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Green Synthesis and Characterization of Nanoparticles Made from *Ocimum sanctum* Leaf Extracts and Assessment of its Antibacterial Activity

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Abstract

Herbal plants have an important role as traditional medicines. *Ocimum sanctum* L. (Tulsi) is a fragrant herb belonging to the Lamiaceae family that has been proven to have immunomodulatory, immunostimulatory properties and antagonistic activity against bacteria, fungi, and viruses. Secondary metabolite "Apigenin" (269.0 m/z), a flavonoid, present in this divine herb, was detected, and quantified by liquid chromatography-electron spray ionisation tandem mass spectrometry (LC-MS/MS) analysis. The objective of this investigation was to synthesize *O. sanctum* leaf extract mediated zinc oxide nanoparticles (ZnO NPs), assess its antimicrobial activity and corelate this activity with the secondary metabolites presents in this herbal extract. In this study, shade-dried leaves of *O. sanctum*, were used for initiation of the biosynthesis of ZnO NPs. The occurrence of ZnO in the NPs was proven by different analytical methods such as Ultraviolet-Visible (UV-Vis) spectrophotometer and Fourier-transform infrared spectroscopy (FTIR). Using field emission-scanning electron microscope (FE-SEM), the average dimension of *O. sanctum* ZnO NPs ranged from 63.3 nm to 89.1 nm. Profound broad spectrum bactericidal activity was noted. This can be corelated to the occurrence of apigenin in the leaf extract and its ZnO NPs. *O. sanctum* is thus, the choice of many ayurvedic formulations.

Keywords

Ocimum sanctum, Apigenin, Nanoparticles, Antibacterial, Ayurvedic formulations

Introduction

Medicinal plants, often known for herbal remedies, are used for the management of specific conditions or to maintain health [1]. Many medicinal plants of Indian origin are explored for their therapeutic properties and are components of ayurvedic formulations [2]. Herbs are well-considered as a viable technique for humans to combat viral illnesses because of their inexpensive cost, simple preparation, and powerful antiviral impact [3]. *O. sanctum* is a commercial basil plant known for its medicinal properties. Some of the well-researched secondary metabolites like oleanolic acid, linalool, sitosterol, rosmalinic acid, caryophyllene, apigenin, and luteolin display properties like febrifuge, antifungal, antibacterial, and antioxidant properties which are responsible for treating a variety of disorders [4-6].

Ancient cultures used traditional herbal remedies to treat or prevent the common cold and influenza. Numerous plants have been successfully utilized as cures for viral respiratory infections [7]. *O. sanctum* leaf extracts have been examined for their ability to heal many diseases from the common cold, malaria, different poisonings, heart disease, headaches, and gastric challenges [8,

9]. The extracts of this plant can function in work as powerful agents for maintaining the shape, dimension, stability by binding to the surface of the NPs [10]. Investigations have directed the development of inexpensive and environmentally benign materials in nanosized in the domains of science, engineering, and biotechnology [11]. Nanotechnology is a newer technology that extracts NPs from medicinal plants for use in conventional applications [12]. *O. tenuiflorum* leaves have been used to make copper, gold, zinc, and silver NPs [13]. The ZnO NPs has demonstrated antibacterial, anticancer and enzyme inhibitory properties, and they also exhibit biological compatibility. The current effort was carried out to generate and characterize ZnO NPs using a water-based extract of *O. sanctum* plant leaves. The antibacterial efficacy of *O. sanctum* ZnO NPs against non-pathogenic bacteria has been reported earlier [14]. *O. sanctum* must be ingested fresh to suppress and prevent the common cold. As *O. sanctum* leaves are bestowed with all these therapeutic activities, a green synthesis of *O. sanctum* ZnO NPs was attempted and subsequently its antimicrobial activity [15]. Because of their antibacterial capabilities, *O. sanctum* ZnO NPs are one of the most extensively employed nanoparticulate materials, although their key principle for action has not been thoroughly explored. was also assessed in the present study.

Material and Methods

Plant material and chemical usage

In this study, leaves of *O. sanctum* were separated from the gathered plant components, cleaned, dried in the shade, and finely crushed. Standard apigenin ($C_{15}H_{10}O_5$; Mol. Wt. 270.24) was procured from Medchem Express LLC (USA). Primary reference stock solutions were prepared as reported earlier [16, 17] and kept at 4 °C.

