Green Synthesis of Silver Nanoparticles Using Mixed Leaves Aqueous Extract of Wild Olive and Pistachio: Characterization, Enhancing Antioxidant, Antimicrobial Potential and Effect on Virulence Factors of Candida

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Abstract

*Olea europaea* subsp. *europaea* and *Pistacia lentiscus* are well known as natural sources of secondary metabolites promising in various fields. Phenolic compounds from Plant are suitable markers to differentiate varieties related to geographical area. This work aimed to enhance the antimicrobial potential of new green silver nanoparticles AgNPs using for the first time the mixed leaves extract of *Olea europaea* subsp. *europaea var. sylvestris* and *Pistacia lentiscus* from natural geographical association and describe their antimicrobial, antibiofilm and their effect on virulence factor of Candida. A rapid, simple environmentally approach for biosynthesis of AgNPs by using mixed plant aqueous extract acts both as reducing and capting agents without any solvent or hazardous reagents. The AgNPs were characterized by UV-Vis spectrophotometer, FTIR spectrum and the X-ray crystallography.

The AgNPs showed superior antioxidant activity by measuring DPPH, Ferric Antioxidant Reducing Power (FRAP) and the total antioxidant activity. It was most richness with flavonoids, tannins, alkaloids and total polyphenols contents compared to plant extract. The new AgNPs possess high bactericidal and fungicidal effects against clinical strains, limit spore's germination of filamentous fungi, announcing high anti-biofilm activity, synergistic effect with the conventional antibiotic’s drugs and affecting virulence factors of Candida (Proteinase, Phospholipase and morphogenesis).

Introduction

Metal nanoparticles are of great importance due to their various applications principally their antibacterial potential. The silver nanoparticles are the most applied [1, 2]. Based on the toxic chemical and physical methods used for nanoparticles synthesis, there is a crucial emergency to generate an alternative non-toxic approach [3]. Green synthesis of nanoparticles has extensive regard since they are ecofriendly and rapid by single step process and relatively reproducible and more stable materials [4]. Currently, green syntheses are more compatible and simpler than the moderate Nano synthesis by microorganisms, because of the need to improve cultivation methods [5]. On the other hand, green synthesis show more advantages, by owing to antibacterial activity of silver nanoparticles because plants extracts contained chemical constituents such as phenols, reducing sugars, ascorbic acids, and others which are responsible for the bio-reduction of metal ions, the stabilization of the nanoparticles and the attachment on surface of nanomaterials. Nowadays biotechnological industries target for new natural antimicrobial drugs[3, 6].

Tunisia is rich on natural medicinal species using in traditional medicine as *Olea europaea* L. (Oleaceae) and *Pistacia lentiscus* L. (Anacardiaceae). In addition, diverse natural associations are frequently and spread in natural ecosystem as wild olive trees (*Olea europaea* subsp. *europaea var. sylvestris*) or oleaster forests with pistachio (*Pistacia lentiscus*). This type of natural association is isolated from all cultural practices. Therefore, the natural ecosystem reflects an extreme environment condition of this natural association. The wild olive trees or Oleaster are native in Tunisia as reported by historical reports.
and confirmed by nuclear and cytoplasmic molecular markers [9, 10]. *Pistacia lentiscus* L. is evergreen shrub widespread in Mediterranean forests [11].

This study described the enhancement of the antimicrobial and antioxidant activities of green synthesis AgNPs by using mixed aqueous extract from oleaster (*Olea europaea* subsp. *europaea* var. *sylvestris*) and pistachio (*Pistacia lentiscus*) leaves. In this context, we focused on the use of oleaster and pistachio leaves as natural source of biomolecules and surface reducers of nanoparticles based on their richness on secondary metabolites as phenolic compounds and their natural association in extreme environmental showing their adaptation to environment and studying their antibacterial, antifungal actions, define the action mechanisms such as biofilm, spores germination, and virulence factor yeast reduction.

**Material And Methods**

**Plant Material and preparation of aqueous extract**

Oleaster (*Olea europaea* subsp. *europaea* var. *sylvestris*) and Pistachio (*Pistacia lentiscus*) leaves were freshly collected from natural association from Tunisia northern forest. The leaves of plant species were surface cleaned with running tap water to remove soil and other contaminated organic contents, followed by double distilled water and air dried at room temperature. About 20 g of the leaves (10 g from each specie) was cut into small pieces and then boiled with 100 mL distilled water for 20 min. The obtained extract after filtration was stored at 4°C for the further use including the analysis of its major chemical constituents.

**Synthesis of nanoparticles**

Aqueous solution (5 mM) of silver nitrate was prepared. The plant extract (10 mL) was added drop by drop to 20 mL of silver nitrate solution (5 mM). The reaction mixture of Ag NO$_3$ and leaf extract was stirred for 2 min. The color changed from yellow to reddish brown color indicating the formation of silver nanoparticles. Then, the AgNPs obtained was purified by repeated centrifugation at 10000 rpm for 10 min. The pellet was collected and dried.

**Characterization of synthesized silver nanoparticles**

The reduction of pure silver ions was confirmed by measuring the UV–vis spectrum of the reaction mixture against distilled water as a blank. The Spectrum analysis was done using a 2802 UV/Vis spectrophotometer (UNICO) in the 250-700 nm region. The Fourier Transform-Infrared Spectroscopy (FTIR) spectrum was recorded in the range 400-4000 cm$^{-1}$ on a Varian FTIR 640 spectrophotometer with KBr pellets. The X-ray powder diffraction (XRD) measurements was performed on a D8 ADVANCE BRUKER diffractometer using Cu-K$_\alpha$ radiations and equipped with Lynxeye accelerator.

