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Research Article

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Role of Cubebin in the Zinc Nanoparticle Synthesis of *Piper nigrum*

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Abstract

Green nanoparticles synthesis is becoming more popular these days due to its low toxicity as compared to chemical methods. To investigate the structure and morphology of nanoparticles, a zinc nitrate solution is used for Zinc nanoparticles (Zn-NPs) synthesis of *Piper nigrum*. This aqueous extract of seed was utilized in the biogenesis of the Zinc nanoparticles to assess its potential as an effective antimicrobial agent. The surface plasma resonance band at 274 nm, revealed the production of Zn-NPs. Fourier transform infrared detected the presence of functional groups similar to the spectra of Cubebin, the secondary metabolite of *P. nigrum*. Energy-dispersive X-ray analysis showed an elemental make-up of 89.4% Zn content in the NPs Cubebin in seed extract may be the eco-friendly green factor responsible in an aqueous medium as a reducing and capping agent. This green biogenic method was quick and easy to implement, and it could quickly replace chemical production. The bactericidal activity of Zn-NP of *P. nigrum* was determined against both gram-positive and gram-negative bacterial strains. The research demonstrated that the secondary metabolites of the dried seed extract can be a suitable capping agent in the synthesis of the nanoparticles as well as responsible for antimicrobial activities.

Keywords

Antimicrobial, Capping, Cubebin, *P. nigrum*, Zinc nanoparticles

Introduction

Green technologies are popular for their high effectiveness and non-toxicity [1]. This eco-friendly technology involves the use of herbal extracts for the preparation of NPs. The properties of the formed NPs can be influenced by plant extracts from the different parts of the plant by improving their usefulness for biomedical applications [2]. This emerging discipline involves combining biologically produced macromolecules with nanoscience to provide more precise therapy alternatives for a variety of disorders [3]. Colloidal suspensions of NPs are considerably altered by capping agents, making them appealing candidates for biological applications. An amphiphilic molecule with both positive and negative charges can be an excellent capping agent [4]. Among the metal oxides examined for antibacterial activity, Zinc oxide (ZnO) NPs have been reported to exhibit an inhibitory role against potential microbial agents [5]. *Tectona grandis* leaf extract was added to ZnO NPs for anti-arthritic, anti-cancer, antioxidant, antibacterial, and *in vitro* cytotoxic action [6].

Piper is a constituent of the Piperaceae family, and its intense aroma has earned it the title of "King of Spices." Many tropical countries, including Brazil, Indonesia, and India, grow black pepper. *P. nigrum* has bioactive chemicals that

are utilized in medicine, preservatives, and fragrance [6]. The Indian native spice black pepper (*P. nigrum*) has been widely used in human cuisines for thousands of years. The harsh and stinging characteristics of the alkaloid piperine are well-known [7]. Piperine and Cubebin are the most cited therapeutically important secondary metabolites reported in *P. nigrum* [8]. Another important metabolite, a lignan, Cubebin extracted from dry seeds of the genus *P. cubeba*, is noted for reducing spasms, inflammation, pain, and its antagonistic activity against trypanosomes, mycobacteria, and other microorganisms [9]. Most *Piper* crude extracts have been shown to have an antagonistic effect against a variety of microorganisms [10]. The objective of the proposed research work was to assess the use of the crude extract of *P. nigrum* seed as a possible therapeutic agent possessing antimicrobial properties with the least toxicity. Some earlier reports showed the presence of cubebin responsible for the therapeutic activity [11]. Hence the possibility of the presence of cubebin in the plant extract using chromatography in *P. nigrum* seed extract was envisaged. Further, it is also questionable whether the secondary metabolite, Cubebin present in *P. nigrum* extract is responsible for facilitating the synthesis of the metallic nanoparticles.

Material and Methods

Collection, identification of plant material, and chemicals used

The *P. nigrum* plant produces seeds after 5-6 years. The harvested seed from an 8-year-old plant of *P. nigrum* (Figure 1e) from Kannur, Kerala was used in the present study. Cubebin ($C_{20}H_{20}O_6$; molecular weight: 356.37) was purchased from Phyto Lab GMbH & Co. KG (Germany). A standard stock solution (1000 µg ml^{-1}) in methanol was prepared and stored at –18 °C.

