Acta Scientific Pharmaceutical Sciences

Volume 1 Issue 3 September 2017

Research Article

Biosynthesis of *Elaeocarpus floribundus* Mediated Silver Nanoparticles with Broad Antibacterial Spectrum

Bijayanta Sircar[1], Manisha Mandal[2], Mohabul Alam Mondal[3] and Shyamapada Mandal[1]*

[1]*Department of Zoology, University of Gour Banga, Malda, India*

[2]*Department of Physiology, MGM Medical College and LSK Hospital, Kishanganj, India*

[3]*Department of Chemistry, University of Gour Banga, Malda, India*

***Corresponding Author:** Shyamapada Mandal, Laboratory of Microbiology and Experimental Medicine, Department of Zoology, University of Gour Banga, West Bengal, Malda, India. E-Mail: samtropmed@gmail.com

Received: July 05, 2017; **Published:** August 26, 2017

Abstract

This study assesses the olive, *Elaeocarpus floribundus*, fruit mediated synthesis of silver nanoparticles (SNPs) having antibacterial activity and determines the HPLC chromatogram of the extracts. The SNPs were synthesized with aqueous extract of olive fruit parts, seed (AqOS) and mesocarp-epicarp (AqOME) and were subjected to antibacterial activity testing by agar-well diffusion method, against gram-positive (*Bacillus cereus, Staphylococcus aureus* and *Listeria monocytogenes*) as well as gram-negative (*Acinetobacter baumannii, Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris* and *Pseudomonas aeruginosa*) pathogenic bacteria. The presence of phytochemicals in the extracts was determined by high performance liquid chromatography (HPLC). The zone diameter of inhibition (ZDI) values of the SNPs synthesized with AqOME and AqOS were 20.8 ± 3.2 mm (range: 16 - 25 mm) and 14.7 ±1.06 mm (range: 14 - 17 mm), respectively, while the respective ZDIs from the action of AqOME, AqOS and silver nitrate solution, against the test bacteria, were 6.7 ± 1.49 mm (range: 6 - 10 mm), 6 ± 0.00 mm, and 13.3 ± 0.82 mm (range: 12 - 15 mm); the values expressed as mean ± standard deviation. The HPLC chromatograms showed the presence of 8 major compounds in AqOME and 11 major compounds in AqOS with retention times 1.45 - 4.84 min and 1.91 - 4.84 min, respectively. Thus, the olive fruit extracts (AqOME and AqOS) demonstrated the capacity to synthesize SNPs possessing broad antibacterial spectrum, suggesting their plausible utilization in combating the bacterial drug resistance and the infections caused by them.

Keywords: Silver Nanoparticles; *Elaeocarpus floribundus*; Antibacterial Activity; Bacterial Pathogen; Zone Diameter of Inhibition; High Performance Liquid Chromatography

Abbreviations

ANOVA: Analysis of Variance; ATCC: American Type Culture Collection; AqOME: olive mesocarp-epicarp aqueous extract; AqOS: olive seed aqueous extract; HPLC: high performance liquid chromatography; MDR: multidrug resistant; MTCC: Microbial Type Culture Collection; SD: standard deviation; SNPs: silver nanoparticles; SPR: surface plasmon resonance; ZDI: zone diameter of inhibition

Introduction

Silver has long been used as an antimicrobial agent and in medicine. However, with the advent of antibiotics the usage of silver, due to its low potentiality as the antimicrobial agent and its adverse effects on application, has been diminished. Nevertheless, the researchers have growing interest on silver to be used as the antibacterial agent, by increasing its efficacy, in order to combat MDR bacterial infection around the world. Because, in the era of antibiotics with their non-judicious usage, the microorganisms including the pathogenic one acquired resistances to such agents thereby causing treatment difficulties and outbreaks of the diseases caused by them [1,2]. In order to combat such problems, plant mediated synthesis of SNPs, with elevated antibacterial capacity, has been on the rise, because of the presence, in different plant parts, of an array of bioactive components [3], displaying both reducing and stabilizing activity during SNPs synthesis [4].

The SNPs are the kind of agents possessing antibacterial activity at very low concentrations that are non-toxic to humans [5]. The SNPs can be synthesized from silver salts, such as $AgNO_3$, precursors, by using reducing and capping agents, in chemical and biological systems [6]. The process of SNPs synthesis is cost effective as well as safe in biological systems that utilized microorganisms and plant materials; however, using plant products/extracts are plausibly advantageous over the utilization of microorganisms [7], since various bioactive components of plant extracts help act effectively the extract as both the reducing and stabilizing agents [8] and help in the reduction of Ag+ (in $AgNO_3$) to Ag^0 resulting into the formation of SNPs and stabilize the nanoparticles [9].