**Antioxidant activities**
DPPH radical scavenging activity

The DPPH (2,2-Diphenyl-1-picryl-hydrazyl) free radical scavenging activity was estimated by colorimetric method. One mL of sample was added to 2 mL of 1,1-diphenyl-2-picrylhydrazyl methanolic solution (1:2) mixture was incubated for 30 min in dark after shaking. The presence of an antioxidant donator of hydrogen, the DPPH radical was reduced in 2,2-diphényl-1-picrylhydrazine (DPPH-H) reflected by the color change. The absorbance was measured at 517 nm. The scavenging activity was expressed as percentage of inhibition (PI) following this formula: PI = ((Ac – As)/As) x 100 [12].

Where Ac is the absorbance of the control and As is the absorbance of the sample.

Total antioxidant activity (TAA)

The mixture reaction containing 0.2 mL of sulfuric acid, sodium phosphate (H$_2$SO$_4$, 0.6M NaHPO$_4$, H$_2$O 28 mL) and ammonium heptamolybdate ((NH$_4$)$_2$MoO$_7$.4H$_2$O 4mM) at acidic pH was prepared. Then, 0.3 mL of sample was added. The reaction was placed in boiled water at 95°C for 90 min. The reduction of Mo was accompanied by green color. The absorbance was measured at 695nm. The total antioxidant activity was expressed as mg of gallic acid equivalent per g of dry matter (DM) sample MS (mg GAE/g DM).

Ferric Reducing Antioxidant Power (FRAP)

The mixture reaction containing 100 µL of the sample solution (silver nanoparticles or plant extract) with 3 mL of FRAP reagent and were incubated at room temperature in dark for 10 min. The FRAP method relies on the reduction by the antioxidants, of the complex ferric ion-TPTZ (2,4,6-tri(2-pyridyl)1,3,5-triazine) accompanied by the apparition of bleu color. The absorbance was measured at 593 nm (Analytik Jena) spectrophotometer. The FRAP value was expressed as mg Ascorbic acid Equivalent Antioxidant Capacity (AEAC) per g of sample [13].

Phytochemical characterization of EA and AgNPs

Total polyphenols Content Total polyphenols content in the sample solution was estimated using the method of Folin Ciocalteu method as described by [14] and the results were expressed as mg of gallic acid equivalent (GAE) per mL (mg GAE/mL of extract).

Total Flavonoids content The flavonoids content was estimated by using aluminum chloride colorimetric technique at 765 nm. Results expressed as mg quercetin equivalents per mL of extract based on a quercetin calibration curve [15].

Tannins content

The tannins content was determined by the vanillin method in acid medium. Tannic acid was served as a standard and Tannin content was expressed as mg tannic acid equivalent per mL of extract. The absorbance was measured at 760nm [16].
Total Alkaloids content

Total alkaloids content was estimated after extraction with glacial acetic acid and ethanol and precipitated with Draggendroff’s reagent. The residue treated with sodium sulfite and thiourea solution. Atropine standard solution was prepared (Sigma chemical, USA) and optical density was measured at 435 nm [17].

Antibacterial and antifungal potentialities of the silver nanoparticles

Well Agar diffusion method for antimicrobial detection

A clinical bacteria and fungi strains from a Tunisian clinical laboratory were used here as fellow: gram-negative bacteria species (Klebsiella pneumoniae; Escherchia coli; Enterobacter cloacae) and gram-positive bacteria (Staphylococcus aureus; Micrococcus luteus). Fungi species are belonging to Candida albicans, Candida parapsilosis, Penicillium spp, and Aspergillus spp. Before use, the AgNPs was diluted in distilled water and adjusted to the appropriate concentration. The cell suspension (0.1 mL) adjusted to $10^7$ CFU/mL for bacteria and $10^5$ spores/mL for fungi were transferred separately into the surface of agar plates and 40 µL of the tested AgNPs were aseptically pipetted into wells (6mm). The plates were incubated at 37°C. The observation of inhibition zone around the wells indicates the antimicrobial activity and the diameter of inhibition zone was measured in mm. Ceftazidime CAZ30 was used as positive control for gram negative bacteria and Vancomycin for gram positive bacteria, Amphotericin B and Fluconazole 25 were used as fungicide standards. All tests were performed in triplicate [18].

Minimum Inhibitory Concentration (MIC) determination

The MIC was determined by broth dilution method by conducting broth culture of pathogen strains in the presence of different concentrations of silver nanoparticles. The incubation was performed in Eppendorf tube containing 1mL of Nutrient broth (NB) and 10CFU/mL of the pathogen strains. The negative control tube contained the NB without AgNPs. High rotational speed of 200 rpm was maintained to avoid the aggregation of the nanoparticles. The absorption was measured at 600nm, to depict bacterial and fungal growth, no increase in absorbance indicates the MIC [2].

Minimal Bactericidal Concentration (MBC) and Minimal Fungicidal Concentration (MCF) determinations

The MBC and MFC were determined by transferring an aliquot of 10µL from the tube corresponding to MIC values on the surface of the appropriate agar plates and the inoculated plates were incubated at 37°C for 24h. The lowest concentration of AgNPs at which no visible colony was observed on the surface of the agar plate was reported as the MBC or MFC according to the modified method [19]. All assays were performed in quadruplicate.