Preparation of crude aqueous extract

Shade-dried *P. nigrum* seed was weighed (2 g) and dissolved in 20 ml of Methanol and Milli Q water (50:50 v/v), mixed well by sonication for 30 min at a temperature of 45 °C to 50 °C, and later the temperature was reduced to 37 °C. After centrifugation of the extract at a high speed (10,000 rpm) for 10 min, the top layer was transferred to a fresh centrifuge tube. This process was repeated two times and the final extract was filtered using a specialized Millipore PVDF filter (Millex -GV Hydrophilic 0.22 µm pore size). For injection into LC-MS/MS system, 1 ml of the filtrate was transferred to injection vials.

Detection of cubebin in the crude seed aqueous extract

Liquid Chromatography-Mass spectrometric analysis using Agilent Technologies 6470 Triple Quad Liquid Chromatography tandem Mass spectrometry (LC-MS/MS) was performed by gradient program to quantify Cubebin, which is most abundant in the *P. nigrum* seed. For the mass spectrometric analysis, a gradient mobile phase consisted of eluent A comprising Formic acid (0.01%) and ammonia (1%) and Eluent B comprising water: methanol in the ratio of 95:5. Multi-step gradient program was applied. In the LC system, an elution program was carried out at a 3 ml min^{-1} flow rate and an injection volume of 2 µl. A C18 column was procured operating in reverse phase (Zorbax RRHD Eclipse Plus C18) with a dimension 1.8 m, 100 x 3.0 mm, and was used for chromatographic separation. The ion-spray source with the following settings: 175 °C for the gas, 15 L/min for the flow, 55 psi for the nebulizer (N_2), 350 °C for the sheath gas, 11 L/min for the flow, 3500 V for the capillary voltage, negative polarity, was used. Five level calibration curve concentrations (500 ng to 10,000 ng) were used for the quantitation method of cubebin. The concentration of cubebin in *P. nigrum* seed extract was also interpolated using this standard curve.

