *C. quadrangularis* showed better antihelmintic activity when compared with the aqueous extract of *C. quadrangularis*. Nanoparticles show less time to cause paralysis and death of worms followed by the aqueous extract. The data obtained on the antihelminthic activities of normal saline, standard drug, different concentrations of the aqueous extract and ZnO nanoparticles overall revealed the concentration dependent nature of the extract and ZnO nanoparticles in bringing out this bioactivity. It was found that the colloidal suspension of ZnO nanoparticles prepared in *C. quadrangularis* extract showed more antihelminthic activity than the aqueous extract of *C. quadrangularis*. From this study it may be concluded that the phytochemical components along with the ZnO nanoparticles have more antihelmintic activity (Table-2). Results of [19] demonstrated the bioavailabilty of ZnO nanoparticles throughout the cross sections of earthworms. This reveals that intact ZnO nanoparticles can be taken by the earthworm from soil and proved to be a good potential of bioremediation into non-toxic forms (Fig. 4).

Antioxidant activity

Hydrogen peroxide scavenging activity: The scavenging ability of aqueous extracts and ZnO nanoparticles of *C. quadrangularis* on hydrogen peroxide is shown Fig. 5, Table-3 and compared with α-tocopherol as standards. 100 µg of extracts of *C. quadrangularis* exhibited 22 % scavenging activity and Synthesized ZnO nanoparticles exhibits 36 % Scavenging activity on hydrogen peroxide. On the other hand, using the same amounts, α-tocopherol exhibited 44.58 % hydrogen peroxide scavenging activity. Hydrogen peroxide itself is not very reactive, but it can sometimes be toxic to the cell because of it may give rise to hydroxyl radical in the cells.Thus, the removing of H_2O_2 is very important for antioxidant defense in cell or food systems.

TABLE-2
ANTIHELMINTHIC ACTIVITY

Group	Treatment	Concentration (mg/mL)	Time taken for paralysis	Time taken for death
1	Normal control	–	–	–
2	Positive control	100	15	20
3	Negative control	–	–	–
4	Plant extract	5	64	70
		25	59	63
		50	43	54
		100	34	42
5	Zinc oxide nanoparticles	5	42	49
		25	39	42
		50	28	36
		100	15	22