Based on the method previously used [20] showing that values of report of MBC/MIC reflects the bactericide and bacteriostatic effects. The values of MBC/MIC $\leq$ 4 showed that the tested compound
was considered as bactericide agent, and the values of MBC/MIC > 4 showed that the compound has bacteriostatic effect.

**Anti-biofilm activity**

The probably effect on biofilm formation of the bacteria species have been tested on Eppendorf tubes by using the method used for *Candida* species [21, 22].

**Spores germination inhibition**

To evaluate the effect of the silver nanoparticles on spore germination of fungi species, the mixed reaction consisting of v/v of the AgNPs and the conidial suspensions (10⁵ spores/mL) mixed in Eppendorf tube containing 1mL of 5% glucose as previously described [23].

**Synergistic antibiotic effect of AgNPs with conventional antibiotics**

In order to enhance the antibacterial effect of the synthesized AgNPs, we have tested their synergistic effect with conventional antibiotics by using the method of agar diffusion of [24].

**Evaluation of AgNPs on the virulence factors of Candida species**

**Phospholipase detection**

The phospholipase activity (Pz) was determined based on the method of [25]. Briefly, the Egg yolk agar medium was used and a volume of 10 µL of yeast suspension adjusted at 10⁷ CFU/mL, was deposit into the wells. Then the plates were incubated for 48h at 37°C. The diameter of colonies and the diameter of zone opacity were measured and the phospholipase activity (Pz) was calculated as follow:

\[
Pz = \frac{\text{Colony diameter (in mm)}}{\text{Zone opacity+colony diameter (in mm)}}
\]

**Proteinase detection**

The bovine serum albumin medium was used and the wells punched on the surface were inoculated by 10µL of Candida suspension (at 10⁷ CFU/mL), after incubation of 48h at 37°C, based on the modified method of Staib as detailed by [26]. The Proteinase activity (Pz) was calculated as detailed above in phospholipase activity assay.

**Morphogenesis change**

The effect of the addition of AgNPs on Candida albicans morphogenesis, was examined by direct microscopic observation of 100µL of *Candida albicans* suspension from 1mL culture in Sabouraud broth
for 48h at 37°C in the presence of AgNPs (10µL of 250µg/mL) and coloration with bleu cotton and compared to control tube culture without the addition of AgNPs.

**Results**

**UV-vis spectroscopy study**

The formation of AgNPs can be visualized by the color change of the mixture which is turned from pale yellow to brown color (Figure 1A). This color change in the reaction mixture strongly indicates the reduction of Ag$^+$ ions to Ag$^0$. UV-Vis spectroscopic analysis elucidates a single absorbance peak at 422 nm (Figure 1B) indicating the formation of AgNPs. Ancient studies suggested that peak located between 410 and 450 nm has been observed for AgNPs and might be attributed to spherical nanoparticles$^{27}$.

**FT-IR analysis**

FT-IR analysis was performed to identify the bond linkages and functional groups of the active components in the obtained AgNPs based on the peaks and the values in the IR region. All the observed intense bands were compared with standard values to identify the functional groups. As shown in figure 3, the intense band of AgNPs at 3226 cm$^{-1}$ denotes the presence of C-H of aromatic compounds$^{28}$. The absorption peak at 1633 cm$^{-1}$ correspond to the C=C bending$^{29}$. Further, the peak emerged at 1322 cm$^{-1}$ corresponds to O-H bending vibrations. Finally, the band at 1012 cm$^{-1}$ can be assigned C-O stretching vibration indicated the presence of phenols and aliphatic amines$^{30}$. (Figure 2)

**Structural studies**

The crystalline nature of nanoparticles was confirmed by X-ray crystallography. The XRD pattern of the synthesized AgNPs is illustrated in Figure 3. The observed 2θ values at 37.01, 45.38, 64.43 and 78.62° corresponding to the (111), (200), (220) and (311) reflections, respectively, which indicated that spherical silver nanoparticles are crystalline in nature with face-centered cubic structure (fcc)$^{29,30}$. The crystallite size was calculated using the Scherer’s formula that depends on the peak position and FWHM of the dominant reflection. Scherer’s equation is $d = k \lambda / \beta \cos \theta$ where, $d$ is the average crystallite size of the nanoparticles, $k$ is the geometric factor equal to 0.9, $\lambda$ is the wavelength of X-ray radiation source equal to 1.54Å, $\beta$ is the angular FWHM (full-width at half maximum) of the XRD peak at the diffraction angle $\theta$$^{31}$. The calculated average crystallite of the AgNPs is 23 nm.

**Antioxidant activities**

Based on the obtained results, mixed leaves extract showed high total antioxidant activity (Figure 4B), this may be due to their richness by various phytoconstituents from both studied plant species. The antioxidant activities using free radical scavenging the DPPH radical, the total antioxidant activity and the Ferric Antioxidant Reducing Power mehtods showed that the AgNPs preserve an important amount of
antioxidant activity (Figure 4) which may be due to the stability of the antioxidant activity in nanomaterials.