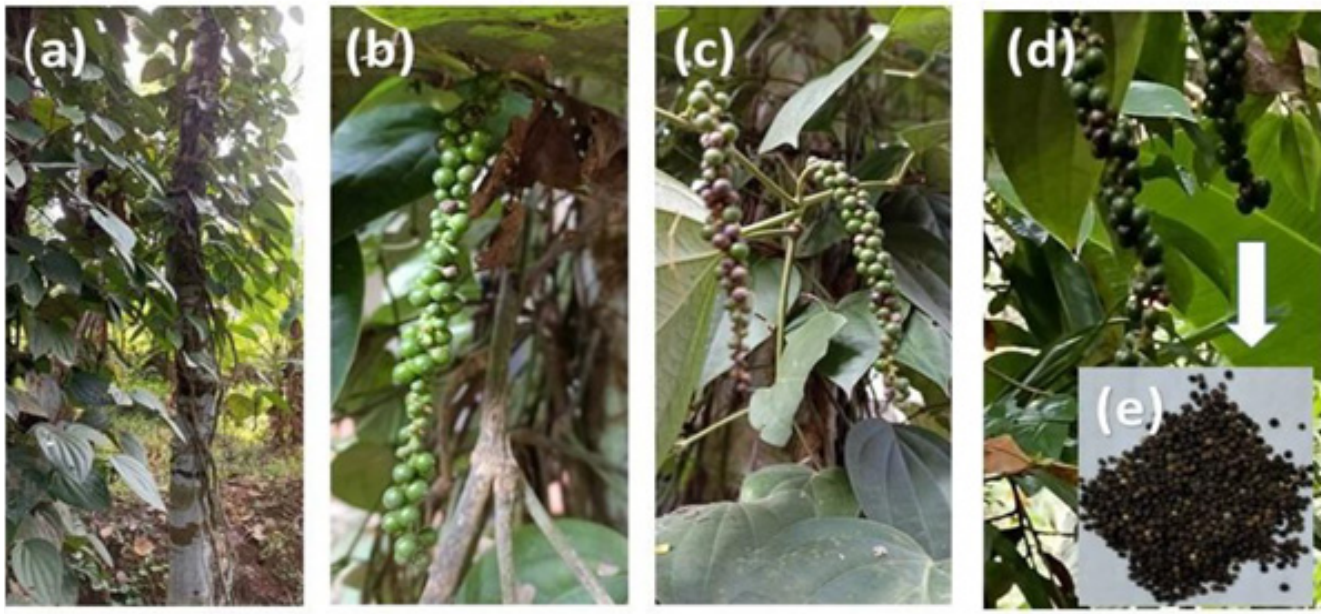

Figure 1: *P. nigrum*, 8 years old, growing at Kannur, Kerala (a) Vegetative growth of the plant bearing seeds in different stages of growth (b) green (c) pinkish green to brown (d) black (e) matured black colored seeds.

Metal analysis using inductively coupled plasma mass spectrometry (ICP-MS)

Fresh seed samples were digested in a Microwave oven and analyzed using ICP-MS according to the protocol followed by Debnath and co-workers [12].

Synthesis of ZnO NPs

Under ambient conditions, 1 mM Zinc sulfate heptahydrate ($ZnSO_4.7H_2O$) was reacted with 1 percent (m/v) aqueous seed extract of *P. nigrum* to produce ZnO-PSE-NPs. The color change from yellow to light yellowish, to white, indicated its biosynthesis. The suspended particles were purified three times by centrifuging the creamy-white colored Zn-NPs solution at 2000 rpm and 18 °C for 30 min. The pale white particles were subsequently washed with 99% ethanol and dried for six hours in a vacuum oven at 60 °C. This solution was also utilized to characterize the *P. nigrum Zn-NPs*.

Characterization of *P. nigrum* seed extract and *P. nigrum* Zn-NPs

The NPs of *P. nigrum* were characterized using UV Spectrophotometer; (Shimadzu UV-2600, Japan), Fourier transform infrared (FTIR) spectrometer (Bruker Optics, Ettlingen, Germany, Model Alpha-T with Eco ATR); Field emission scanning electron microscopy (JEOL make JSM-7610FPlus, Japan; FESEM) and Energy-dispersive X-ray diffraction (EDAX AMTEX, USA).

Antimicrobial activity of *P. nigrum* seed extract

Antimicrobial activity was performed according to the agar cup method [13]. Certified non-pathogenic bacterial strains *Pseudomonas aeruginosa* (MTCC1688) *Bacillus subtilis,* (MTCC 6633) *Staphylococcus aureus* (MTCC 737), and *Escherichia coli* (MTCC 1687) were used in this process. The antimicrobial activity was expressed as the degree of inhibition zone on the agar plate.

Results and Discussion

Quantitative analysis of Cubebin in *P. nigrum* using LC-MS

Peaks corresponding to the molecule "Cubebin" (Figure 2) present in the extract of *P. nigrum* seed were identified by LC-MS/MS analysis. The main characteristics for identification were their charge signal responses (m/z), molecular formula, and molecular mass. Electrospray ionization in negative mode was chosen for the analysis (Figure 2). Along with the calibration standard Cubebin, seed extract of *P. nigrum* was also injected into the column, and the content of Cubebin present in the seed extract was quantified against the calibration standard.

It was observed that Cubebin was eluted at the retention time of 9.8 +/- 0.1 min. (Figure 3 a-d) and the peak eluting displayed a parent mass to fragment mass ion ratio as 355.1 -> 177.1 (Figure 3 e-f). The calibration curve showed a linear regression correlation coefficient of 0.996. During mass analysis, it was evident that the aqueous extract of *P. nigrum* seed contains approximately 443.80 µg/L of the bioactive compound, Cubebin. The standard Cubebin and the Cubebin present in the seed extract showed similar properties and confirmed their unique presence in the extract.

Phytochemical analysis of the seed extract of *P. nigrum*

The concentration of the metals present in the extract of *P. nigrum* seed was assessed and quantified using ICP-MS. It was found that the seed extract showed the presence of some metals as shown in figure 4a. WHO guidelines [14] states that medicinal plants used as raw materials for final goods may be tested for the presence of heavy metals, and the permitted level of lead (Pb), is 10 mg/kg, Similar results were also noted earlier too [15]. These results showed a possibility of the use of the seed extract as a possible therapeutic agent considering it low concentration of metals below permissible limits.