Extraction of metabolites from of *O. sanctum*

For the removal of contaminants and dust, the shade dried plant components were ground in a motor pestle and transferred to a 1 L flask, containing 800 ml of distilled water. The contents were heated in a water bath at 50 °C for 5 h. The resultant extract was cooled, filtered, and dried in the hot air oven.

Preliminary analysis of aqueous extract

To determine whether secondary metabolites were present in the crude *O. sanctum* leaf extract, qualitative analysis was carried out. Using common chromogenic reagents as per the standard protocol, the aqueous extract of the plant was subjected to secondary metabolite investigation in order to find the presence of steroid, alkaloid, flavonoid, saponin, and tannin. These qualitative tests yielded analytical results for precipitate generation and changes in color intensity [16].

Chromatographic analysis of leaf extract of detection of flavonoid apigenin

The sample preparation and method for analysis of apigenin in the aqueous extract of *O. sanctum* leaf was performed using LC-MS/MS, in the same way as previously discussed [17].

Quantitative evaluation of metal content in *O. sanctum* leaves

Toxic content of heavy metal compound present in the plant extract was be evaluated by ICP-MS (Inductively coupled plasma mass spectrometry) [18]. After completion of digestion in microwave digester, the clear sample solution was filtered, diluted, and injected through ICP-MS for the analysis of the heavy metals. A stock concentration of 1 ppm of a mixture of lead, cadmium, copper, cobalt, zinc, arsenic, nickel, and mercury was prepared from 1000 ppm standard metal solutions.

Biosynthesis and purification of *O. sanctum* leaf extract mediated ZnO NPs

The *O. sanctum* mediated ZnO NPs biosynthesis was performed by agitation of 100 ml of a 1 mM zinc sulphate heptahydrate ($ZnSO_4.7H_2O$) solution and the 1 percent (m/v) aqueous leaf extract for an hour at 60 °C and 700 rpm. Following the drop wise addition of 25 ml of leaf extract, color changes were observed. After measuring the pH, 1 M NaOH solution was added to and adjusted to pH 12. Production of *O. sanctum* ZnO NPs was noted and visualized as particles with cream-white appearance. Following a night of incubation at room temperature, it was further incubated in the same condition for further 120 min. The cream-colored suspension of ZnO NPs was filtered thrice by centrifuging it at 2000 rpm for half an hour. 99 percent ethanol was used to wash the creamy white-colored particles in order to remove impurities from the completed products [19].

Characterization of *O. sanctum* ZnO NPs

O. sanctum ZnO NPs generated by biological activities were assessed using a diversity of analytical characterisation methods. The *O. sanctum* mediated ZnO NPs synthesis was initially verified by means of a UV-Vis spectrophotometer depending on their morphology, shape, and size, ZnO NPs can emit light with a wavelength that varies from 300 to 400 nm according to surface plasmon resonance (SPR). Using FE-SEM, the topological morphology of the created NPs was investigated.

Antimicrobial studies of *O. sanctum* ZnO NPs

To cultivate bacteria, nutrient broth and nutrient Agar medium was used to grow the selected microorganisms for the present investigation. *Staphylococcus aureus* (MTCC No. 9542), *Escherichia coli* (ATCC 39403), *Bacillus subtilis* (MT678921), and *Pseudomonas aeruginosa* (MZ348930) were administered against the 50 µl leaf extract (1 mg/ml) and NPs during the antimicrobial assay. Using a sterile glass spreader, 40 mm of 24 h old culture inoculum was applied to nutrient agar plates. The medium was then divided into 4 diameter wells, which were subsequently filled with 50 µl (1 mg/ml) leaf extract, *O. sanctum* ZnO NPs, ZnO and distilled water, and allowed to diffuse for an hour at room temperature. Additionally, these plates were left untouched during their 24 h incubation period at 35 - 37 °C. The inhibitory zone was established using the well diffusion approach and the diameter size was evaluated immediately after the incubation period [19].

Results and Discussion

Qualitative analysis

A simple biochemical analysis of the leaf extracts was carried out to detect the bioactive metabolites present in it. *O. sanctum* leaf extract lacks anthraquinone but contains secondary metabolites such as saponin, steroid, flavonoid, and tannins. Similar reports of leaf extract of *O. sanctum* were also reported earlier [20].