**Phytochemical characterization of plant extract AE and AgNPs**

Results showed that the synthesized nanoparticles were more richer in tannins, total polyphenols and flavonoids compared to the mixed leaves aqueous extracts, (Table 1), the observed data can explain the enhancement of the antimicrobial properties of the AgNPs compared to the plant extract one.

| Phytoconstituents                  | Screening          |
|-----------------------------------|--------------------|
| Tannins content (mg TAE/mL)       | Plant extract 5b   |
|                                  | AgNPs 11.6a        |
| Total Polyphenols content (mg GAE/mL) | 1.35c     |
|                                  | 1.48c              |
| Flavonoids content (mg QE/ml)     | 0.54d              |
|                                  | 0.55d              |
| Alkaloids content (mg/ml)         | 0.93cd             |
|                                  | 1.58c              |

TAE: tannic acid equivalent; GAE: gallic acid equivalent; QE: quercetin equivalents

**Antibacterial and anti-candida screening**

The synthesized silver nanoparticles (NP) showed an antibacterial activity against all tested gram positive and gram negative clinical bacteria strains, unlike the aqueous extract which was unable to limit the growth of used bacteria strains. The observed results indicate that the increase of zone inhibition expressed in mm given by the silver nanoparticles compared to AgNO$_3$ and the development of the sensibility of strain *Escherichia coli* which was resistant to Ag NO$_3$ (Figure 5A)

The present results mentioned that the synthesized AgNPs (NP) gave more inhibitory activity against *Candida* species compared to Ag NO$_3$. The aqueous plant extract was unable to limit any Candida species. Our synthesized nanoparticles were more effective than the fungicide amphotericin B with 19.66 and 17.33mm compared to 19mm and 10mm against Candida albicans and *Candida parapsilosis*, respectively by the AgNPs and Amphotericin B (Figure 5B.) MIC values ranged from 31 to 500 µg/mL related to the tested pathogens strains. Based on calculated value of MBC/MIC ≤ 4, AgNPs showed bactericide and fungicide effects against all tested pathogens strains. In the present work, we have investigated the silver nanoparticles effect on filamentous fungi genus *Penicillium* and *Aspergillus*, and the finding show that only the synthesized silver nanoparticles can affect mycelium growth compared to
the plant extract or Ag NO₃. Superior inhibitory activity was given against spores germination of *Penicillium* with 98.4%.

**Anti-biofilm activity**

The investigation of the synthesized AgNPs effect on biofilm formation elucidate that AgNPs used at the corresponding MIC value were qualified to limit biofilm formation of all tested gram positive and gram negative bacteria with value ranging from 32.74 to 83%, but they were more efficient on biofilm of *Candida* albicans strains with (87%) and *Candida parapsilosis* with 63% (Table 2).

| Microorganisms pathogens          | Biofilm inhibition (%) |
|----------------------------------|------------------------|
| *Klebsiella pneumoniae*          | 56.75±0.29b            |
| *Enterobacter cloacae*           | 76.64±1.33a            |
| *Escherchia coli*                | 73.64±6.39a            |
| *Staphylococcus aureus*          | 83.00±1.41a            |
| *Micrococcus luteus*             | 32.74±1.75c            |
| *Candida albicans*               | 87d                    |
| *Candida parapsilosis*           | 63d                    |

**Antibiotic synergistic effect of AgNPs**

According to the observed results, the synthesized nanoparticles booster the effect of the tested conventional antibiotic currently used or make bacteria strains more sensitive to used antibiotic. For example, both bacteria strains were sensible to NA but they were sensitive when it was associated with AgNP, and TM against *Micrococcus* (Figure 6.)

**Silver nanoparticles on virulence factor of Candida strains**

In order to illustrate the ability of the silver nanoparticles on factor virulence of yeast species, here we reported the effect of AgNPs on hydrolytic enzymes production and yeast morphogenesis. As a result Table 3, mentioned that, the enzymes hydrolase proteinase and phospholipase were higly reduced in Candida species growth additioned with silver nanoparticles. Morover, Figure 7, illustrated that in vitro co-culture model of *Candida* strains, confirmed the superior inhibitory effect of the AgNPs used at MIC of 250µg/mL, on *Candida albicans* morphogenesis compared to untreated *Candida albicans* culture, the observation indicate the absence of any morphogenesis key virulence change for biofilm resistance such as: germ tube, chlamydomspore and filamentous hyphae, as well as the alteration of the scare Blastospore
in the presence of AgNPs. For both *Candida* species, the results indicate that the addition of AgNPs in co-cultural model, affect hardly the number of Blastospore observed (so affect the cell multiplication phenomena).

Table 3
Comparison between the virulence factors expressed in *Candida* species in the absence and the presence of AgNPs after 48h of incubation with or without AgNPs.

| Key virulence          | Candida albicans | Candida parapsilosis |
|------------------------|------------------|-----------------------|
|                        | Untreated strains | AgNPs | Untreated | AgNPs |
| Hydrolytic enzymes     |                  |     |           |       |
| Proteinase             |                  |     |           |       |
| (Pz in mm)             | 0.569            | 0.78 | 0.38      | 0.68  |
| Phospholipase          |                  |     |           |       |
| (Pz in mm)             | 0.68             | 0.88 | 0.63      | 0.887 |
| Biofilm                |                  |     |           |       |
| Morphogenesis          | +++              | -   | ++        | -     |

Pz= 1 (negative); Pz (0.9-0.99:+); Pz: (0.8-0.89 (++); Pz: (<0.7:+++++)

Biofilm morphogenesis change (+++); absence of any morphogenesis change (-)

**Discussion**

It is well known that the green synthesis of nanoparticles materials has attracted the attention of many studies due to their advancement over other methods as single step process, cost effective and emerged as ecofriendly environment [4]. This report demonstrated for the first time the biosynthesis of AgNPs using aqueous extract from the equal mixture of *Olea europaea* subsp. *europaea* var. *sylvestris* and *Pistacia lentiscus* leaves. Here we proved that firstly, two *Olea europaea* subsp. *europaea* var. *sylvestris* and *Pistacia lentiscus* natural associated species can be used as natural sources to synthesize silver nanoparticles by enhancing their antimicrobial potential compared to other AgNPs synthesized from individually plant as reported previously in literature. For example as reported by the *P lentiscus* leaf extracts which possess moderate antimicrobial activities and scare works focused on the biosynthesis of silver nanomaterial from this species [32].

