Nano World Journal

https://doi.org/10.17756/nwj.2022-s1-007

Research Article

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Comparison of the Phytochemical Constituents and Antimicrobial Activity of Zinc Nanoparticles of Two Varieties of Olive

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Received: August 23, 2022
Accepted: October 07, 2022
Published: October 09, 2022

Citation: Sisodiya S, Debnath M. 2022. Comparison of the Phytochemical Constituents and Antimicrobial Activity of Zinc Nanoparticles of Two Varieties of Olive. *NanoWorld J* 8(S1): S32-S38.

Published by United Scientific Group

Abstract

Olive (*Olea europaea* L.) leaf extract (OLE) has a repository of secondary metabolites with a unique potential for anti-inflammatory, anticancer, antidiabetic, antimicrobial, antiviral, hypoglycaemic, hypolipidemic, and antioxidant activity. As Olive leaves are bestowed with all these therapeutic activities, green synthesis of Zinc Oxide (ZnO) OLE nanoparticles (NPs) was attempted, and subsequently, its antimicrobial activity was also assessed in the present study. The Arbequina and Coratina varieties of Olives, showed a marked difference in oleuropein and hydroxytyrosol content. The leaves of both the varieties were assessed for their NPs biosynthesis, characterization, and their antimicrobial activity. The antimicrobial activity of the Olive variety, Coratina was more against both *Bacillus subtilis* and *Staphylococcus aureus* in comparison to Olive cultivar Arbequina. Profound bacteriocidal activity was also observed by the NPs produced by the Cortina in comparison to the Arbequina variety of Olive. The antimicrobial activity of the Olive and its NPs may be due to the secondary metabolite content. Using OLEs, the ZnO NPs formed can be used, in the future for the synthesis of cosmetic formulations.

Keywords

Oleuropein, Hydroxytyrosol, Olive leaf extract, *Olea europaea* L, Zinc nanoparticles

Introduction

In the twenty-first century, nanotechnology is the most forward-thinking field. NPs have sparked a lot of interest because of their unique and captivating qualities in a variety of applications [1]. Environmentally safe and cost-effective, green NPs synthesis outperforms physical and chemical processes [2]. Nanotechnology is described as the study and management of matter at rough dimensions scales of 1 - 100 nanometres, allowing for the development of innovative applications as a result of nanotechnology's unique phenomena [3]. They have extraordinarily high surface-to-volume ratios due to their small size, allowing them to constrain electron motions within limitations, improving photocatalytic activity [4]. ZnO is a non-toxic that aids in disinfection [5]. Metallic NPs can be manufactured organically with a range of plants and extracts that are widely available. The plants and extracts are non-toxic and good for the environment [6].

Plant extracts have been used in the production of metal NPs in recent studies. The changes in secondary metabolite content in different varieties can show differential therapeutic activity. The NPs produced from such different cultivars will also show different characteristics. More than 25 varieties of Olives *O. europaea* are grown all over the world. A wide range of alcohols, secoiridoids, phenolics, and flavonoids are found in OLE [7]. Phenolic compounds are amongst the most important families of active chemicals in plants, accounting

for the majority of plant bioactivity. OLEs' biocompatibility appears to be linked to the antimicrobial properties and different phenolic chemicals found in the leaves [8]. The phenolic profiles of olive leaves can be influenced by a number of variables, including cultivar/genotype, developmental stage, climate, season, and post-processing variables including the drying conditions, temperature, light exposure, and oxygen exposure. The cultivar/genotype will have a big impact on the kind and quantity of phenolic chemicals [8] The antibacterial properties of the plant are attributed to phenolic components identified in crude extracts of olive leaves [9]. The principal olive oil phenolic compounds, Hydroxytyrosol, Tyrosol, Oleuropein, Oleocanthal, and Oleacein have biological activity, metabolism, and bioavailability, with the most notable being their antiatherogenic, cardioprotective, anticancer, neuroprotective, and endocrine properties [10]. Oleuropein and Hydroxytyrosol present in olive leaves and olives have a variety of therapeutic applications against a variety of diseases, including melanoma, type 2 diabetes, nephropathy, and neurotrophic and myocardial infarctions. This is most likely due to their putative antioxidant and anti-inflammatory activity [8]. The biological characteristics of phenolic compounds are well established [3]. Oleuropein has been well researched for its anti-inflammatory and antioxidant capabilities and for apoptosis activation, Alzheimer's development suppression, as well as its anti-cancer activity [11]. It has been demonstrated that the bitter iridoid Polyphenol oleuropein, which was derived from the olive, prevents *Bacillus cereus* from sporulating. Recent research has shown that the hydroxytyrosol, is effective against clinical human pathogenic strains of *Staphylococcus aureus, Moraxella catarrhalis*, *Salmonella typhi, Moraxella influenzae*, and *Salmonella typhi* [12]. This research work aims to understand the phytochemical difference and the antimicrobial activity of ZnNPs formed using the leaf extract of two varieties of Olive (Arbequina and Coratina).