Several authors [10,11] reported the synthesis of SNPs from $AgNO_3$ using extracts of different parts of various plants and detected the existence of SNPs in the reaction mixture by visual inspection of its colour change as well as determining the absorbance spectrum, and reported antibacterial activity against potential human pathogenic bacteria: *Staphylococcus aureus (S. aureus)*, Pseudomonas *aeruginosa (P. aeruginosa)* and *Escherichia coli (E. coli)* [12,13]. The SNPs synthesized with *Chamaemelum nobile* extract exhibited significant antibacterial activity, against *E. coli, S. typhimurium, S. aureus* and *B. subtilis,* as has been reported by [14]. The earlier authors [8,15] utilized olive leaf extracts too, in order to synthesize the SNPs having bacterial growth inhibition property against *S. aureus, P. aeruginosa* and *E. coli* [8].

However, no report has been accessible, in literatures, in utilizing the Indian olive, *E. floribundus*, fruits, from our part of the globe, in green synthesis of SNPs, and thus on their antibacterial activity. Therefore, in the current study, the olive fruits, collected from locally available niches (West Bengal state, India), were utilized aiming to synthesize as well as characterize SNPs and to assess the antibacterial efficacy of the synthesized SNPs against clinical bacteria and to determine the HPLC profiles of the fruit phytochemicals.

Materials and Methods

Bacterial Strains

The gram-positive (*Bacillus cereus* and *Staphylococcus aureus*) and gram-negative (*Acinetobacter baumannii, Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris* and *Pseudomonas aeruginosa*) clinical bacterial isolates were used in the study. The standard bacterial strains utilized were *Listeria monocytogenes* MTCC 657 and *Pseudomonas aeruginosa* ATCC 27813.

Preparation of plant extract

The wild mature olive, *E. floribundus*, fruits collected from Uttar Dinajpur district, West Bengal state, India, washed with distilled water, and the seeds and mesocarp-epicarp parts were separated. The shed-dried mesocarp-epicarp part and seeds were grinded separately. A 10g of granules, both seeds and mesocarp-epicarp parts, were boiled separately in 60 ml of double distilled water for 30 min, in order to prepare the aqueous extract. The prepared extracts (AqOS and AqOME: 166 µg/µl), after filtration, were stored at 4^0C.

Synthesis of Silver Nanoparticles

The synthesis of SNPs was done following the protocol of Paralikar [10], with slight modifications. Briefly, in 9 ml of silver nitrate solution (1 mM in double distilled water), 1 ml of AqOME was mixed and incubated for 72h at room temperature (≈ 24^0C). Thereafter, the color change, in the reaction mixture was recorded through visual examination; the color change was indicative for reduction of Ag^+ (in $AgNO_3$) to silver atom (Ag^0), and thus formation of SNPs [10,16]. The absorbance patterns of the reaction mixture (AqOME- silver nitrate solution) was recorded, at 24h, 48h and 72h, in between the wavelengths 200 nm and 700 nm, in order to ensure the formation and existence of SNPs in the mixture [10], by UV-Vis spectrophotometric analysis using a spectrophotometer (LABARD instruments, Microprocessor UV-Vis Double Beam Spectrophotometer LIM-332).

The same steps were followed for AqOS mediated SNPs synthesis by preparing the reaction mixture with 5 ml of $AgNO_3$ solution (1 mM in double distilled water) and 5 ml of AqOS. The AqOME, AqOS, and silver nitrate solution were kept in similar experimental setting, as the controls.

Antibacterial Activity

The SNPs synthesized with olive fruit parts extracts (AqOME and AqOS) were tested for antibacterial activity against clinical bacterial isolates: gram-positive (*B. cereus* and *S. aureus*) and gram-negative (*A. baumannii, E. coli, K. pneumoniae, P. vulgaris* and *Ps. aeruginosa*) as well as the standard bacterial strains (Listeria monocytogenes MTCC 657 and *Pseudomonas aeruginosa* ATCC 27813), following agar-well diffusion method [17], the details of which was described elsewhere [18]. The concentration of SNPs utilized was 50-µl/well (6 mm, diameter), on nutrient agar plate swabbed inoculated with the individual test bacteria. After 24h incubation, at 37°C, the ZDI values (nearest whole) obtained around the wells loaded with the synthesized SNPs, were measured, and interpreted as highly active with ZDIs ≥ 15 mm, less active (ZDIs: ≤ 10 mm) or moderately active (11 - 14 mm) [18].