Secondly, here the AgNPs synthesis were completely free from any chemical solvents and hazardous reagents similar to the green synthesis described previously [33], based on the use of two cultivars (Leccino and Carolea) of *Olea europaea* growing in the same pedoclimatic conditions for green biosynthesis of silver nanoparticles with new properties as antibacterial potential against, and were able to induce toxicity in breast cancer cell lines. In addition, here the mixed leaves extract enhance the
biosynthesis of AgNPs, on one hand, the size of silver nanoparticles of 23nm, were smaller than those obtained only by Pistachio leaves extract as reported by [34]. show that the Ag NPs from Pistachia ethanol extract ranged from 24 to 26nm. On the other hand, the mixed leaves from both plant species enhance the antibacterial potential as compared to those obtained by silver nanoparticles from Pistachio which not exceed 13mm of zone inhibition against *Escherichia coli* and *Staphylococcus aureus* [34].

Face to the emergence of multidrug resistant bacteria and biofilms producer’s strains to currently used antibiotics, AgNPs may play a crucial role compared to the conventional antibiotics by action of multiple antagonism mechanism. In this work, we revealed a significant antibacterial and antifungal efficacy of AgNPs against all tested clinical strains. This finding may be attributed to its richness with secondary metabolites from both natural associated plant species. On the other hand, Olive leaves are polyphenol rich compounds that are known to have antioxidant, antimicrobial, and anti-inflammatory activities. In literature the anti-inflammatory and antibacterial effect against gram positive bacteria is due to the Olive component Oleuropein [35]. Furthermore, here the enhancement of AgNPs from the mixed leaves extract observed by the superior antibacterial activity by exhibiting maximum antibacterial effect against both gram negative and gram positive bacteria.

In addition, the results mentioned that the susceptibility of Gram positive and Gram negative bacteria to biosynthesized AgNPs was found to vary from study to other, related to the pathogen strains tested [36] and the concentration of the inoculum or solvent used [37]. For example, here the zone inhibition given by the AgNPs against *Staphylococcus aureus* and *Escherichia coli* were 18.66mm and 15.33 mm respectively, these diameters were superior to those obtained by the AgNPs from *Pistachia lenticus* with 13mm and 13mm respectively against the same bacteria strains, these data could be due to the maximum richness of the mixed metabolites from both plant leaves [38]. Few works aimed the antifungal potential of AgNPs, here we successfully describe the high antifungal potential of our AgNPs against *Candida* species and *Penicillium*. The observed results on filamentous fungi confirmed that the silver nanoparticles enhanced the antifungal behavior against mycelium and spores [39]. Numerous works, described the antifungal potential of Olive plant against filamentous fungi like *Rhizopus, Fusarium* and *Alternaria* as reported by [40]. These funding mentioned that the antifungal behavior of the biosynthesized silver nanoparticles from mixed extracts could be due to the olive composition, which was able to affect the spores and the mycelium fungi growth. In literature, the major active components in Olive leaf are known to be Oleuropein and its derivatives as owing superior antifungal potential against fungi [41]. It is essential to note that, the new AgNPs exhibited novel bactericidal and fungicidal potential which may be highly relevant in infections caused by filamentous fungi and MDR bacteria strains. Consequently, the broad spectrum killing caused by AgNPs, have encouraged their use as antimicrobial drugs including multidrug strains MDR [2, 42].

Numerous studies have shown that nanoparticles generally improved the pharmaceutical characteristics of antifungals, as lower toxicity and enhancing antifungal potential, and the possibility of prolonged action [40]. The reported data show that the presence of AgNPs in the Candida growth can limit the virulence factor as enzyme production and biofilm formation.
In literature, several mechanisms have been reported for antimicrobial activity of AgNPs such as disruption of the bacterial cell membrane, interference in the respiratory electron transport chain formation of reactive oxygen species (ROS) [43, 44]. Nanoparticles exhibited new or improved properties depending upon their size and morphology. The pathogenicity of Candida species was attributed to the factor virulence such as enzyme hydrolases to invade host cells and biofilm formation to adhere to solid surface. The present work was the first to highlight the effect of silver nanoparticles from mixed leaves from Olive and Pistachia on Candida key virulence factors by means of enzyme production (proteinase, phospholipase) and morphogenesis reduction. Our results illustrated that the addition of silver nanoparticles can reduce the enzyme production and the germ tube and filamentous hyphae. Recently scare works, reported the reduction of enzyme and biofilm by Candida albicans by the addition of green silver nanoparticles. However, the exact mechanism of action on biofilm by silver nanoparticles is not known. In addition, the inhibition of yeast morphogenesis; like germ tube and filamentous hyphae lead to the suppression of biofilm formation in Candida strains [45, 46].

Furthermore, a combination of conventional antifungals with natural compounds can also minimize the toxicity of these drugs by reducing the dose request. Therefore, the focus of this study was to explore the new AgNPs by enhancing the antibacterial potential of conventional antibiotic drugs tested on clinical strains. Here the association of silver nanoparticles ameliorate the action of conventional antibiotic or make resistant bacteria more sensible against it. After demonstrating the simple method of synthesis, the antioxidant potential of AgNPs by testing DPPH radical scavenging, Ferric Antioxidant Reducing Power (FRAP) as well as the total antioxidant activity was determined, thus the combination of the antioxidant effects and the antibacterial and antifungal activities encourage the use of the green biosynthesized AgNPs in pharmaceutical field.