The seed extract was also studied using a UV-Vis spectrophotometer. The highest absorbance noted during spectrophotometric analysis in the seed extracts was in the region of 212 nm which corresponds to the standard cubebin at 230 nm (Figure 4b). This spectral investigation confirms the presence of Cubebin in *P. nigrum* [15].

The FTIR spectroscopy analysis exhibited absorption peaks of *P. nigrum* extract between 4000 cm^{-1} and 400 cm^{-1} in the FTIR spectrum (Figure 4c). The stretching of O-H alcohol and phenol group vibrations is assigned to the bands detected at 3315 cm^{-1}. The N-H stretching alkane could be assigned to the bands detected at 2920 cm^{-1}, whereas the bands detected

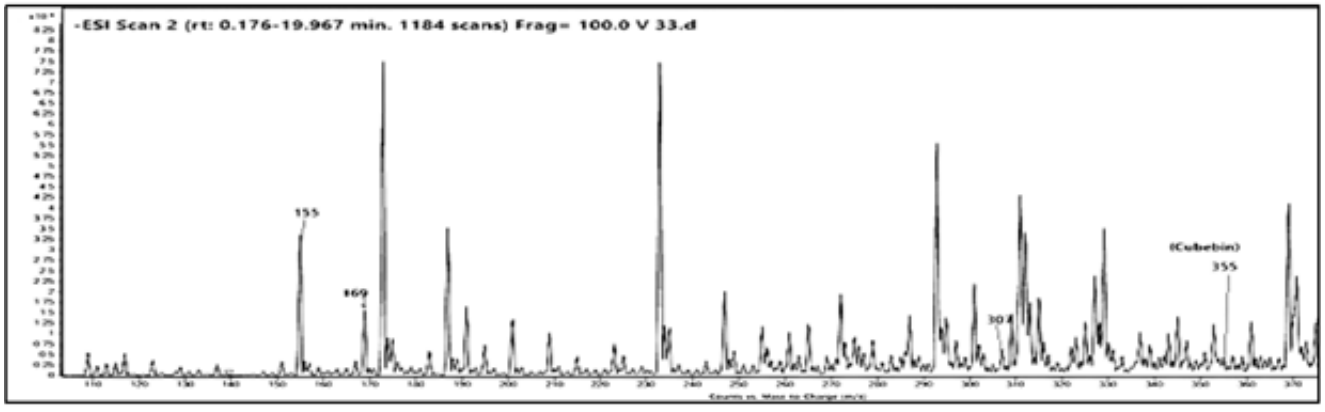

Figure 2: Total ion chromatogram of the seed extract of *P. nigrum* in the negative mode of Electrospray ionization.