Quantitative analysis of apigenin in leaf extracts of *O. sanctum*

LC-MS/MS was used for the detection of the chosen metabolite present in flavanoid. The electrospray ionization (ESI) in the negative mode was opted for the analysis of "Apigenin", one of the active principle present in *O. sanctum*. A precursor ion [M-H] showing m/z 268.9 - >116.9 at 3.4 +/- 0.1 min was amongst the important peaks (Figure 1). These peaks were recognized as matching to the chemical "Apigenin" (Figure 1) acquired by LC-MS/MS. Along with the calibration standard, leaf extract of *O. sanctum* was introduced in the column. Apigenin was calculated by calibrating it against the procured standard chemical "Apigenin". It was noted that the chemical based standard apigenin (Figure 1), and plant extracted apigenin (Figure 1), both were eluted at 3.4 min +/- 0.1 as hence was recorded as their retention time. Both the chemical and extract containing apigenin showed similarity in their mass to ion (m/z) transition as 268.9 - >116.9 apigenin (Figure 1).

Quantitative analysis of metal content in *O. sanctum* leaves

A detailed list of 22 elements as depicted in figure 2, were found from the results of the analysis of *O. sanctum* leaf extract using ICP-MS. As shown in figure 2, toxic metals like Pb, Cd, Co, Ni and Hg were below the permissible limit as compared to the value reported in World Health Organization (WHO) [18], whereas a non-carcinogenic health risk to the consumer associated with the consumption of *O. sanctum* marketed in study areas.

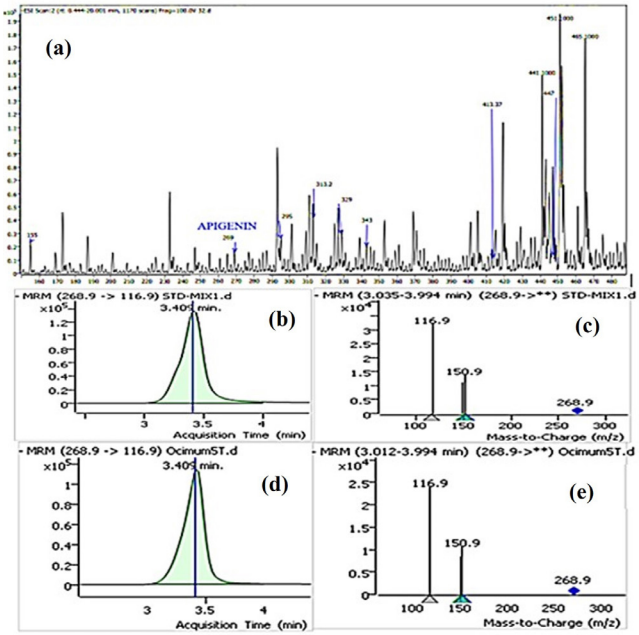

Figure 1: LC-MS/MS analysis of *O. sanctum* leaf extract showing **(a)** Total ion chromatogram of the crude extract of *O. sanctum*, **(b)** Chromatogram of standard apigenin, and **(d)** Apigenin present in the leaf extract of *O. sanctum* showed similar retention time of 3.409. **(c)** Mass fragment apigenin standard and in **(e)** sample extract showed similar mass fragments of 116.9 and 150.9 molecular weight, respectively.

UV-Vis spectroscopy analysis

O. sanctum leaf extract and apigenin was utilized to endeavor the role of apigenin in NPs synthesis. Apigenin ardently absorbed UV-Vis light and showed figure 3a absorption maxima at 202 nm and 320 nm. A UV-4000 UV-Vis spectrophotometer was employed to measure the zinc ion reduction in the resulting solution at wavelengths ranging from 200 nm to 800 nm. Figure 3b shows that the *O. sanctum* ZnO NPs matched the ZnO solution and that the SPR was between 340 nm and 350 nm. The absorption maxima at 350 nm in figure 3b can be correlated to the SPR and indicates the production of *O. sanctum* ZnO NPs. According to previous study [21] SPR of the bioinspired ZnO NPs from *O. tenuiflorum* synthesized NPs peak was observed at 380 nm.

