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BIOSYNTHESIS AND CHARACTERIZATION OF ZnO NANOPARTICLES USING *AZADIRACHTA INDICA* SEED HUSK EXTRACT

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ABSTRACT

Green synthesis of nanoparticles has been suggested as an alternative to physical and chemical methods which involve the use of toxic chemicals and pose detrimental effects on humans and the environment. The purpose of this investigation is to study the green synthesis of ZnO nanoparticles by co-precipitation method using *Azadirachta indica* seed husk extract as the reducing and stabilizing agent. Preliminary phytochemical screening was carried out on the aqueous *Azadirachta indica* seed husk extract which revealed the presence of important phytochemicals: alkaloids, flavonoids, terpenoids, tannins, reducing sugars, saponnins, suggested to be responsible for reducing divalent zinc to zero-valency. The green synthesized products were characterized by FTIR and SEM-EDX to study surface morphology, elemental composition and purity of the synthesized nanoparticles. The FTIR results revealed the presence of phenols, polyphenols and primary amines and zinc oxide. The synthesized ZnO nanoparticles were pure, predominantly spherical in shape with sizes ranging from 35 to 60 nm. This route of nanoparticle synthesis is safe, economical and eco-friendly.

Key words: *Azadirachta indica*, Biosynthesis, Zinc Oxide nanoparticles.

INTRODUCTION

Nanotechnology has been enriched with nature directed biosynthesis of nanoentities. To distinguish this green approach of nano-synthesis from the conventional chemical or physical methods, the term ‘green synthesis’ (GS) has been coined for this new scientific discipline [1].

Green chemistry is the fabrication of products and methods that reduce or eliminate the use of toxic chemicals and ultimately the disposal of hazardous substances out of the environment [2]. Passed in 1990 as a Pollution Prevention Act in the United States of

America, green chemistry is a new approach for dealing with pollution by preventing environmental problems [2].

Green synthesis employs the 12 green commands which according to Anstas et al [3] are important for the synthesis of nanoparticles as an alternativeto the physical and chemical approaches that involve the use of toxic and expensive chemicals. This method utilizes naturally occurring molecules for the formation of nanoparticles with distinctive properties. It has adopted the principles of green chemistry by minimizing the use of unsafe reagents and maximizing the efficiency of chemical processes [4]. The green synthesized nanoparticles induce a less detrimental effect on the environment and human health compared with those by chemical methods. Until now, this approach has brought about tremendous applications of nanoparticles in various fields such as medicine, agriculture and environmental remediation [5]. The naturally occurring materials for biogenic synthesis include plant extracts, plants, and microorganisms [6, 7].

In the biosynthesis of metallic nanoparticles using plant extract, three important materials are metal salt, a reducing agent, and a stabilizing or capping agent for controlling the size of nanoparticles and preventing their aggregation [6]. Many biomolecules in plants such as proteins/enzymes, amino acids, carbohydrates, alkaloids, terpenoids, tannins, saponins, phenolic compounds, reducing sugar and vitamins [6] could be involved in bioreduction, formation and stabilization of metal nanoparticles. The reduction of metal ions by plant extracts depends on the presence of polyphenols, enzymes, and other chelating agents present in plants. Also, it has critical effects on the amounts of nanoparticle produced [8]. Terpenoids are surface-active molecules that help to stabilize the nanoparticles [9].

Zinc oxide (ZnO) belongs to a class of inorganic metal oxides available with a wide range of nanostructures. Moreover, ZnO has been recognized safe by the U.S. Food and Drug Administration (21CFR182.8991) [10]. The advantages of nanostructured ZnO particles over other metal nanoparticles are due to their lower cost, UV blocking properties, high catalytic activity, large surface area, white appearance and their remarkable applications in the field of medicine and agriculture [4].

In this present study, preparation of crude plant extract and preliminary phytochemical screening of extract to determine the presence of important phytochemicals responsible for reducing and stabilizing nanoparticles.

Biosynthesis of ZnO nanoparticles was carried out by co-precipitation method using *Azadirachta indica* seed husk extract. Also, characterization of synthesized nanoparticles by UV-Vis spectroscopy, FTIR, SEM-EDX, TEM for surface morphology, structural and elemental composition was carried out.