The test bacterial strains were treated also with the control agents: silver nitrate solution (0.15 µg/µl), AqOME (16.6 µg/µl) and AqOS (83 µg/µl), by dispensing 50 µl of the agents per well, in order to justify their antibacterial efficacy.

HPLC Analysis

The chemical components of olive fruit extracts, AqOME and AqOS, were separated in HPLC system (YL 9100 HPLC system). The samples, 10 µl, each of AqOME and AqOS, were injected into the HPLC linked to a C18 column (5 µm; 100Å; 4.6 × 250 mm), at 35°C. The column was eluted at a flow rate of 1.0 ml/min using the mobile phase consisting of acetonitrile (solvent A) and water (solvent B), in 2:3 ratio, and the eluting compounds were detected at 230 nm.

Statistical Analysis

The test results were expressed as the mean ± SD, and assessed by one-way ANOVA using MS Excel 2010 software, while comparison of the mean values was assessed using the Tukey's test, and statistical significance was estimated at $p < 0.05$.

Results and Discussion

The current research was intended to develop SNPs, with broad antibacterial spectrum, assisted with extracts of edible fruits from olive, *E. floribundus*, plant, the antibacterial property of which has already been explored [19]. The capacity of SNPs synthesis of olive seed aqueous extract has been reported earlier by Khadri., *et al.* [20], and in addition to the seed extract we have utilized the epicarp-mesocarp (from olive fruits) aqueous extract, in SNPs fabrication, too. Herein, the mixing of olive fruit aqueous extracts into the silver nitrate solution caused a change in colour of the colourless silver nitrate solution to milky white in presence of AqOME, and bright violet in presence of AqOS, at 24h, signifying the synthesis of SNPs in the mixture (Figure 1), and the colour remain unchanged after 24h, and through 72h.

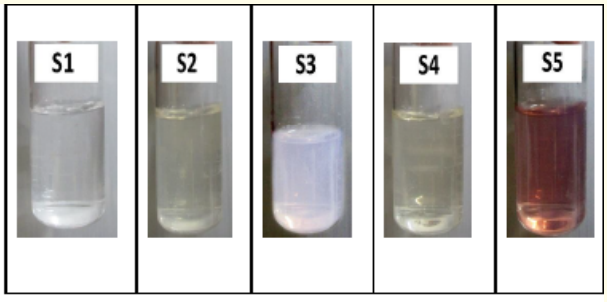

Figure 1: Synthesis of SNPs using Aqueous Olive Fruit Parts Extracts. S1: Silver Nitrate Solution (1 mM); S2: AqOME (16.6 µg/µl); S3: SNPs Synthesized with AqOME; S4: AqOS (83 µg/µl); S5: SNPs Synthesized with AqOS.

The colour change of the silver nitrate solution on mixing with the plant extract has been reported as an indication of SNPs formation in the reaction mixture [10,21].

The alteration of colour in the reaction mixture, and consequently the development of SNPs, was corroborated with the UV-Vis absorbance spectrum (of SNPs existed in the solution), which has extensively been considered as one of the important criteria to detect the presence of SNPs synthesized in the plant extract mixed silver nitrate precursor solution [22,23]. Characteristically, the SNPs displayed absorption peaks within UV-Vis range of wavelengths (λ = 300 - 500 nm), and thus absorbance in between 300 nm and 500 nm has particularly been a predetermined indicator to validate the reduction of Ag^+ to Ag^0, thereby producing SNPs [24]. In the current study, at room temperature (25°C), the absorbance of 2.839, corresponding to λ_{max} = 320 nm, was displayed by the SNPs synthesized with AqOME at 24h, while the peak values were 2.775 and 2.737, respectively at 48 and 72h (Figure 2A), as projected from the SPR of SNPs, while in case of AqOS mediated synthesized SNPs, during 24 - 72h, the pronounced peak values (absorbance) ranged 2.245 - 2.378, at λ_{max} = 310 nm (Figure 2B). Also, characteristically, the SNPs displayed absorption peaks within a range of wavelengths 380 - 490 nm, depending on their shape and size, as has been reported by earlier authors [25-27]. As per the report of Khadri., *et al.,* [20], the UV-Vis spectrum from the mixture of silver nitrate solution and aqueous olive seed extract displayed distinct peak, after 6 hours, at 419 nm. However, the generation of non-spherical (triangular) SNPs has been the evidence from the SPR peaks of SNPs at the shorter (348 - 373 nm) wavelengths, as has been reported by Pal., *et al* [28]. Shet., *et al* [29] reported the fruit extract (*Citrus aurantifolia, Citrus sinensis* and *Solanum lycopersicum*) mediated fabrication small sized SNPs, the SPR peaks for which were appeared in between wavelengths 300 nm and 350 nm. Mlalila., *et al.* [30] demonstrated the synthesis of SNPs with SPR peaks at around 320 nm, due to the non-spherical particles shapes, corresponding to the pronounced SPR peaks of the SNPs at 320 nm, in the instant case (Figure 2). The UV-Vis spectra of the synthesized SNPs were recorded further through 72h, but the colour intensity of the reaction mixture was not intensified beyond 24h, validating the completion of reaction by 24h, and also, the control silver nitrate solution did not develop any coloration as well as display the characteristic band (λ_{max} at 320 nm), indicating the absence of abiotic reduction of silver nitrate under the current experimental setting, as has also been demonstrated earlier by Ibrahim [31].