In conclusion to our knowledge, this is the first study evaluating the antioxidant and antimicrobial effects of silver nanoparticles biosynthesized from Olea europaea subsp. europaea var. sylvestris and Pistacia lentiscus leaves. The synthesized AgNPs is rich in secondary metabolites and has an antioxidant activity. The reported AgNPs exhibited markedly bactericidal and fungicidal effects against clinical pathogen strains. The synergistic interaction with the conventional antibiotic as well as the effect on bacteria biofilm and the spores of filamentous encouraged their formulation in pharmaceutical and medical purposes.

Declarations

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The authors declare no conflict of interests

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Authors' contributions

**Essghaier Badiaa**: Microbial Methodology, Data curation, writing original draft, **Rihab Dridi, Hannachi Hedia, Ben khedher Ghada**: methodology, validation, **Med Faouzi Zid, Chaffei Chiraz**: Supervision.

References

1. Jyoti K, Baunthiyal M, Singh A. Characterization of silver nanoparticles synthesized using Urtica dioica Linn leaves and their synergiostic effects with antibiotics. *J of Radiation Research and Applied Sciences* 2016; 9 (3): 217-227 https://doi.org/10.1016/j.jrras.2015.10.002.

2. Bharti S, Mukherji S, Mukherji S. Enhanced antibacterial activity of decahedral silver nanoparticles. *J Nanopart Res* 2021; 23 (2): -36, https://doi.org/10.1007/s11051-020-05106-z

3. Gomathi M, Rajkumar PV, Prakasam A, Ravichandran K. Green synthesis of silver nanoparticles using Datura stramonium leaf extract and assessment of their antibacterial activity *Res Efficient Technologies* 2017; 3(3):280-284, https://doi.org/10.1016/j.reffit.2016.12.005

4. Mittal J, Batra A, Singh A, Sharma MM. Phytofabrication of nanoparticles through plant as nanofactories. Advances in Natural Sciences:Advances in Natural Sciences, *Nanoscience and Nanotechnology* 2014; 5 043002, http://dx.doi.org/10.1088/2043-6262/5/4/ 043002.

5. Morones-Ramirez JR, Winkler JA, Spina CS, Collins JJ. Silver enhances antibiotic activity againstgram-negative bacteria. *Sci Trans Med* 2013; 5: 181−190, http://doi.org/10.1126/scitranslmed.3006276

6. Shakeel Ahmad S, Mudasir AS, Swami BL. Green synthesis of silver nanoparticles using Azadirachta indica aqueous leaf extract. *J of Radiation Research and Applied Sciences* 2016; 9: 1-7: DOI: 10.1016/j.jrras.2015.06.006

7. Camps-FabrER H L’olivier. 1ère partie, In L’olivier et l’huile dans l’Afrique romaine. Gouvernement général de l’Algérie. Direction de l’intérieur et des beaux arts In: Encyclopédie Mondial de l’Olivier, C.O.I. (Eds.), 1997 ; 30–33p.

8. Camps-FabrER H. 1ère partie, La culture de l’olivier en Afrique du Nord, Evolution et histoire. In L’olivier et l’huile dans l’Afrique romaine. Gouvernement général de l’Algérie. Direction de l’intérieur et des beaux arts. Service des Antiquités. Imp. Off., Alger, (1953) pp 1–93.
9. Hannachi H, Breton C, Msallem M, Ben El Hadj S, El Gazzah M, Bervillé A. Differences between local and introduced olive cultivars as revealed by morphology of drupes, oil composition and SSR polymorphisms: a case study in Tunisia. *Scientia Horticulturae* 2008; 116:280-290, DOI: 10.1016/j.scienta.2008.01.004

10. Hannachi H, Breton C, Msallem M, Ben El Hadj S, El Gazzah M, Bervillé A. Genetic Relationships between Cultivated and Wild Olive Trees (Olea Europaea L. Var. Europaea and Var. Sylvestris) Based on Nuclear and Chloroplast SSR Markers. *Natural Resources journal scirp* 2010;1 95-103, DOI: 10.4236/nr.2010.12010

11. Gardeli C, Vassiliki P, Athanasios M, Kibouris T, Komaitis M. Essential oil composition of *Pisatacia lentiscus* L. *Myrtus communis* L.: evaluation of antioxidant capacity of methanolic extracts. *Food Chemistry* 2018; 107:112-1130, https://doi.org/10.1016/j.foodchem.2007.09.036

12. Chan EWC, Lim YY, Omar M. Antioxidant and antibacterial activity of leaves of *Etlingera* Species (Zingiberaceae) in Peninsular Malaysia. *Food Chemistry* 2007; 104 (4):1586–1593, DOI: 10.1016/j.foodchem.2007.03.023

13. Athavale A, Jirankalgikar N, Nariya P, Des S. Evaluation of in-vitro antioxidant activity of panchagavya: a traditional ayurvedic preparation. Int j Pharma *Sciences and research* 2012; 3(8): 2543–2549.

14. Singleton VL, Rossi JA. Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents. *Am J Enol Vitic* 1965;16:144-158.

15. Harris LJ, Ray SN. Determination of plasma Ascorbic acid by 2, 6-dichorphenol indophenols titration. Lancet; 1935 pp462.