EXPERIMENTAL

Reagents

Sodium hydroxide (NaOH) pellets, Ethanol, Zinc acetate dihydrate salt precursor, Deionized water were purchased from Sigma-Aldrich and of analytical grade. All glasswares were properly cleaned with deionized water and dried in an oven before use.

Collection and preparation of *Azadirachta indica* Seed Husk

Azadirachta indica seed husk were collected from Zaria Local Government Area, Kaduna State and authenticated by a botanist at the Herbarium of the Department of Plant Biology, Ahmadu Bello University, Zaria, Nigeria. The seed husk was separated from the kernel and washed repeatedly with deionized water to remove particulate matter and other impurities on it, shade dried to constant weight, then pulverized into coarse powder using mortar and pestle, and stored in closed containers for further use.

Preparation of Aqueous *Azadirachta indica* Seed Husk Extract

A. indica aqueous extract was prepared by slight modifications of the method described by Nava *et* al [11]. Briefly, about 5 g *A.indica* coarse powderwere weighed and added into 250 mL beaker containing 50 mL of deionized water, and was stirred and heated for about 4 hours in water bath at 40^{o}C. After cooling, the extracts were filtered through muslin cloth and through Whatman No. 1 filter paper. The yellowish filtrate was stored at 4^{o}C in a refrigerator until further use.

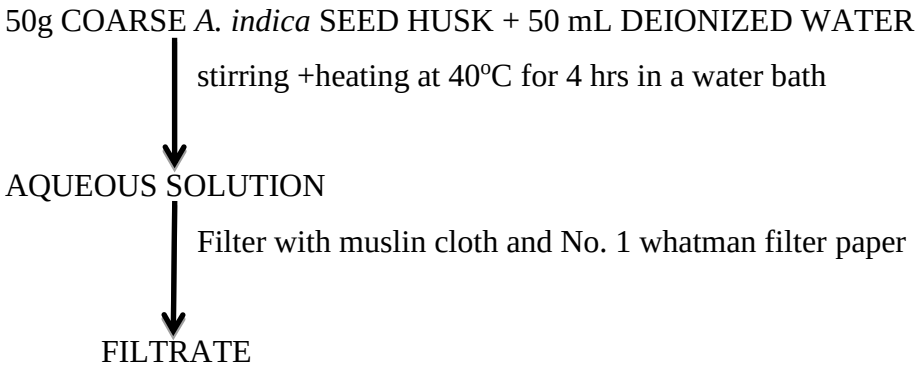

Fig.1: Schematic diagram for aqueous extraction of *A. indica* seed husk extract

Preliminary Phytochemical Screening

Crude aqueous extracts were evaluated to determine the presence of alkaloids, flavonoids, phenols, saponins, terpenoids, steroids, tannins, anthraquinones according to standard methods [12]. Any change of colours or the precipitate formation was used as indicative of positive response to these tests.

Testing for alkaloids

Each extract (10 ml) was dissolved in 2 mL of hydrochloric acid 5%, after mixing and filtering, three aliquots were taken. Drops of Wagner, Mayer, Bouchardat and Dragendorff reagents were added to each. A red-brown precipitate (Wagner), yellowish-white precipitate (Mayer), brown precipitate (Bouchardat) and red–orange precipitate (Dragendorff) indicated the presence of such metabolites.

Testing for flavonoids

Shinoda test

About 1 mL of absolute ethanol and 3 drops of concentrated hydrochloric acid were added to 0.5 mL of diluted extract in isopropyl alcohol. Formation of red color indicated the presence of aurones and chalcones. In cases where no colour change was observed, pieces of metallic magnesium were added. The formation of orange, red or magenta colouration indicated the presence of flavones and flavonols, respectively.

Sodium hydroxide (10%) test

About 3 drops of sodium hydroxide 10% were added to 1 mL of diluted extract in isopropyl alcohol. Formation of yellow-red, coffee-orange, purple-red or blue coloration indicated the presence of xanthones and/or flavones, flavonols, chalcones and anthocyanins, respectively.

Testing for saponins

Foam height test

About 1 mL of distilled water were added to 10 drops of the extract dissolved in isopropyl alcohol (20 mg/mL) in a test-tube, shaken vigorously to froth, and then allowed to stand for 10 min. Saponin content was measured as follows: no froth (absence); froth less than 3 mm high (poor); froth 6 mm high (moderate) and froth greater than 8 mm high (abundant).