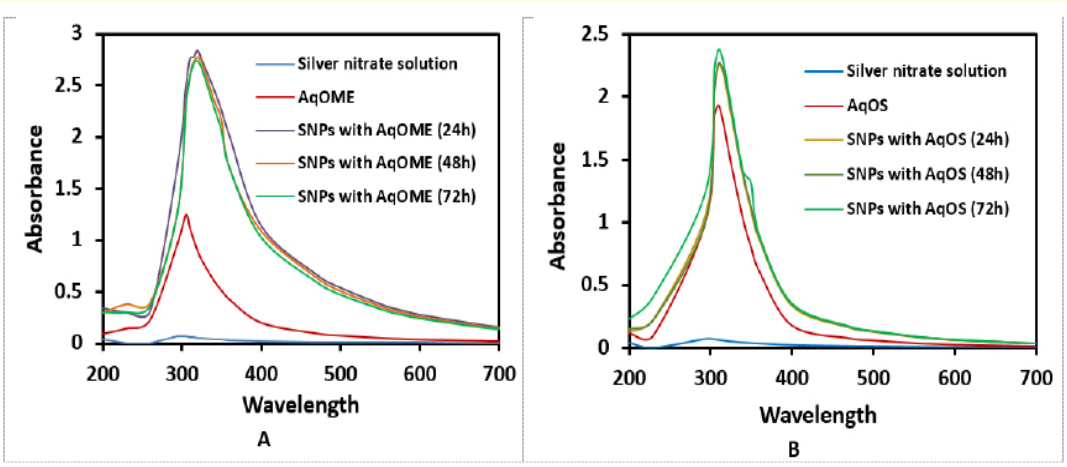

Figure 2: UV-Vis Spectra of Synthesized SNPs using Olive Fruit Parts Extracts. A: SNPs Synthesized with AqOME; B: SNPs Synthesized with AqOS.