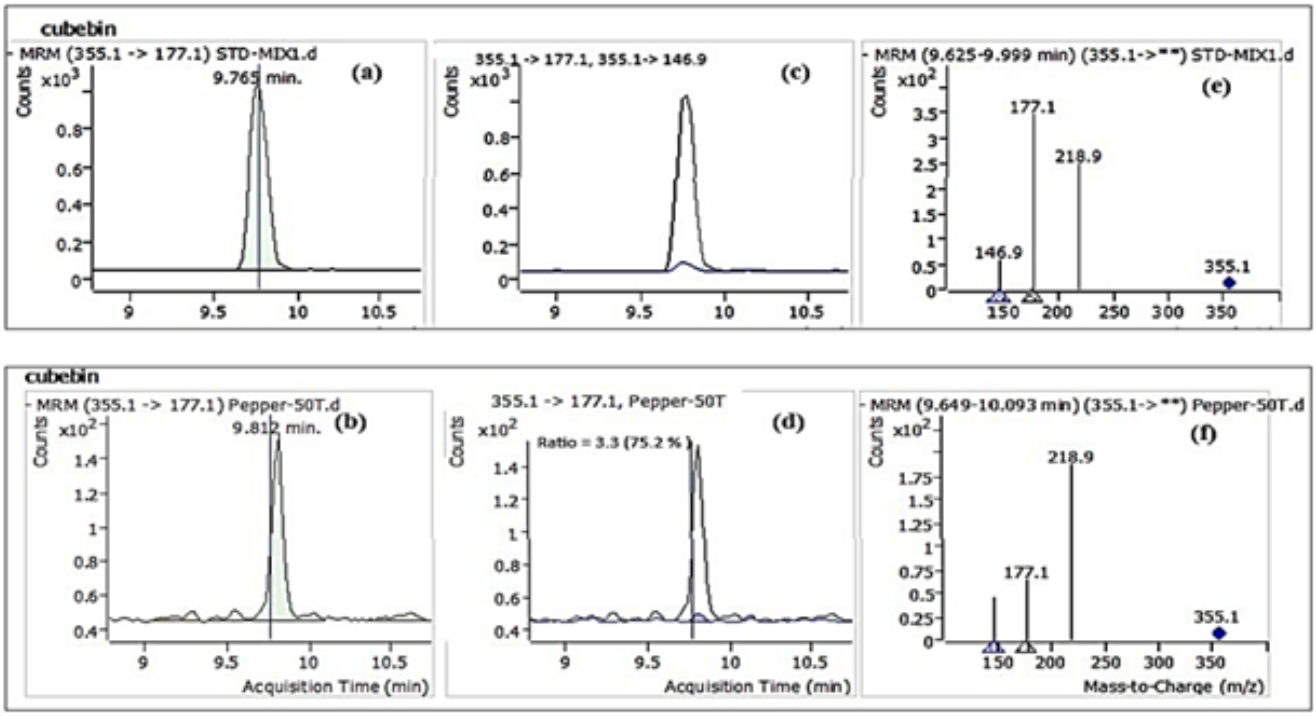

Figure 3: Liquid chromatography-electrospray ionization tandem mass spectroscopy analysis of sample seed extract of *P. nigrum*. (a, b) Multiple reaction monitoring scans showing the retention time of 9.76 of cubebin standard similar to the (c, d) Multiple reaction monitoring of the cubebin present in the seed extract showing the similar retention time of 9.81. (e) Similar daughter ion mass fragment 177.1 in standard cubebin and (f) in sample seed extract respectively.