Material and Methods

Collection of plant material

Fresh green leaves of Olives *O. europaea* variety Arbequina and Coratina were harvested from Rajasthan Olive cultivation private limited, Bassi, Rajasthan, India. The leaves were rinsed three times with running water to remove dust particles. Until being placed on blotting paper to dry, the leaves were gently washed with sterile distilled water.

Preparation of crude aqueous extract

The Olive cultivar dried leaves were properly crushed with a motor pestle and stored at room temperature in a sealed beaker. To make the extract, 50 g of crushed olive leaves were combined with 1000 ml of Milli-Q water and heated for 6 h at 60 °C. As a result of this procedure, filtered leaf extract was generated. The extract was then utilized to create ZnNPs.

Qualitative analysis of the aqueous extract

Qualitative analysis was performed to check the presence of the secondary metabolites in the 10% (w/v) crude extract of olive leaves in a liquid solution of both varieties. According to Harborne [13], a phytochemical investigation of the crude aqueous extract of the plant was performed using conventional chromogenic reagents to detect anthroquinone, alkaloids, flavonoids, saponins, and tannins. Analytical responses to these qualitative tests included color intensity and precipitate formation [14].

Quantitative analysis of Oleuropein and Hydroxytyrosol content in aqueous extract

The secondary metabolite of Oleuropein and Hydroxytyrosol, which is most prevalent in the aqueous extract of olive leaves, was quantified by Liquid chromatography mass spectrometric analysis (Agilent 6470 triple quadrupole LC/MS). Based on the total ion current (TIC) chromatogram, the LC-MS/MS analysis was performed according to the protocol as described by Majumder and co-workers [15].

Synthesis of Olive ZnO NPs

Olive leaf extract ZnNPs were produced using 100 ml of a 1 mM Zinc sulfate heptahydrate ($ZnSO_4.7H_2O$) solution and 1 percent aqueous leaf extract. The solution of 1 mM $ZnSO_4.7H_2O$ solution was continuously stirred for 1 h at 60 °C and 700 rpm. Leaves extract (25 ml) was added dropwise and again stirred continuously in a magnetic shaker till the color change was noted. The pH was finally adjusted to 12. Olive ZnO nanoparticles finally appeared as a cream-white, cloudy suspension. This suspension was left for 120 min at room temperature and later kept for overnight incubation or in the same condition at 37 °C [16]. The suspension was centrifuged at 2000 rpm for 30 min. The pellet was subsequently washed with ethanol and dried for 6 h in a hot air oven at 60 °C. This powder was finally utilized to characterize NPs [2].

Characterization of ZnO Olive NPs

ZnNPs generated by biological activities were assessed using characterization techniques, including Ultraviolet-Visible spectroscopy, Fourier transform infrared spectrometer (FTIR), Field emission scanning electron microscope (FE-SEM), and energy dispersive atomic spectrometry (EDAX). For preliminary confirmation of the ZnNPs production, a UV–Vis spectrophotometer (Shimadzu UV-2600, Japan) was used. The presence of functional groups involved in ZnNPs bioreduction, capping, and stability was noted using FTIR (Bruker Optics, Ettlingen, Germany, Model Alpha-T with Eco ATR). To analyze the surface morphology of synthesized NPs, a FESEM (JEOL make JSM-7610FPlus, Japan) was employed. The elemental content in the NPs was also analyzed using energy dispersion X-ray spectroscopy (EDAX AMTEX, USA), operating at 20 kV using the software EDAX APEX™ V1.3.1.