The bacterial growth inhibition property of the olive fruits extracts, AqOME and AqOS, was assessed by agar-well diffusion method, the results of which are represented in Figure 3 and Figure 4. The antibacterial activity, in terms of ZDI values, expressed as mean ± SD, due to the action of SNPs synthesized with two different extracts from olive fruits (AqOME and AqOS), solution of silver nitrate salt, and AqOME and AqOS have been recorded (Figure 5). The ZDIs of the SNPs synthesized with AqOME and AqOS were 20.8 ± 3.2 mm (range: 16 - 25 mm) and 14.7± 1.06 mm (range: 14 - 17 mm), respectively, and hence, the SNPs synthesized with AqOME was highly active against all the gram-positive and gram-negative test bacterial pathogens (ZDIs: > 15 mm), while the SNPs synthesized with AqOS was highly active against *S. aureus, A. baumannii* and *E. coli,* only (ZDIs: ≥ 15 mm). The AqOME mediated synthesized SNPs, compared to the SNPs synthesized with AqOS, thus, had higher antibacterial activity, and, a significance difference was observed between their activities (p value: < 0.001). The respective ZDIs from the action of AqOME, AqOS and silver nitrate solution, against the test bacteria, were 6.7 ± 1.49 mm (range: 6 - 10 mm), 6 ± 0.00 mm, and 13.3 ± 0.82 mm (range: 12 - 15 mm); Thus, the olive, *E. floribundus*, fruit extracts (AqOS and AqOME), which were used in the synthesis of SNPs, were less active when applied alone against the test bacterial pathogens (ZDIs: ≤ 10 mm); the silver nitrate solution alone had activity against a clinical isolate of *S. aureus* (ZDI: 15 mm), only and hence, significance differences in the action between SNPs and SNPs precursors (AqOME, AqOS or silver nitrate solution) were noted (p values: < 0.005 to < 0.001). Khalil., *et al.* [8] observed a fact that the aqueous olive leaf extract had no activity against the test bacteria (*S. aureus, P. aeruginosa* and *E. coli*), at concentrations, which were used in the preparation of SNPs. Vanaja., *et al.* [21] reported that the *Solanum trilobatum* leaf extract and silver nitrate solution had ZDIs 6 - 7 mm and 9 - 13 mm, respectively, while the synthesized SNPs showed higher growth inhibitory activity (ZDIs: 11 - 15 mm) against the test pathogenic bacteria (*Bacillus subtilis, Streptococcus* sp., *Serratia* sp., *E. coli, Klebsiella planticola K. pneumonia*). The SNPs synthesized with *Chamaemelum nobile* extract exhibited significantly increased antibacterial activity (ZDIs: 13 - 14 mm), compared to the $AgNO_3$ solution (ZDIs: 7.6 - 8.3 mm), against *E. coli, Salmonella typhimurium, S. aureus* and *B. subtilis* [14]. According to the earlier report, by Pal., *et al.* [28], that the non-spherical SNPs had higher efficacy in inhibiting the growth of *E. coli* than the spherical SNPs, which have been synthesized at lower wavelengths. Shet., *et al* [29] reported the fruit extract mediated synthesis of non-spherical SNPs, with SPR peaks at the wavelengths of 300 - 350 nm, expressing excellent antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*, while very high antibacterial activity of SNPs (with SPR peak at 340 nm) have been noted due to their non-spherical shapes, compared with spherical-shaped SNPs, peaked at 420 nm, as has been demonstrated by Dong., *et al.* [32]. In the present study, the synthesized SNPs demonstrating the SPR peaks at wavelengths 310 - 320 nm, showed pronounced antibacterial activity.

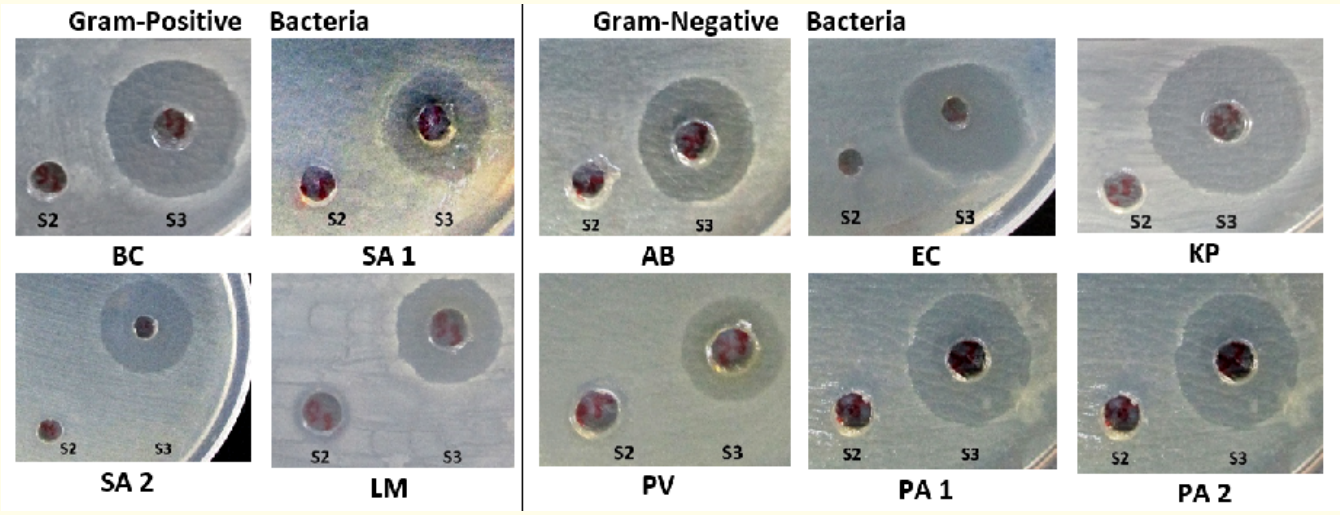

Figure 3: Antibacterial activity of SNPs Synthesized with AqOME by Agar-Well Diffusion Technique against Gram-Positive (BC: Bacillus Cereus; SA1: *Staphylococcus aureus* 1; SA2: *Staphylococcus aureus* 2) and Gram-Negative (AB: *Acinetobacter baumannii*; EC: *Escherichia coli*; KP: *Klebsiella pneumoniae*; PA1: *Pseudomonas aeruginosa* 1; PV: *Proteus vulgaris*) Clinical Bacterial Isolates, and Standard Bacterial Strains (LM: *Listeria monocytogenes* MTCC 657; PA 2: *Pseudomonas aeruginosa* ATCC 27813). S2: AqOME (16.6 µg/µl); S3: SNPs Synthesized with AqOME. Clear Halos around the wells on the Plates Indicate Bacterial Growth Inhibition.