16. Rodríguez-Romero M, Godoy-Cancho B, Calha IM, Passarinho JA, Moreira AC. Allelopathic Effects of Three Herb Species on Phytophthora cinnamomi, a Pathogen Causing Severe Oak Decline in Mediterranean Wood Pastures. *Forests* 2021; 12: 285, https://doi.org/10.3390/f12030285

17. Srividya N, Mehrotra S. Spectrophotometric Method for the estimation of Alkaloids Precipitable with Dragendroff’s reagent in plant materials *Journal of AOAC international* 2003; 86 (6): 1124–1127.

18. Essghaier B, Naoura A, Abdelhak J, Zid MF, Sadfi zouaoui N. Synthesis, crystal structure and potential antimicrobial activities of di (4-sulfamoyl-phenyl-ammonium) sulphate. *Microbiol Res* 2014; 169:504-510, http://dx.doi.org/10.1016/j.micres/2013/11/005.

19. *Ruparelia* JP, *Chatterjee* AK, *Dutta Gupta* SP, *Mukherji* S. Strain specificity in antimicrobial activity of silver and copper nanoparticles. *Acta Biomater* 2008; 4(3): 707-16, https://doi.org/10.1016/j.actbio.2007.11.006

20. Okou OC, Yapo SE, Kporou KE, Baibo GL, Monthaut S, Djaman AJ. Evaluation de l’activité antibactérienne des extraits de feuilles de *Solanum torvum* Swartz (Solanaceae) sur la croissance in vitro de 3 souches d’entérobactéries. *J of Appl Biosciences* 2018 ; 122 :1282-1290.

21. Kim MK, Zhao A, Wang A, Brown ZZ, Muir TW, Stone HA, Bassler BL. Surface attached molecules control *Staphylococcus aureus* quorum sensing and biofilm development, *Nat Microbiol* 2017; 17080, https://doi.org/10.1038/nmicrobiol.2017.80
22. Gulati M, Lohse MB, Ennis CL, Gonzalez CL, Perry AM, Bapat P, Arevalo AV, Rodriguez DL, Nobile CJ. In vitro culturing and screening of Candida albicans Biofilms *Curr Protoc Microbiol* 2018; 50: 60, https://doi.org/10.1002/cpmc.60.

23. Sarangi N, Athukorala P, Dilantha Fernando WG, Rashid KY, Kievit TD. The role of volatile and non volatile antibiotics produced by Pseudomonas chlororaphis strain PA23 in its root colonization and control of Sclerotinia sclerotiorum. *Biocontrol Science and Technology* 2010; 20(8): 875-890.

24. Danielli LJ, Pippi B, Duarte JA, Maciel AJ, Lopes W, Machado, et al MM. Antifungal mechanism of action of *Schinus lentiscifolius* Marchand essential oil and its synergistic effect in vitro with terbinfine and ciclopirox against dermatophytes. *Journal of Pharmacy and Pharmacology* 2018; 70 (9):1216–1227, https://doi.org/10.1111/jphp.12949

25. Samaranayake YH, Dassanayake RS, Jayatilake JS, Cheung BK, Yau JY, Yeung KS, et al. Phospholipase B enzyme expression is not associated with other virulence attributes in *Candida albicans* isolates from patients with human immunodeficiency virus infection. *J Med Microbiol* 2005; 54:583–593.

26. Lara HH, Dulce G. Romero-Urbina, Pierce C, Jose L. Lopez-Ribot, M. Josefina. Arellano-Jiménez and M. Jose-Yacaman. Effect of silver nanoparticles on Candida albicans biofilms: an ultrastructural study J Nanobiotechnol 2015; 13:91 DOI 10.1186/s12951-015-0147-8

27. Vidhu VK, Aromal SA, Philip D. Spectrochim. Acta Mol. Biomol. Spectrosc 2011; 83: 392–397.

28. Zia F, Ghafoor N, Iqbal M, Mehboob S. Green synthesis and characterization of silver nanoparticles using Cydonia oblong seed extract, Appl Nanosci 2016; 6:1023-1029, http://doi.org/10.1007/s13204-016-0517-z.

29. Dyah W, Rengga P, Yufitasari A, Adi W. Synthesis of Silver Nanoparticles from Silver Nitrate Solution Using Green Tea Extract (Camelia sinensis) as Bioreductor. *JBAT* 2017; 6 (1): 32-38, DOI 10.15294/jbat.v6i1.6628

30. Mahiuddin Md, Saha P, Ochiai B. Green Synthesis and Catalytic Activity of Silver Nanoparticles Based on Piper chaba Stem Extracts. *Nanomaterials* 2020; 10(9):1777, doi: 10.3390/nano10091777

31. Zhuang Z, Huang L, Wang F, Chen Z. Effects of cyclodextrin on the morphology and reactivity of iron-based nanoparticles using *Eucalyptus* leaf extract. *Ind Crops Prod* 2015; 69: 308–313, DOI : 10.1016/j.indcrop.2015.02.027

32. Ibrahim HMM. Green synthesis and characterization of silver nanoparticles using banana peel extract and their antimicrobial activity against representative microorganisms. *Journal of Radiation Research and Applied Sciences* 2015; 8(3):265-275, https://doi.org/10.1016/j.jrras.2015.01.007.

33. Rajkumar PV, Ravichandran K, Baneto M, Ravidhas C, Sakthivel B, Dineshbabu N. *Mater. Sci. Semicond Process* 2015; 35: 189–196, https://doi.org/10.1016/j.mssp.2015.03.010.