Antimicrobial studies

Staphylococcus aureus (MTCC-9542) and *Bacillus subtilis* (MTCC-6633) were the bacteria tested for antimicrobial resistance. The well diffusion method was used to define the inhibition zone. With the help of a sterilized glass spreader, 100 µl inoculum of 24 h old cultures of both bacteria were spread on two different nutrient agar (NA) plates. Following that, wells with a diameter of 20 mm were made into the medium and filled with 50 µl of leaf extract of both cultivars and

ZnNPs of both extracts. ZnO was taken as a positive control and water were considered as a negative control. They were allowed to diffuse for 1 h at room temperature and later left undisturbed in an incubator at room temperature for the whole night. The zone of inhibition was noted after every 24 h for 3 days [17].

Results and Discussion

Qualitative analysis

Saponin, steroid, polyphenol and tannin were present and anthraquinone, flavanoid was absent in both the leaf extract analyzed for both the varieties (Arbequina and Coratina). Rahman and his group also reported similar type of results with the aqueous leaf extract of *O. europaea* [14].

Quantitative analysis of *O. europaea* Oleuropein and Hydroxytyrosol using LC-MS

TOleuropein and Hydroxytyrosol were detected via mass/charge analysis using electrospray ionisation for the detection of the secondary metabolite (Figure 1). The peak eluting for hydroxytyrosol displayed at a precursor ion transition of 152.8 -> 123.1 at 1.273 +/- 0.1 min. (Figure 1a). The acquisition time and m/z ratio were same for both the varieties Arbequina and Coratina varieties of Olive. These peaks correspond to the secondary metabolite "Oleuropein and Hydroxytyrosol".

Along with the calibration standard, leaf extract of *O. europaea* was injected in LC-MS/MS. Oleuropein and Hydroxytyrosol were quantified against the calibration standard. It was observed that Oleuropein was eluted at the retention time of 8.582 min and the mass to ion transition was recorded as 538.9 -> 275.1 (Figure 1b) with a linear regression correlation coefficient of 0.996. Based on this data, the quantitative analysis showed that the Arbequina and Coratina leaf aqueous extracts contained oleuropein 205.96 mg/kg in the Coratina variety and 3060 mg/kg in Arbequina variety. Hydroxytyrosol was present in 12.19 mg/kg in Coratina variety whereas, 132.4 mg/kg in Arbequina variety.

UV-Vis spectroscopy analysis

According to the findings, the highest absorbance of both the metabolites were found in the range of 212 - 280 nm. This investigation of the spectrum showed that the absorption maxima of both oleuropein and hydroxytyrosol matched with that of the OLE. The prepared OLE ZnNPs on drying was also characterized (Figure 2a). As shown in figure 2b, the ZnNPs matched the ZnO NPs and the surface plasmon resonance was between 345 nm to 368 nm respectively**.** This variation in metallic Zn reduction is entirely dependent on the reducing capacities of leaf extract from the different varieties, as evidenced by differences in their color intensities [3].

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

The FTIR spectroscopy analysis exhibited absorption peaks of *O. europaea* leaf extract between 4000 cm^{-1} and 400 cm^{-1}. The major functional groups found in OLE, Oleuropein,