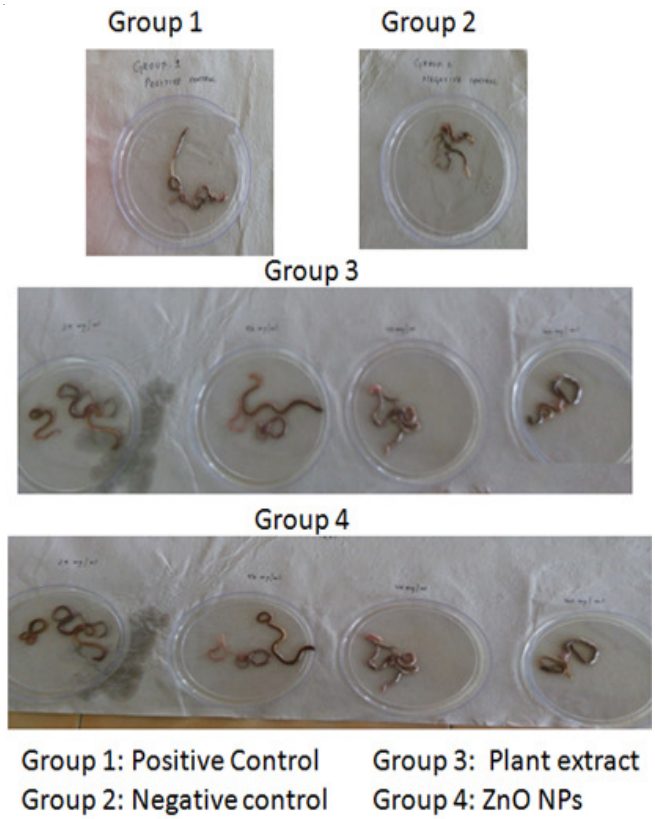

Fig. 4. Antihelminthic activity of zinc oxide nanoparticles

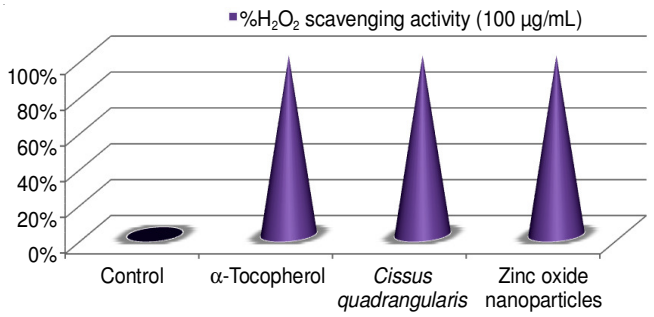

Fig. 5. Antioxidant activity

TABLE-3
ANTIOXIDANT ACTIVITY

Samples	% H_2O_2 Scavenging activity (100 µg/mL)
Control	0
α-Tocopherol	44.58
Cissus quadrangularis	22
Zinc oxide nanoparticles	36

Antiarthritic activity

Bovine serum protein denaturation method: In *in vitro* antiarthritic activity by bovine serum protein denaturation method the plant extract *C. quadrangularis* at concentration of 25, 50, 75, 100 µg/mL showed 62, 74 and 89 and 90 % inhibition whereas ZnO nanoparticles showed 74, 82, 90, 93 % inhibition of denaturation of bovin serum whereas, standard diclofenac sodium at 100 µg/mL showed 96.91 % inhibition of denaturation of bovine serum (Table-4).

TABLE-4
ANTIARTHRITIC ACTIVITY (BOVINE SERUM PROTEIN DENATURATION METHOD)

S. No.	Drug	Concentration (µg/mL)	Inhibition (%)
1	Control	–	–
2	Plant extract	25	62
		50	74
		75	89
		100	90
3	Zinc oxide nanoparticles	25	74
		50	82
		75	90
		100	93
4	Diclofenac sodium	100	96

Egg albumin denaturation method: In *in vitro* antiarthritic activity by egg albumin denaturation method at concentration of 25, 50, 75, 100 µg/mL of aqueous extract of *C. quadrangularis* showed 75.00, 80.31, 84.15 and 89.02 % inhibition of Egg Albumin denaturation whereas the ZnO nanoparticles showed 77.08, 82.11, 87.11, 91.08 % inhibition of egg albumin denaturation and the standard diclofenac at 100 µg/mL showed 95.77 % inhibition of egg albumin denaturation (Table-5).

TABLE-5
ANTIARTHRITIC ACTIVITY
(EGG ALBUMIN DENATURATION METHOD)

S. No.	Drug	Concentration (µg/mL)	Inhibition (%)
1	Control	–	–
2	Plant extract	25	75.00
		50	80.31
		75	84.15
		100	89.02
3	Zinc oxide nanoparticles	25	77.08
		50	82.11
		75	87.77
		100	91.08
4	Diclofenac sodium	100	95.77

Conclusion

The results obtained from this study will be facilitating a pathway to extend the performance of ZnO nanoparticles in biomedical applications to produce suitable drugs, so that it will be beneficial for animals and human beings to abstain from bacterial infections, intestinal worms, rheumatoid arthritis *etc*. The present method is simple, green, environment-friendly, economical, nontoxic and free of the use of any organic solvents, surfactants and specialized instruments. The phytochemical screening of plant extracts confirms the presence of secondary metabolites such as carbohydrates, tannins, steroids, protein, alkaloids, phenol *etc*. It shows that some present in high quantity and some are in less quantity. The biomedical applications like antibacterial, antiarthritic, antioxidant and anthelminthic activities were done and concluded the remarkable efficacy of plant extract and synthesized nanoparticles. The plant contains the most of the pharmacological properties such as anticancer, antioesteoporosis, anthelminthic, anti-inflammatory, antioxidant properties as reported in literatures. The comparable study of pharmacological properties of plant extract and synthesized nanoparticles has deduced that the ZnO nanoparticles contain some of the properties better than the plant extract and can be used in biomedical applications.

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