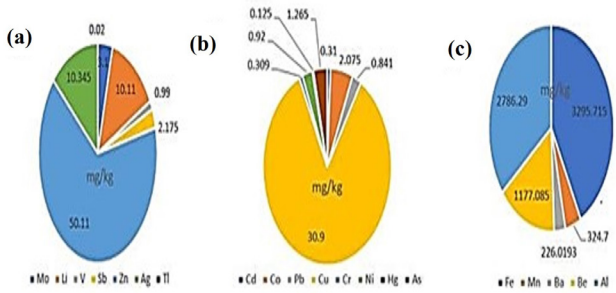

Figure 2: (a - c) Metal content in *O. sanctum* as analyzed by the ICP-MS study were below the permissible limit as compared to the value reported in WHO [18], whereas a non-carcinogenic health risk to the consumer associated with the consumption of *O. sanctum* marketed in study areas.

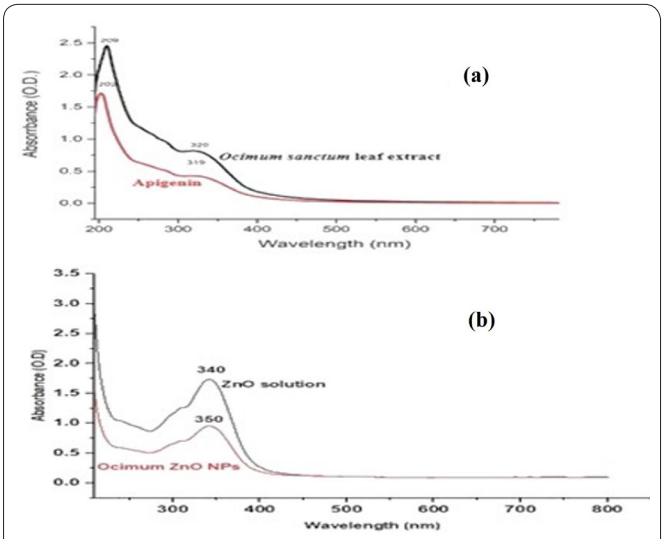

Figure 3: UV-Vis spectroscopy analysis of **(a)** *O. sanctum* leaf extract and apigenin **(b)** ZnO solution and *O. sanctum* ZnO NPs.

FTIR spectroscopic analysis of the crude extract confirmation of formation of NPs

The primary absorption bands in the FTIR spectrum of *O. sanctum* leaf extract as shown in figure 4a, are 3338 and 2968 cm^{-1}, 3338 cm^{-1}, a prominent absorption band indicates stretching of the -NH band of amino groups and the -OH group mostly from phenolic compounds, 873 cm^{-1} showing C-H bending for 1,3-disubstituted cyclohexane and 1690 cm^{-1} C=O stretching primary amide, respectively. Three prominent peaks were also noted at 1634 cm^{-1} C=C stretching 873 cm^{-1} alkene, and 1041 cm^{-1} CO-O-CO stretching representing anhydride. The presence of carboxylic acid groups is indicated by the band 2968 cm^{-1} O-H stretching and C-H bending of the aldehyde group are respectively attributed to the bands at 1389 cm^{-1}. The 2884 cm^{-1} band represents -CH stretching and the band 523 cm^{-1} represents the characteristics of the C-I and C-Br stretching vibration [22-24]. Shah and Patel. examined the presence of organic components in FTIR spectral analysis in *O. sanctum* leaf extract. According to previous research similar functional groups have been discovered in apigenin compounds that are present in *O. sanctum* leaf extract [24]. The presence of 1,3-disubstituted cyclohexane was found in both apigenin and in *O. sanctum* leaf extract. This may be the active principle contributing to the antimicrobial activity as also cited in previous studies.

O. sanctum ZnO NPs FTIR spectra are displayed in figure 4a. The purity and composition of the metal NPs were determined using FTIR studies. The absorption bands that metal oxides often produce, which are caused by inter-atomic vibrations, are in the spectral region, or below 1000 cm^{-1}. The vibration of the N-H stretching was given the band number 3785 cm^{-1}. The OH of the phenolic group of the plant extract helps in capping activity, is denoted by a wide band at 3736 cm^{-1}. The bands at 1627 and 1537 cm^{-1} can be designated as stretching vibrations of the C=C and N-O stretching, respectively. The distinct peak observed at 3736 cm^{-1} may be due to O-H deformation and stretching, which are attributed to the attachment of the water molecule on the metallic surface of the NPs. Peaks at 1627 cm^{-1}, can be marked as Zn-O stretching and deformation vibration. The small band at 517 cm^{-1}, 566 cm^{-1} is ascribed to metal-oxygen stretching, demonstrating the production of ZnO NPs [23]. According to previous reports [23], 413 cm^{-1} represent ZnO peaks. The correlations between ZnO, *O. sanctum* leaf extract and biosynthesized *O. sanctum* ZnO NPs revealed minor changes yet confirming the presence and role of ZnO in the reduction mixture. The reduction of metal ions in the present study is probably facilitated by flavonoids, specially apigenin present in crude leaf extract. The study looked for putative functional groups involved in ZnO NPs bio-reduction, capping, and stability using FTIR.