Testing for quinones and anthraquinones

Borntrager's test

About 3 mL of each extracts were treated with 3 mL of chloroform and the chloroform layer were separated. To this 5% potassium hydroxide dissolution were added. Occurrence of red color in alkaline phase indicated the presence of quinones. Those samples showing yellow color with Green fluorescence where treated with one drop of 6% hydrogen peroxide, formation of red color was considered positive for anthrones derivatives.

Ammonium hydroxide test.

One drop of concentrated ammonium hydroxide was added to 10 mg of each extract, previously dissolved in isopropyl alcohol. After two minutes, formation of red color indicated the presence of anthraquinone.

Sulphuric acid test.

One drop of concentrated sulfuric acid was added to 10 mg of each extract dissolved in isopropyl alcohol. Formation of red color indicated the presence of quinones.

Testing for steroids and / or triterpenoids

Salkowski test

About 2 mL of chloroform and 1 mL concentrated sulfuric acid were added to 10 drops of the extract dissolved in isopropyl alcohol, slowly until double phase formation. The presence of a dish-brown color in the middle layer was indicative of steroidal ring.

Lieberman Bouchard test

About 1 mL of anhydrous acetic acid and 3 drops of concentrated sulphuric acid were added to 2 mL of the extract dissolved in isopropyl alcohol. After 5 min a blue-green color middle layer was indicative of sterols, but pink, red, magenta or violet color revealed the presence of terpenoids.

Testing for tannins

About 10 mg of each extract were dissolved in 1 mL of ethanol, then 2 mL of distilled water was added followed by 4 drops of ferric chloride aqueous solution 10% w/v. Formation of a blue or green color indicated the presence of phenols.

Green Synthesis of Zinc Oxide Nanoparticles Using *Azadirachta Indica* Husk Extract

The zinc oxide nanoparticles were biosynthesized by following the co-precipitation method described by Bhuyanet al [10]. Zinc acetate dihydrate [Zn $(CH_3COO)_2$] $.2H_2O$ and sodium hydroxide were used as precursors. Briefly, zinc acetate (2M) was prepared in 50 mL of deionized water under constant stirring conditions. After complete dissolution of the mixture, 60ml of 25% seed extract was added. When the solution is heated up to 50ºC, 50mL of 2M NaOH was added drop wise to the prepared solution of zinc acetate and seed extracts. The mixture was stirred continuously for 2 h on magnetic stirrer resulting in white precipitate. The precipitate was filtered and washed repeatedly with deionized water followed by ethanol in order to remove the impurities. Finally, a white powder was obtained after overnight drying of the purified precipitate at 60℃ in oven overnight which was further annealed at 200ºC and kept for further characterization.

2M $Zn(CH_3COO)_2.2H_2O$ in 50 mL deionized H_2O

↓ Stirred magnetically for 10 mins

SOLUTION

↓ + 30 mL A. indica extract + Heating at 50^0C

SOLUTION

↓ + 50 mL of 2M NaOH added dropwise for 2hrs

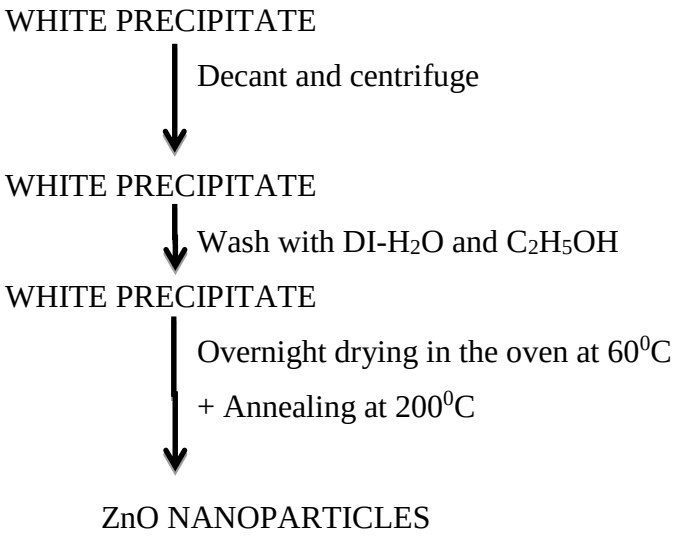

Fig. 2: Schematic Diagram for the Green synthesis of ZnO NPs

Characterization

The green synthesized ZnO NPs was characterized by FTIRfor presence of phytochemicals and zinc oxide using FTIR-8400s (Shimadzu, Japan) Fourier transform infrared spectrophotometer at National Research Institute for Chemical Technology, Zaria, Scanning Electron Microscope (SEM) for the morphology and structure of particles, and Energy Dispersive Xray Spectroscopy (EDX) for the elemental composition and purity of nanoparticles using JOEL-JSM 7600F (JEOL, USA) Scanning Electron Microscope.