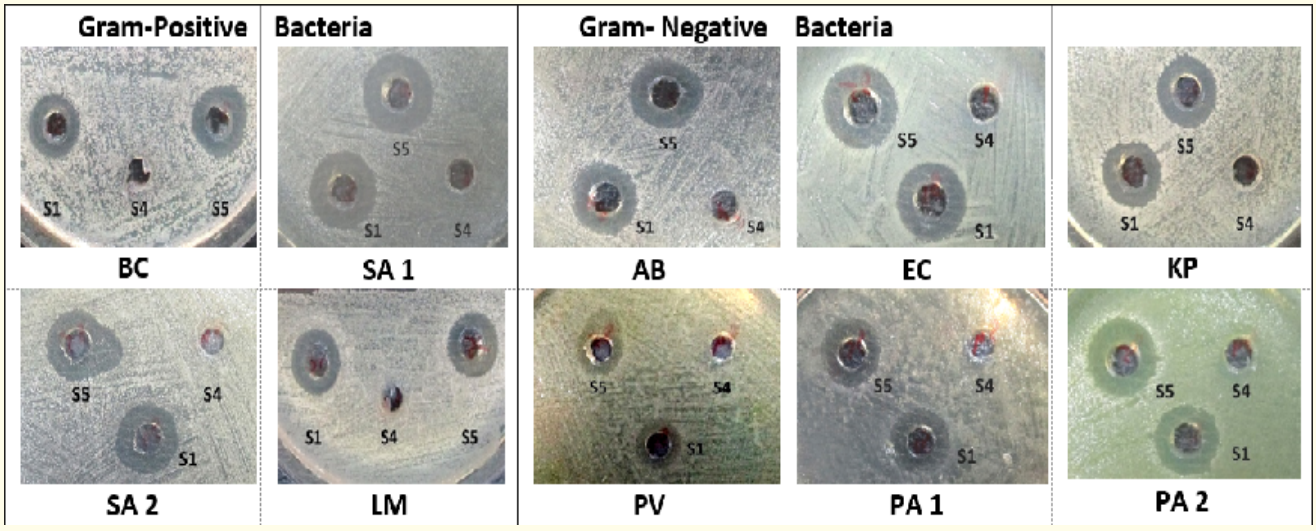

Figure 4: Antibacterial Activity of SNPs Synthesized with AqOS by Agar-Well Diffusion Technique against Gram-Positive (BC: Bacillus Cereus; SA1: *Staphylococcus aureus* 1; SA2: *Staphylococcus aureus* 2) and Gram-Negative (AB: *Acinetobacter baumannii*; EC: *Escherichia coli*; KP: *Klebsiella pneumoniae*; PA1: *Pseudomonas aeruginosa* 1; PV: *Proteus vulgaris*) Clinical Bacterial Isolates, and Standard Bacterial Strains (LM: *Listeria monocytogenes* MTCC 657; PA 2: *Pseudomonas aeruginosa* ATCC 27813). S1: Silver Nitrate Solution (1 mM); S4: AqOS (83 µg/µl); S5: SNPs Synthesized with AqOS. Clear Halos Around the wells on the Plates Indicate Bacterial Growth Inhibition.