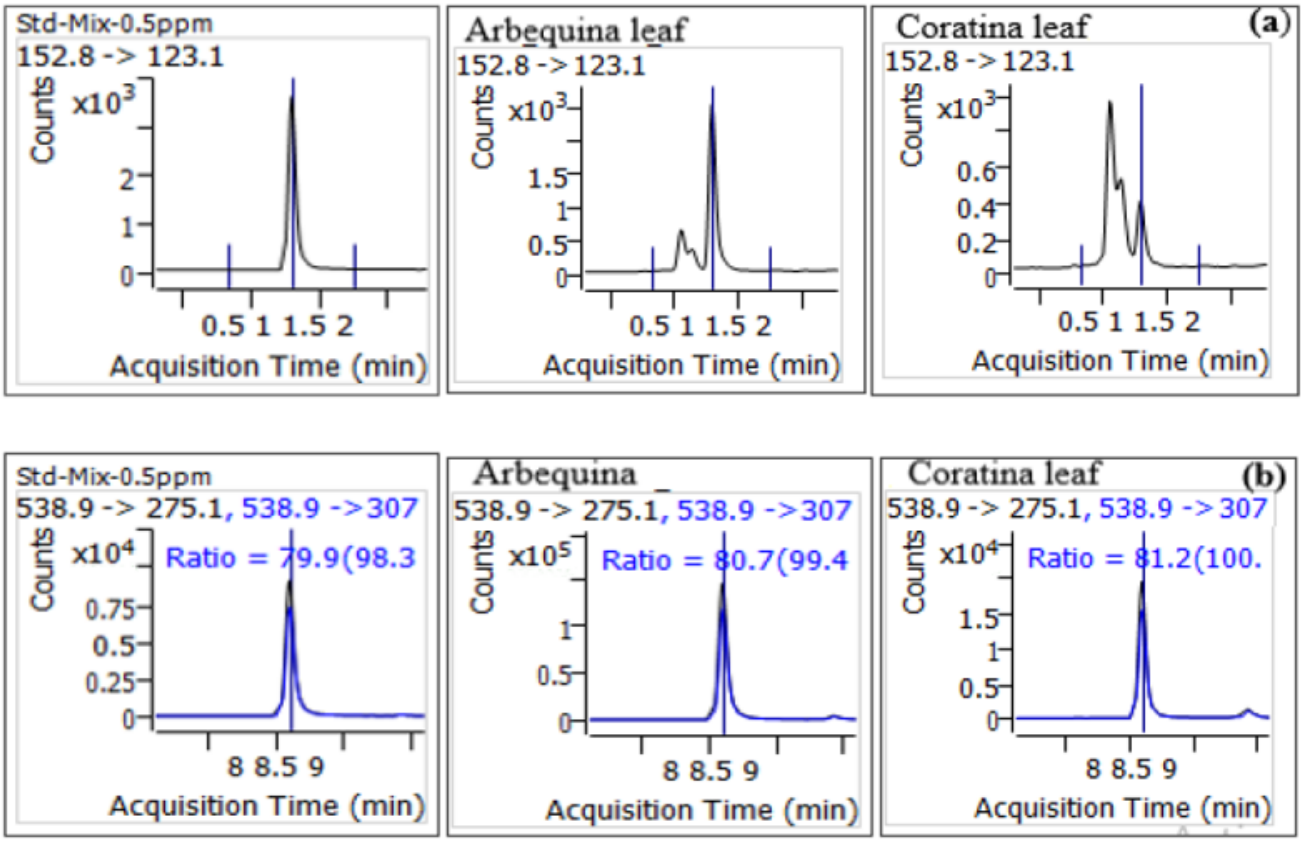

Figure 1: Quantification of oleuropein and hydroxytyrosol in the leaf extract of *O. europaea* (a) Hydroxytyrosol and (b)Oleuropein.