RESULTS AND DISCUSSION

Preliminary phytochemical screening of aqueous *A. Indica* extract

The aqueous extract of *A. Indica* was tested for the presence of secondary metabolites which acts as reducing and stabilizing agent for the subsequent biosynthesis of ZnO nanoparticles.Capping agents are frequently used in nanoparticle synthesis to prevent overgrowth and aggregation as well as to control the size of nanoparticle in a precise way. Stabilizing agents were added to the initial solution to prevent agglomeration of the nano-flowers as in agreement with previous literatures [2, 8, 10, 13-15].

The preliminary phytochemical screening revealed the presence of secondary metabolites such as alkaloids, flavonoids, terpenoids, tannins, reducing sugars, saponnins which are proposed to

be responsible for reducing and stabilizing the zinc oxide nanoparticles which is in agreement to previous work reported by Bigoniya et al [16].

The observed colour change confirmed the formation of ZnONPs. Observed colour change was due to the excitation of Surface Plasmon Resonance of ZnONPs.

This approach was considered green as it involved the use of agricultural waste to replace toxic chemicals such as surfactants or polymers (to control the growth of particles), universal green solvent, water (H_2O) instead of volatile organic solvent (VOC) and low energy consumption (reaction proceeded in room temperature) that fulfilled the principles of green chemistry[3].

The synthesis of ZnO NPs involved a redox process. At initial phase zinc acetate was dissolved in water and heated to give zinc cation (Zn^{2+}) in the solution.*A.indica* seed husk extract was first added into salt of zinc acetate dihydrate aqueous solution that reduced Zn(II) to Zn(0) and maintains the size of particles formed in nano scale by capping them from coming into contact with each other. A cloudy solution formed indicates the occurrence of reduction reaction. Sodium hydroxide was added as an accelerant to enhance the rate of reduction and nucleation process by direct precipitation of Zn^{2+} to $Zn(OH)_2$ in alkaline condition, pH 12 followed by loss of water to form ZnO NPs. This leads to the formation of nanoparticleswhich is being stabilized by phytochemicals secreted by *A.indica* seed husk extract [10].

Synthesized ZnO NPs using *A.indica* seed husk extract contains impurities on the particle surface. Higher purity was obtained through washing with water followed by ethanol several times during vacuum suction filtration to remove the water soluble and also water insoluble impurities present on the surface of synthesized nanoparticles.