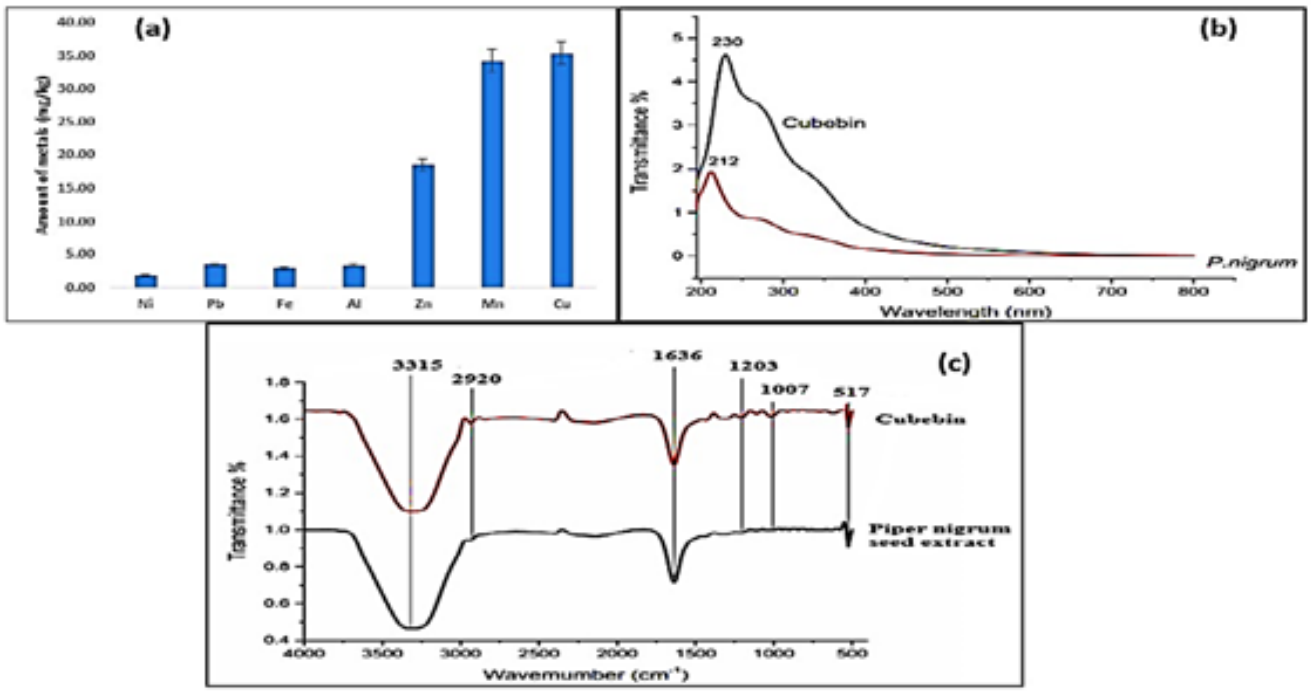

Figure 4: Phytochemical analysis of *P. nigrum* aqueous seed extract (a) Distribution of heavy metal in the sample (b) UV–Visible absorption spectra of sample and standard Cubebin (c) FTIR spectrum of sample and standard cubebin.

at 1636 cm^{-1} may be assigned to stretching cyclic Alkene. 1248 cm^{-1} could be C-N stretching amine can be assigned to the bands 1007 cm^{-1}. Allocating C-I stretching halo molecules is a possibility for 517 cm^{-1}. Krishnan et al. [16] also reported a similar type of observation. in the *P. nigrum* seed extract. All the above results of the *P. nigrum* aqueous extract confirmed the presence of cubebin in the sample.

Characterization of NPs of the crude seed extract of *P. nigrum*

The synthesis of ZnO NPs was confirmed after assessment of the surface plasmon resonance property, at 344 nm which also corresponded to 345 nm in the green synthesized ZnO NPs of *P. nigrum* (Figure 5a). Green produced ZnO NPs of aqueous extract *P. nigrum* seed. The absorption peak is caused by the NPs surface plasmon resonance property, which is caused by the vibrations of unpaired electrons on the NPs interface whenever it coordinates in resonance with the intensity of illumination [17]. The FTIR spectrum of aqueous *P. nigrum* seed extract ZnO NPs and ZnO NPs was investigated (Figure 5b). The O-H extending alcohol could be attributed to the bands detected at 3732 cm^{-1}, whereas the bands detected at 2879 cm^{-1} may be attributed to C-H extending alkane, and 2352 cm^{-1} attributed to O=C=O Carbon dioxide. 1385 cm^{-1} denoted C-H bending alkane,1248 cm^{-1} could be C-N extending amine, or bands found in 1156 cm^{-1} assigned to C-O extending aliphatic, C-N stretching amine can be assigned to the bands 1064 cm^{-1} and 812 cm^{-1} identified as C-H bending, 1,2,3,4-tetrasubstituted [17].

P. nigrum ZnO NPs had a spherical morphology, with an average size of 50.4 - 97.2 nm, as demonstrated by FESEM analysis, validating *Piper* seed extract's ability to create ZnO NPs (Figure 5c). The predicted zinc and oxygen percentages are shown in the EDX results (Figure 5d). It may be noted that Cubebin can be regarded as a potential green capping agent for the stability contributing to the formation of a NPs.

Antimicrobial activity of *P. nigrum* seed extract

P. nigrum (black pepper) seed extracts showed various antibacterial activities against the microorganisms listed. The antimicrobial activity of ZnO NPs of *P. nigrum* was pronounced against, *B. subtilis, P. aeruginosa, E. coli,* and *S. aureus* (Figure 6a-c). Results show that *B. subtilis* has more antimicrobial activity than any other microorganisms. In the comparison of the activities of the ZnO NPs of *P. nigrum* against different bacterial strains it was found that seed extract containing Cubebin had a profound effect on both gram-positive and gram-negative bacterial species. Initially, they inhibited the growth of the bacteria but later their activity with due course of time was bacteriostatic in nature against all these bacteria tested. Antibacterial activity of *P. cubeba* Linn. in fruit extracts and *P. nigrum* seed extract was noted against selected bacterial species [13,15]. ZnO also inhibited the bacterial species. Antimicrobial activity can be explained by the oxygen species that are released on the surface of ZnO and inflict lethal harm to microorganisms. They produce hydrogen peroxide (H_2O_2) molecules when they combine with hydrogen ions. The H_2O_2 produced can pass through the cell membrane and destroy germs. The surface area of ZnO has a considerable influence on the formation of H_2O_2, which results in more oxygen species on the surface and stronger antibacterial activity of the smaller nanoparticles [18].