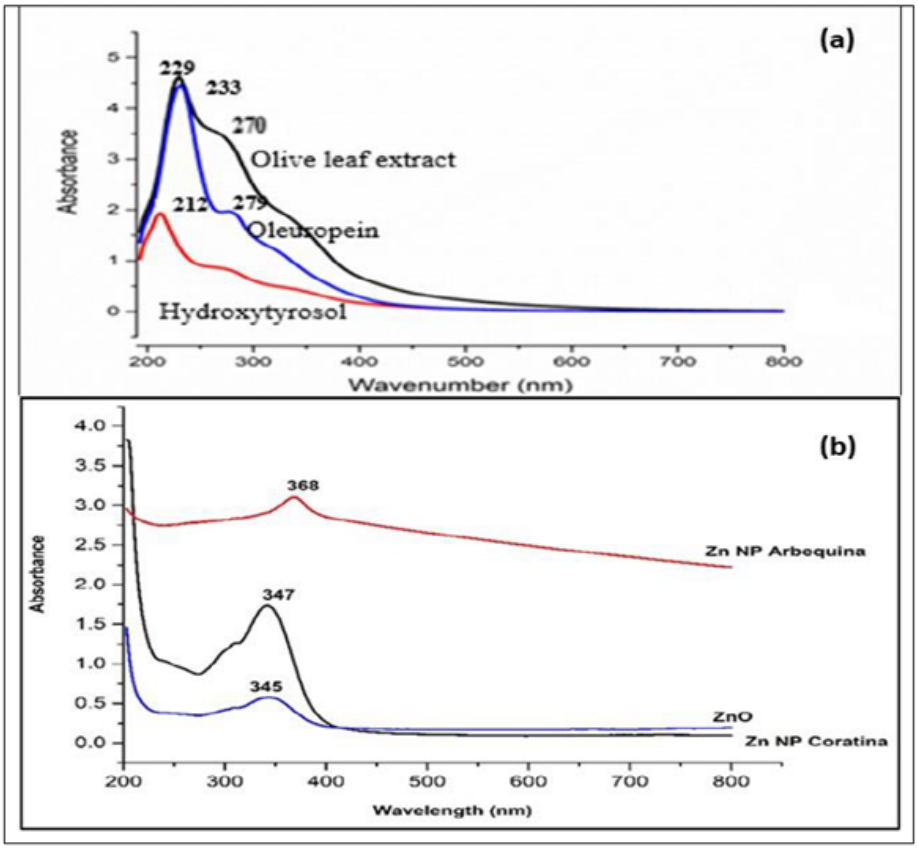

Figure 2: UV–Visible absorption spectra of an olive leaf extract and ZnNPs of OLE of (a) Arbequina variety and (b) Coratina variety.

and Hydroxytyrosol are depicted in the FTIR graph. The extending of C-H stretching alkyne group vibrations is assigned to the bands detected at 3333 cm^{-1}. The N-H stretching amine could be assigned to the bands detected at 2933 cm^{-1}, whereas the bands detected at 2828 and 1636 cm^{-1} may be assigned to C-H stretching aldehyde and C=C alkene, respectively. Allocating C-Br stretching halo molecules is a possibility for 1020 cm^{-1} and 522 cm^{-1}. Krishnan et al. also reported on this type of observation. According to earlier reports [18], similar functional groups are also reported in Oleuropein and Hydroxytyrosol compounds are present in the OLE.

NPs (ZnO) and OLE synthesized ZnNPs (ZnO- OLE) were studied for their FTIR spectra (Figure 3b). O-H and C- H were shown to be involved in stretching vibrations at 3740 and 2870, 540 cm^{-1}. C = C and N –H were found to be responsible for stretching vibrations at 1679 and 1535 cm^{-1} and C-Br halo compound stretching vibrations at 650 cm^{-1}, respectively. Flavonoids, glycosides, proteins, phenols, and terpenoids with functional groups of alcohols and ketones were detected in the bands 1679 cm^{-1} and 1535 cm^{-1}, indicating that they were involved in bio-reduction activities. Alcohols and ketones were found in the bands at 1679 cm^{-1} and 1535 cm^{-1}, respectively, indicating that they were engaged in bio-reduction activities. According to previous reports [18], 540 and 650 cm^{-1} represent ZnO peaks. The correlations between the ZnO and OLE with produced ZnNPs for both varieties show some minor differences in peak location and the absence of some peaks, indicating the presence of residual ZnNPs in the reduction mixture.

Ultra-microscopic examination of the OLE ZnNPs

FESEM examination allowed us to understand the difference among both the ZnNPs from the two olive varieties (Figure 4). Spherical-shaped NPs with an average particle size of 26.8 nm to 45.6 nm were observed. Some earlier published investigations support our findings [3, 16]. The elemental makeup of materials can be determined using EDAX. The EDAX spectrum revealed the presence of Zn with a weight of 63.5 percent (Figure 4). Furthermore, the absence of any agglomerates suggests that ZnNPs have been stabilized, which was made possible by the capping agent capabilities of plant phytochemical Oleuropein and Hydroxytyrosol.