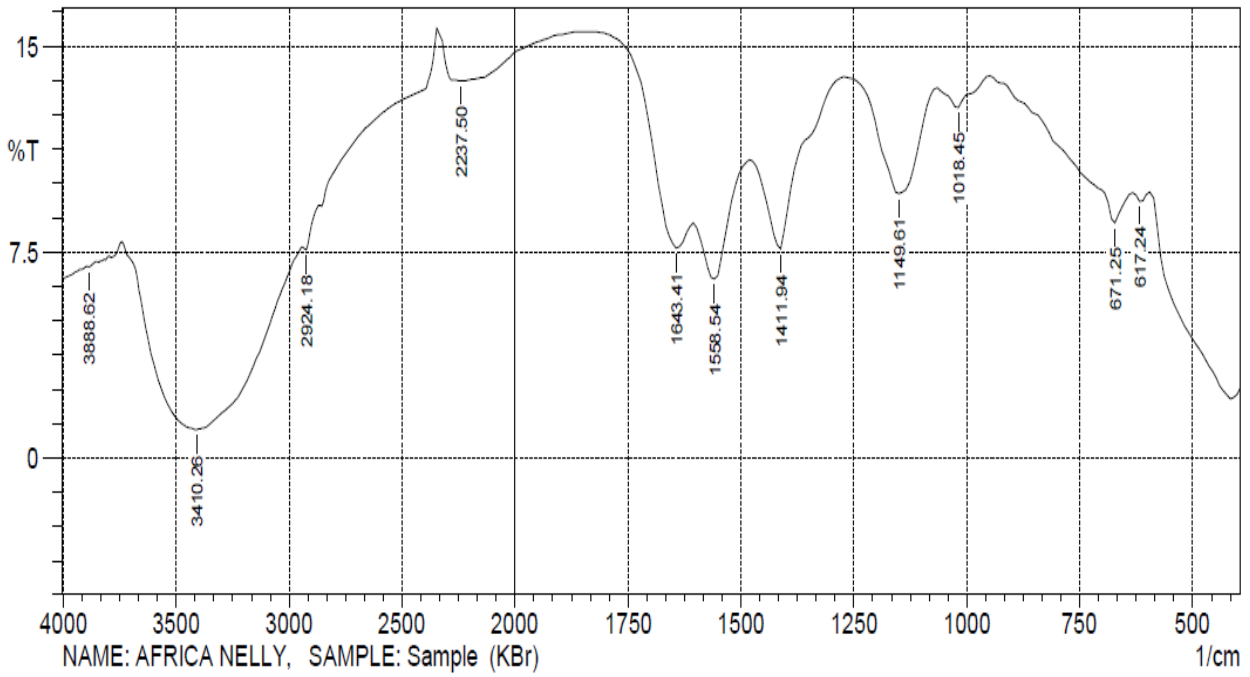

Fig 3: FT-IR Spectra of green synthesized ZnONPs

FT-IR Spectra studies were carried out in order to ascertain the purity and nature of the nanoparticles and also the presence of phytochemicals in the extract. The phytochemicals such as alcohols, phenols, amines and carboxylic acids can interact with the zinc surface and aid in the stabilization of ZnONPs. The peaks that were observed at 1634 and (671, 617, 450) cm−1 correspond to Zn–O stretching and deformation vibration, respectively. Metal oxides generally give absorption peaks in the regions between 600 and 400 cm−1. The Zn–O frequencies observed for the synthesized ZnONPs are in accordance with literature values [17].

The spectral data study showsthat synthesized nanoparticles exhibit (broad, sharp) peaksat 3410.26 cm^{-1} corresponding to stretching bonds of primary O–H groups from phenols, polysaccharides and protein. The peak at 3888.62 cm^{-1} corresponds to the O-H stretching vibration of the intra-molecular hydrogen bond. The peaks at 2924.15 cm−1are attributed to the assymetric and symmetric stretching vibrations of $–CH_2$ group respectively. The peak at 2237.50 cm^{-1} reveals the presence of C–H stretching vibrations of an aromatic aldehyde. The peaks of doublets at 1643.41–1558.54 cm^{-1} correspond to C=O stretching vibrationof primary amines in agreement with earlier results reported by Nethahavanani [2]. Absorption peaks in the region covering 1411.94–1149.61 cm^{-1} imply the presence of an aromatic ring. An absorption peak

found at 1018.45 cm^{-1} corresponds to saturated primary alcohol C–O stretching. The above inference justifies the fact that the presenceof phenols, polyphenols and primary amines in the plantextract could be implicated for capping and stabilization of ZnONPs. The peaks at 671 and 617.24 cm^{-1} indicate the stretching vibrations of ZnO nanoparticle which is consistent with that reported in the previous works of Bhuyan et al [10], and Nethahavanani [2]. The region between 400 and 600 cm^{-1} is assigned for metal-oxygen bond. The FTIR spectrum, absorption at 400 cm_1 to 600 cm^{-1} identifies the presence of zinc oxide nanoparticles which further confirms the formation of zinc oxide nanoparticles by using plant sources. In addition to the absorption bands of the biomolecules used as reduction and stabilization (capping agents), the absorption peak at 450 cm^{-1} indicates the presence of ZnONPs in agreement with previous reports of Suresh et al [18] and Olaitan Ogunyemi et al [19].

The shape, structure and size of the synthesized ZnONPs were determined by the SEM analysis (Figure 4a). The micrograph of ZnONPs proved that they had nano-sized range, spherical shape and uniform distribution.