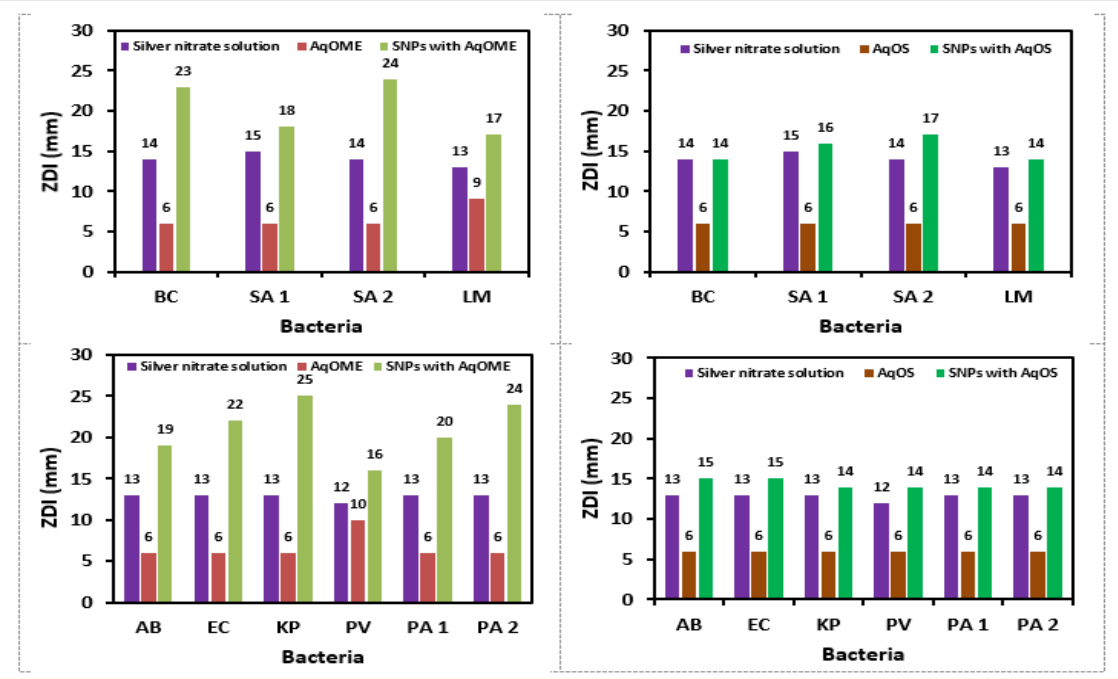

Figure 5: The ZDI values due to the Action Of Olive Fruit Parts Extracts Mediated Synthesized SNPs by Agar-Well Diffusion Method Against Gram-Positive (BC: *Bacillus cereus*; SA1: *Staphylococcus aureus* 1; SA2: *Staphylococcus aureus* 2) and Gram-Negative (AB: *Acinetobacter baumannii*; EC: *Escherichia coli*; KP: *Klebsiella pneumoniae*; PA1: *Pseudomonas aeruginosa* 1; PV: *Proteus vulgaris*) Clinical Bacterial Isolates, and Standard Bacterial Strains (LM: *Listeria monocytogenes* MTCC 657; PA 2: *Pseudomonas aeruginosa* ATCC 27813). AqOME: Aqueous Olive Mesocarp-Epicarp extract; AqOS: Aqueous Olive Seed Extract.

The HPLC analysis showed the presence of 8 major compounds in AqOME and 11 major compounds in AqOS with retention times 1.45 - 4.84 min and 1.91 - 4.84 min, respectively (Figure 6). Among the large number of phenolic compounds present in olive leaves and fruits, a major one has been identified as oleuropein [33], which has been detected by HPLC at different retention times (RT: 3.5 - 26.91), with various mobile phases [19,34,35], The chromatogram of the standard oleuropein solution has been detected with RT of ≈4 min [34], which was equivalent to the compound 9, as detected in AqOS with RT of 4.03. Faria., et al. [36] reported the presence of galloyl-glucose ester, a phenolic acid, with RT of 4.2 min detected in Syzygium cumini fruit, in water-acetonitrile mobile phase, while detecting non-anthocyanin phenolic compounds, which was equivalent to the compound 10, as detected in AqOS with RT of 4.26. The phenolic compounds in olive fruit extracts have been identified by HPLC [37], where 10 main peaks were found, and three compounds: gallic acid (RT: 5.75 min), ferulic acid (RT: 41.63 min) and rutin (RT: 45.95 min), were identified. The UV-Vis spectrometric analysis of *Svensonia hyderobadensis* leaf extracts gave λ_{max} at 262 nm, and the peak extended from 254 nm to 300 nm [38]. It has been reported that phenols and phenolic acids demonstrate λ_{max} in between wavelengths 250 nm and 290 nm, cinnamic acid derivatives in the range of 290 - 330 nm, and flavones and flavanols at 250 - 350 nm [39]. In the present study, the AqOME and AqOS had absorption maxima at the wavelengths 295 - 315 nm and 295 - 340 nm, respectively. The differences in the UV-Vis spectrophotometric features in different plant extracts are due to the presence of varied bioactive components, including phenolic compounds, possessing the capacity to act as the reducing as well as stabilizing agents, during antibacterial SNPs biosynthesis, and thus, rule out the need of chemical SNPs synthesis.