34. El-Chaghaby GA, Ahmad AF. Biosynthesis of silver Nanoparticles using *Pistacia lentiscus* leaves Extract and investigation of their Antimicrobial Effect. *Oriental journal of Chemistry* 2011; 27 (3): 929-936
35. DeMatteis V, Rizzelio L, Ingrosso C, Liatsi-Douvitsa E, De Giorgi ML, De Matteis G, Rinaldi R. Green synthesis and characterization of silver nanoparticles using banana peel extract and their antimicrobial activity against representative microorganisms. *Nanomaterials* 2019; 9(11) 1544, https://doi.org/10.3390/nano9111544

36. Ghadir A, Elchaghaby, Abeer F. Biosytheiss of Silver Nanoparticels using Pistahcia lentiscus leaves extract and investigation of their antimicrobial effect. Oriental J of Chemistry 2011; 27 (3):929-936.

37. Qabahe K, AlRimawi F, Qasem A, Naser AS. Oleuropein is responsible for the major anti-inflammatory effects of Olive leaf extract. *J Med Food* 2018; 21(3):302-305. doi:10.1089/jmf.2017.0070. Epub 2017 Nov 3.

38. Nagajyothi PC, Lee KD. Synthesis of plant-mediated silver nanoparticles using Dioscorea batatas rhizome extract and evaluation of their antimicrobial activities J. nanomaterials 2011; 1 (9) https://doi.org/10.1155/2011/573429

39. Mosconi N, Monti L, Giulidori C, Williams PAM, Raimondi M, Bellü S, Rizzotto. Antifungal, phyto, cyto,genotoxic and lipophilic properties of three complexes of sulfadimethoxine (HSDM) with Ag(I). Synthesis and characterization of [Ag3SDM(SCN)2 ]·H2O and [Ag2(SDM) ophenanthroline]-H2O Polyhedron 2020; https://doi.org/10.1016/j.poly.2020.114965

40. Renzi DF, de Almeida Campos L, Miranda EH, Mainar des RM, Abraham WR, Grigoletto DF, Khalil NM. Nanoparticles as a Tool for Broadening Antifungal Activities. *Current Medicinal Chemistry* 2021; 28 (9) http://dx.doi.org 10.2174/0929867327666200330143338

41. Korukluoglu M, Sahan Y, Yigit A.Antifungal properties of olive leaf extracts and heir phenolic compounds. *J of Food Safety* 2008; 01 Fev 2008 https://doi.org/10.1111/j.1745-4565.2007.00096.x

42. Farag RS, ElBaroty GC, Basuny AM. Safety evaluation of olive phenolic compounds as natural antioxidants. Int J Food Sci Nutr 2003; 54 :159-174.

43. Mlalila NG, Swai HS, Hilonga A, Kadam DM. Antimicrobial of silver nanoparticles in surface plasmon resonance bands against Eshcerchia coli Nanotechnol *Sci Appl* 2017;10 1-9, doi: 10.2147/NSA.S123681. eCollection 2017.

44. Bharti S, Agnihotri S, Mukherji S, Mukherji S. Effectiveness of immobilized silver nanoparticles in inactivation of pathogenic bacteria. *J Environ Res Dev* 2015; 9: 849–856.

45. Mai T, Hilt JZ. Mangetic nanoparticles ; reactive oxygen species generation and potential therapeutic applications *J Nanopart Res* 2017; 19:253, https://doi.org/10.1007/s11051-017-3943-2.

46. Eladly A, and Shabana I. (2018). Antimicrobial Activity of Green Silver Nanoparticles against Fluconazole-resistant Candida albicans in Animal Model. *Egypt. J. Bot* 2018; 58: (1):119 – 132.

47. Jalal M, Aazam Ansari M, SG Ali, Alzohairi MM, HM Khan, A Almatroudi, MI Siddiqui. Anticandidal activity of biosynthesized silver nanoparticles: effect on growth, cell morphology, and key virulence attributes of Candida species. *International Journal of Nanomedicin* 2019; 14: 4667–467

**Figures**
Figure 1

A Plant extract and synthesized AgNPs, B: UV–vis spectrum of synthesized AgNPs.
Figure 2

FTIR spectrum of AgNPs
Figure 3

X-ray diffraction (XRD) pattern of synthetized AgNPs
Figure 4

Antioxidant activity of plant extract and silver nanoparticles by Free radical scavenging activity (A), the total antioxidant activity (B) and the Ferric Antioxidant Reducing Power (FRAP) (C).
Figure 5

A. Antibacterial activity of silver nanoparticles (NP) against clinical bacteria strains compared to antibiotic standard, AgNO3 and Olea europaea var. Sylvestris and Pistacia lentiscus leaves aqueous extract. B: Silver nanoparticles effects, on Candida species compared to fungicides (Fluconazole 25 and amphotericin B) and plant extracts. Values expressed diameters in mm. Bars represent Standard errors.
Figure 6

Assessment of the individual and synergistic effects of the synthesized silver nanoparticles (AgNPs) and the conventional antibiotics: acide nalidixique NA, tobramycine™ and vancomycine VA

Figure 7

(A) Candida albicans +AgNPs  (B) Candida albicans
Microscopic observation of Candida albicans growth culture of 48h, after coloration with bleu cotton of (A): Silver nanoparticles effect's on Candida albicans morphogenesis compared to untreated Candida albicans growth without the addition of AgNPs (B). (gr X100).