Analysis of micrographs and elements present in biosynthesized NPs

The FE-SEM micrographs demonstrate the positive synthesis of *O. sanctum* ZnO NPs and revealed that the biosynthesized NPs has a nano range, and a spherical shape (Figure 5a). $ZnSO_4$ was used as a precursor to create the round ZnO NPs, and as the NPs matured and accumulated, they took the form of bundles resembling flowers as shown in figure 5a. FE-SEM analysis proved that *O. sanctum* ZnO NPs had a spherical morphology and a typical size of 63.3 nm to 89.1 nm, demonstrating the prospective *O. sanctum* extract to produce ZnO NPs [24].

From figure 5b, the EDX (Energy dispersive X-ray spectroscopy) analysis showed the composition of the synthesized *O. sanctum* ZnO NPs, endorsed the manifestation of zinc and the synthesis of pure metallic ZnO NPs. Carbon and oxygen peaks, sourced from secondary metabolites, act as protectors on the exterior of ZnO NPs. In addition, no additional peaks were noted to be associated with components other than 'zinc', carbon and 'oxygen' were identified, verifying the chemical purity of *O. sanctum* ZnO NPs.

Antimicrobial studies of the biosynthesized NPs

Most of the microbial strains remained responsive to

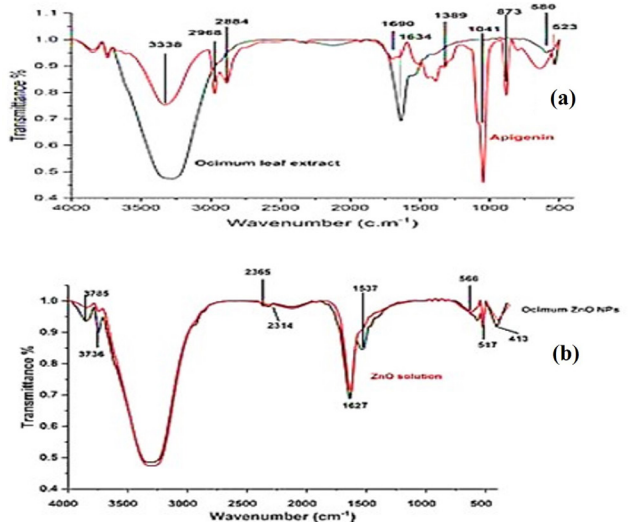

Figure 4: Detection of functional group using FTIR analysis of **(a)** *O. sanctum* leaf extract and apigenin and **(b)** ZnO and *O. sanctum* ZnO NPs.

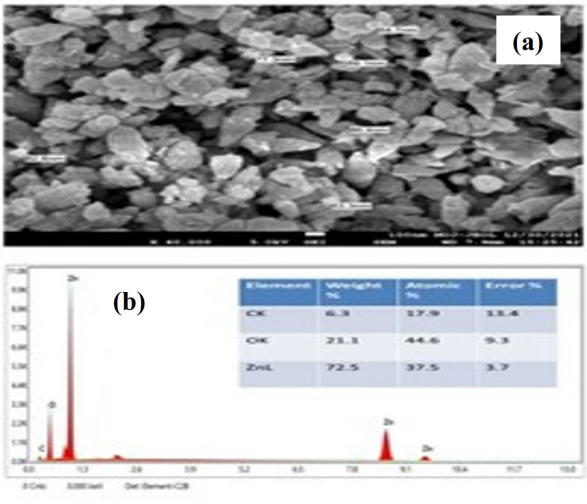

Figure 5: FE-SEM and EDX image of *O. sanctum* ZnO NPs. **(a)** FE-SEM showing NPs of *O. sanctum* ZnO NPs ranging from 63.3 to 89.1 nm and **(b)** EDX image of *O. sanctum* ZnO NPs showing 72.5% zinc.