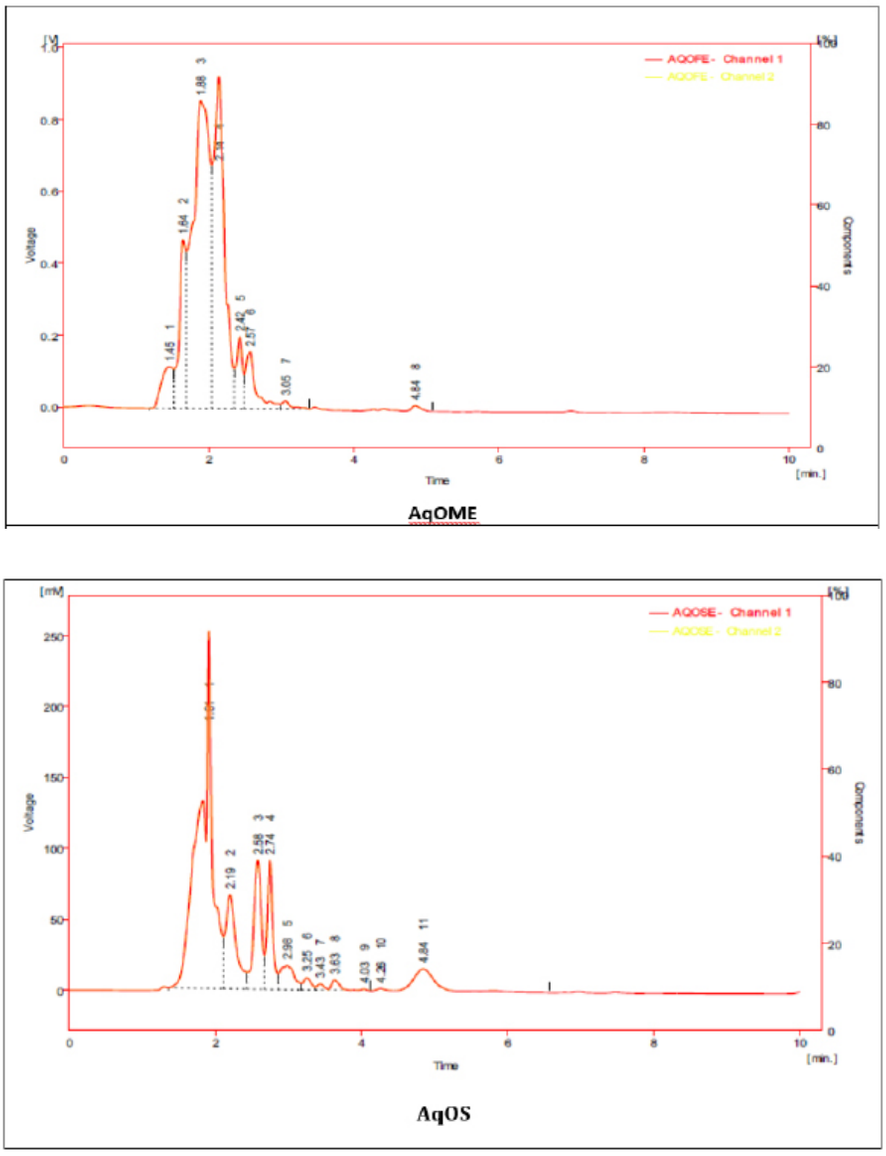

Figure 6: The HPLC Chromatograms of Olive, *Elaeocarpus floribundus*, Fruit Parts Extracts, AqOME and AqOS.

Conclusion

Compared to the synthesized SNPs, the aqueous olive, *Elaeocarpus floribundus*, fruit parts extracts alone had less or the moderate antibacterial activity (at concentrations utilized in the synthesis of SNPs). The olive fruit parts, especially the mesocarp-epicarp, might be utilized as the valid candidates of natural reducing and stabilizing agents in the green synthesis of silver nanoparticles, to be applied as the non-antibiotic therapeutic agents, against bacteria causing several life-threatening infections to humans. To the best of our acceptability, this study was the first to explore the fabrication of silver nanoparticles with extracts of olive fruits available in the local niches of this part of the globe (West Bengal, India).

Nevertheless, pharmacokinetics studies are required in unravelling the toxicity of the silver nanoparticles synthesized, following such green ways with a natively grown edible fruit, olive, and in determining the therapeutically effective dosage.

Acknowledgements

The first author and the corresponding author acknowledge the financial support from the Biotechnology Industry Research Assistance Council (BIRAC) and Gandhian Young Technological Innovation (GYTI), India, for the current study (Order No.: BIRAC SRISTI PMU - 2016/0093 dated 4th May 2016).

Conflict of Interest

There was no conflict of interest.

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