Conclusions

The present study shows that the biogenic production of ZnO nanoparticles is more environmentally sustainable, easy, and effective than the standard technique, and uses fewer harmful chemicals. The green synthesis of the NPs using *Pip-*

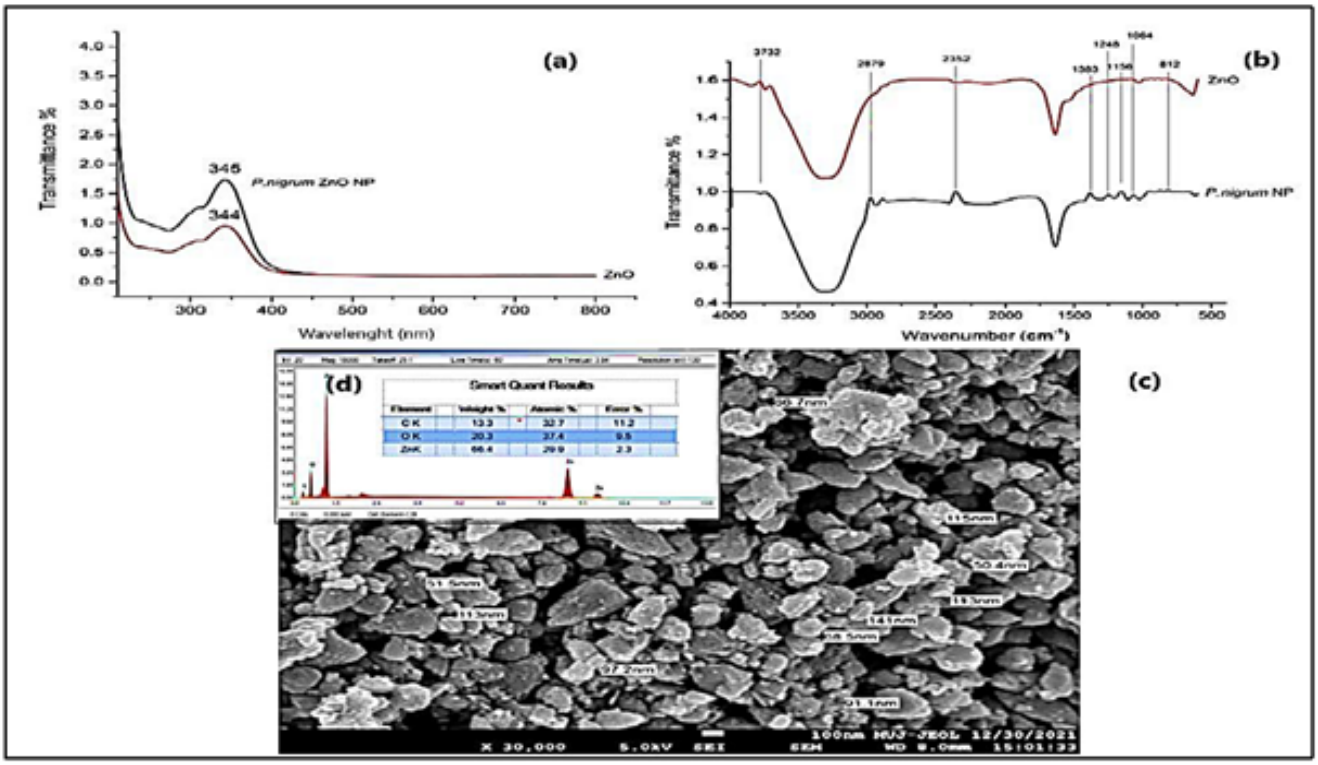

Figure 5: Analysis of the ZnO NPs of *P. nigrum* aqueous seed extract. (a) UV–Visible absorption spectra (b) FTIR spectrum of a sample of ZnO NPs of seed extract and ZnO (c) FESEM image of sample (d) EDAX analysis of a sample containing Cubebin.