The SEM image of the synthesized ZnONPs is shown in Figure 4(a). The image shows few large clusters as well as many quasi-spherical shaped ZnO nanoparticles agglomerated together. The cause of the agglomeration could be due to the polarity and electrostatic attraction of ZnO nanoparticles rising from the biological material. The particle size distribution of the nanoparticles was estimated from the Image J software by treating the nanoparticles as spheres and thus calculating the particle size distribution from the deduced area. Figure 4a.shows the average diameter of the prepared nanoparticles to be ranging from ~35 to 60 nm [2].

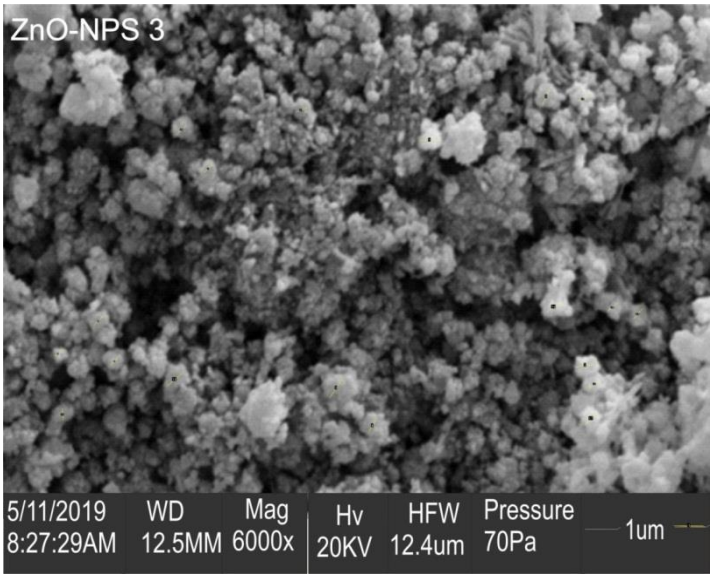

Fig. 4a. SEM MICROGRAPH FOR ZnONPs

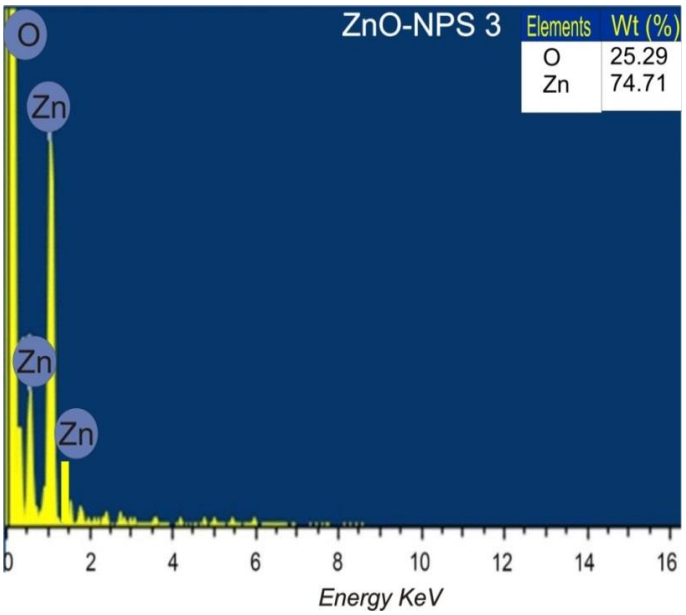

Fig. 4b EDX PLOT FOR ZnONPs

The elemental composition analysis of the ZnONPs from the EDX plot of the SEM images is shown in Figure 4b. The emission of strong signals from zinc and oxygen confirms the presence of zinc and oxygen atoms in the biosynthesized ZnO nanoparticles. The EDX spectra revealed that the required phase of Zn and O is present in the samples with high values of zinc (74.71%)

and oxygen (25.29%), respectively. and confirmed high purity for the synthesized ZnONPs. It confirms the presence of pure ZnO nanostructures.

CONCLUSION

The ZnO NPs were successfully biosynthesized by co-precipitation method using *Azadirachta indica L*. seed husk extract as a reducing and stabilizing agent. The preliminary phytochemical screening of the aqueous extract revealed the presence of alkaloids, flavonoids, terpenoids, tannins, reducing sugars, saponnins. The FTIR spectra revealed the presence of phenols, polyphenols and primary amines in the plant extract and the functional groups of stretching bands of ZnO NPs around 600-400 cm^{-1}. The SEM-EDS analysis showed the morphology of spherical and hexagonal nanoparticles with the compositions of Zn and O elements with no impurities.

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