Antimicrobial studies

ZnNPs derived from OLE demonstrated antimicrobial properties against two bacterial strains *B. subtilis* and *S. aureus*. OLE of variety Coratina showed significantly higher inhibitory action than Olive cultivar Arbequina against the gram-positive bacterial strains *B. subtilis* and *S. aureus* [17]. ZnO showed pronounced antibacterial activity (Figure 5a, b), in comparison to the OLE of variety Coratina, the ZnO NPs of OLE of variety Coratina showed higher inhibition against both *B. subtilis* and *S. aureus* as well as against Olive cultivar Arbequina and ZnO NPs of OLE of variety Arbequina. When the activity against both the bacterial species was studied for ZnO NPs of OLE of variety Coratina, it was noted that it showed high bactericidal activity against *B. subtilis* in comparison to *S. aureus*. The high activity of the ZnO NPs of OLE of both varieties may be due to the additive effect

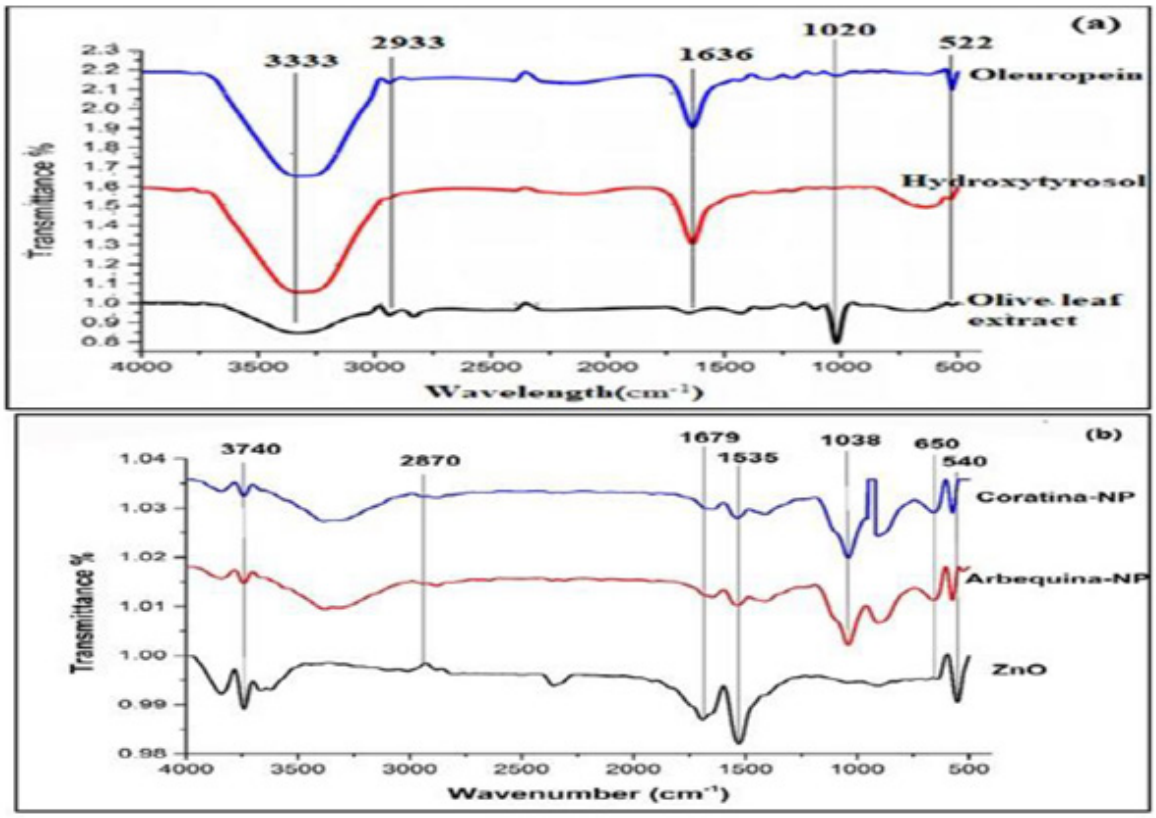

Figure 3: Detection of functional group using FTIR analysis (a) OLE, Oleuropein, and Hydroxytyrosol and (b) ZnO and OLE synthesized ZnNPs from the two varieties of Olive, Arbequina and Coratina.