ZnO NPs and were considerable antibacterial against the tested these strains (Figure 6a to 6d and 6a' to 6d'). Hence, it was concluded that the activity of the *O. sanctum* ZnO NPs showcased broad antimicrobial activity. Based on zone of inhibition produced by the *O. sanctum* ZnO NPs against the tested organism, the order of inhibition was *P. aeruginosa* < *B. subtilis* < *E. coli* < *S. aureus* (Figure 6a to 6d and 6a' to 6d'). In accordance with the activity of ZnO, the maximum antimicrobial activity was noticed in *B. subtilis*, followed by *P. aeruginosa, E. coli*, and *S. aureus*, respectively.

The *O. sanctum* ZnO NPs exhibited strong bactericidal activity when checked against a period of 72 h against *B. subtilis*. Gram positive bacteria are covered by a peptidoglycan layer, ZnO NP attack on the cell wall [25, 26]. *B. subtilis* is a Gram +ve bacteria, and it is possible to break the peptidoglycan layer of the cell wall of the bacteria and inhibit growth followed by cell death of this harmful bacteria. According to Tilahun et al. [21], observed that *O. lamifolium* leaf extract shows highest zone of inhibition against gram positive bacteria. They show that at 100 µg/ml concentration of *O. lamifolium* leaf extract inhibits the growth of bacteria. The presence of 1,3-disubstituted cyclohexane was found in both apigenin and in *O. sanctum* leaf extract. This may be the active principle contributing to the antimicrobial activity as also cited in previous studies [27]. Apigenin has inhibited a wide range of pathogenic bacteria and exhibit broad spectrum of antimicrobial activity [28-31]. Apigenin affects cell membrane organization through modifying the consistency and alignment of lipids in the membrane, resulting in vesicle rupture [29]. Numerous investigations have found that the antibacterial effects of these flavonoids primarily entail inhibition of nucleic acid synthesis, regulation of cytoplasmic membrane function that affects accessibility, and associations with multiple crucial enzymes [30-31].

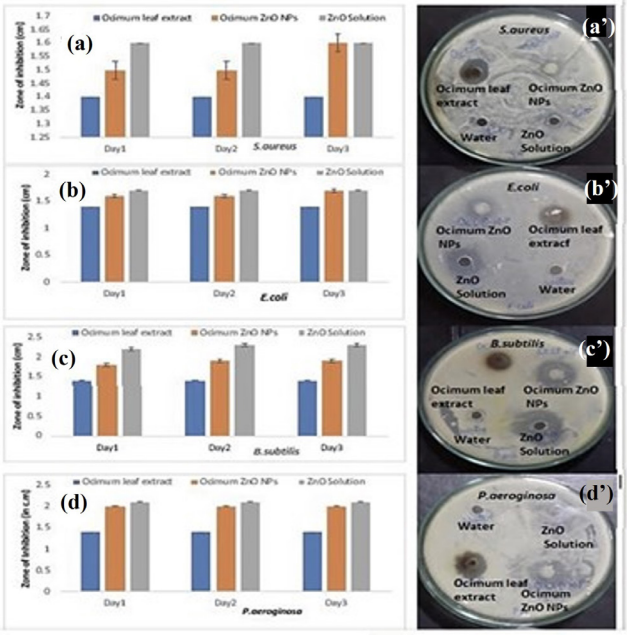

Figure 6: Antimicrobial activity of the *O. sanctum* ZnO NPs, ZnO, *O. sanctum* extract was evident against the different bacterial species **(a)** *S. aureus*, **(b)** *E. coli*, **(c)** *B. subtilis*, and **(d)** *P. aeruginosa*. The well diffusion method was used to check the antimicrobial activity against the non-pathogenic bacteria **(a')** *S. aureus*, **(b')** *E. coli*, **(c')** *B. subtilis*, and **(d')** *P. aeruginosa.*

Conclusion

ZnO NPs mediated *O. sanctum* leaf extract were biosynthesized using an ecofriendly inexpensive, and simple technique. The ability of this metallic NPs exhibited enhanced bactericidal activity against gram +ve and gram -ve bacteria. LC-MS/MS and FTIR spectroscopy revealed that the apigenin present in the plant extract helped in maintaining the configuration of the biosynthesized NPs. Antimicrobial activity was probably due to the same secondary metabolite, apigenin. In future *O. sanctum* leaf extracts and its NPs can be a material of choice for various formulations.

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Conflict of Interest

None.

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