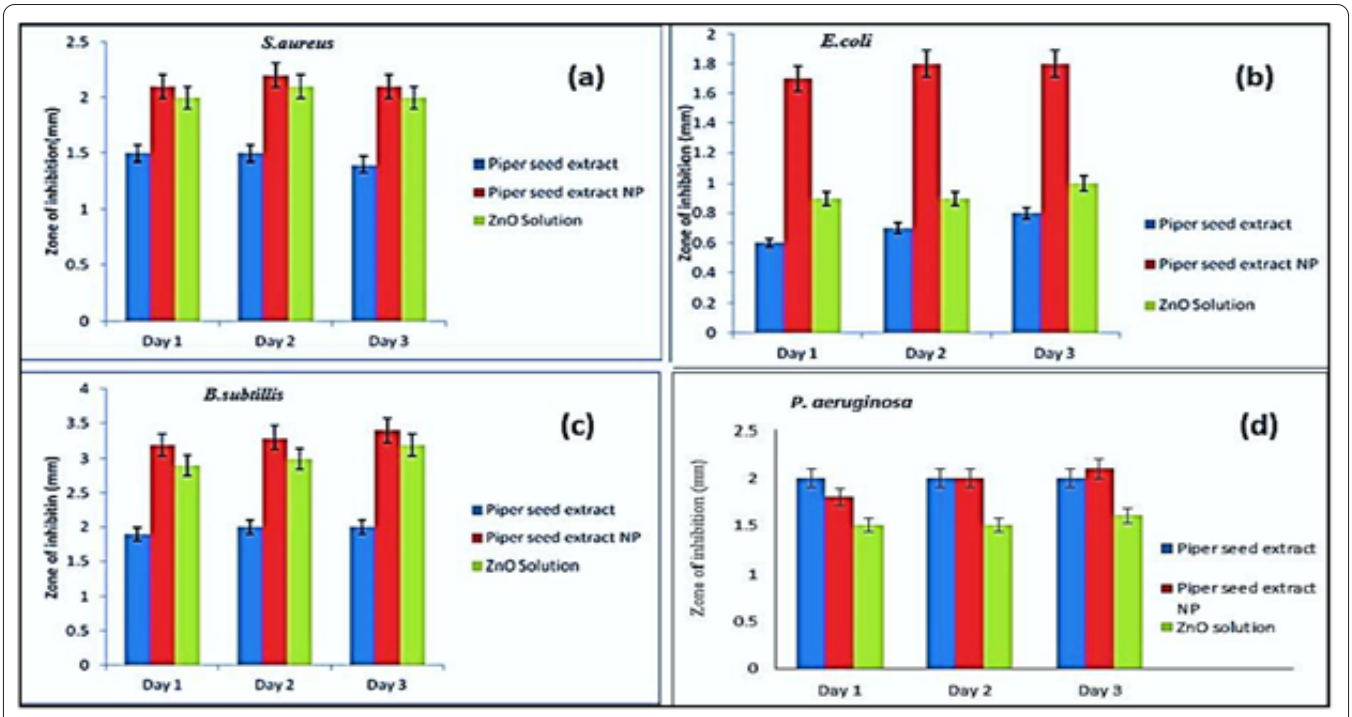

Figure 6: Antimicrobial activity of ZnO NPs of *P. nigrum* seed extract against selected non-pathogenic bacterial isolates. (a) *S. aureus* (b) *E. coli* (c) *B. subtilis* (d) *P. aeruginosa.*

er nigrum seed extract was successfully achieved. *Piper nigrum* seed extract and the Zn NPs showed maximum inhibition of the growth of *B. subtilis* and *P. aeruginosa.* The activity was optimum against *E. coli* and *S. aureus*. The role of secondary metabolites in the antimicrobial effect was established by finding the presence of cubebin in the seed extract. Evidence, as noted in *P. cubeba* Linn for cubebin as the possible active reagent for antimicrobial activity, can be correlated with the similar activity as seen in *P. nigrum.* Cubebin can be regarded as a potential green agent during the formation of the NPs.

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