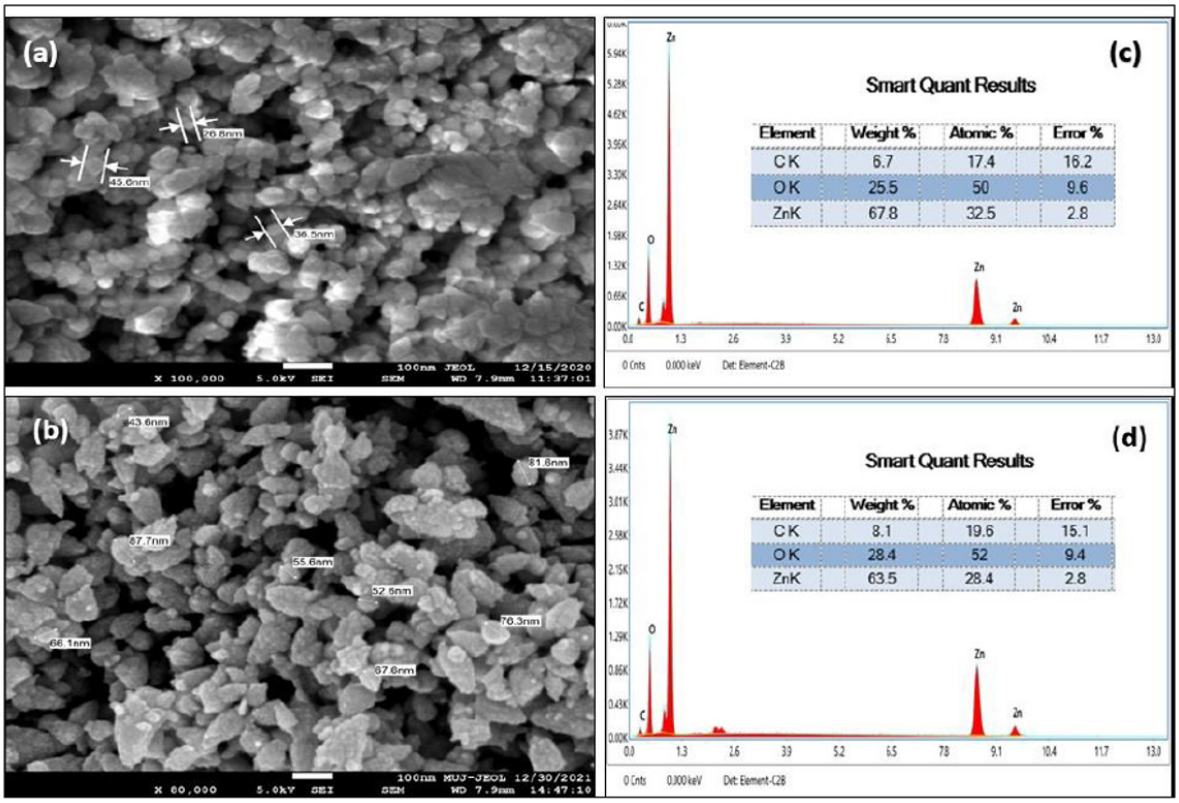

Figure 4: FESEM and EDAX of the ZnNPs of OLE of two olive varieties. Surface morphology of ZnNPs OLE of variety (a) Arbequina and (b) Coratina. EDAX was done showing concentration of Zn in NPs of OLE of variety (c) Arbequina and (d) Coratina.

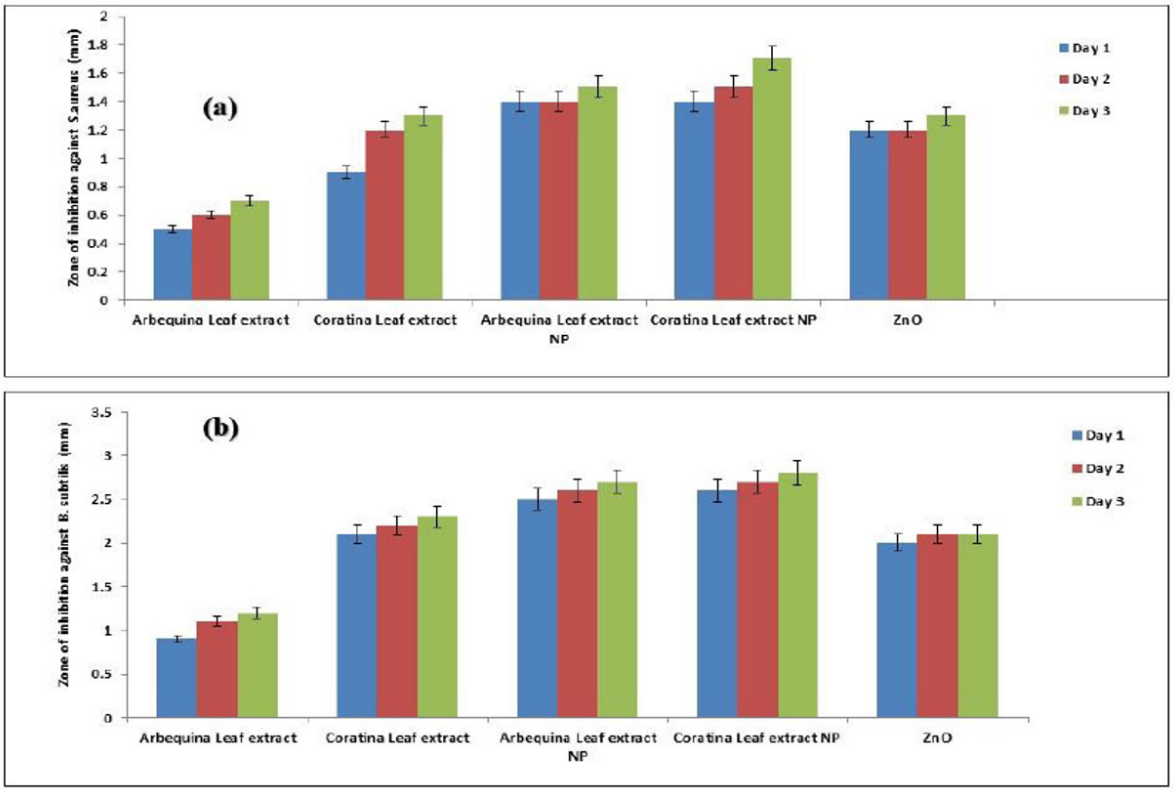

Figure 5: Antibacterial efficiency of OLE of Arbequina, Coratina, ZnO and ZnO synthesized NPs of OLE of both varieties (Arbequina and Coratina) against (a) *S. aureus* and (b) *B. subtilis*.

of the ZnO and the metabolites present in the OLE. Hence Coratina may be a suitable variety for further studies in the formulation of cosmeceuticals.

Conclusion

Green approach is defined as an effective and imaginative search for more sustainable materials to manufacture nanomaterials. Green synthesized Olive ZnNPs have been successfully applied in a variety of fields including biomedicine, textiles, food, cosmetics, and agriculture. *O. europeae* seems to be a rich source of bioactive compounds including Oleuropein, Hydroxytyrosol, Oleocanthal or Squalene, and others, which indicates their potential as a medicinal agent as well as for forecasting the prominence of the Olive plant. Compounds with bioactivity Oleuropein and Hydroxytyrosol, both derived from plants, are effective in reducing, encapsulating, and stabilizing ZnNPs. The present study confirms these two secondary metabolites are responsible for the differential activity of antimicrobial behavior in Arbequina and Coratina varieties. The use of ZnNPs developed from Olive leaves showed their ability to inhibit gram-positive bacteria. Using OLEs ZnO NPs can be used in the future for the synthesis of cosmetic formulations. Thus, leaves that were once discarded as by-products of tree pruning are now considered a valuable commodity.

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