**Characterising biosynthesised silver nanoparticles from *Salvia rosmarinus* and assessing their in vitro antifungal and cytotoxic activities against phytopathogens and cervical cells**

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**Abstract**

Nanotechnology is revolutionizing world agriculture dramatically through engineered nanomaterials, which contribute to enhancing agricultural production by controlling fungal and bacterial phytopathogens, and consequently minimizing crop loss. Biosynthesised nanoparticles ranging 1-100 nm in size are incorporated in agricultural practices to control various emerging fungal phytopathogens. In the present study, silver nanoparticles were biosynthesised using aqueous extracts of organically grown *Salvia rosmarinus* leaves (RM- AgNPs). The synthesis was confirmed by visual examination when the colour changed to brown. Further, the synthesised nanoparticles were characterised and examined with ultraviolet-visible spectroscopy, energy dispersive spectroscopy (EDX), Fourier transform infrared (FTIR) spectroscopy, dynamic light scattering (DLS), and transmission electron microscopy (TEM). The results obtained show a characteristic peak with ultraviolet-visible spectroscopy, and the nanoparticle size ranged between 7 nm -58 nm, as revealed by TEM. The biosynthesised nanoparticles were tested against fungal phytopathogens with the poison food technique. Further, these particles were tested for their anticancer properties by an MTT assay against the cervical HeLa cell line. Our findings show significant inhibition of tested fungi, but in a variable manner. The strongest inhibition was shown by *Fusarium oxysporum* (61%), followed by *Alternaraia alternate* (50%). Lastly, in vitro cytotoxicity against cervical cancer cells demonstrated potential inhibition with an IC50 of 11.28 ± 0.33 µg/ml.

**Key words:** *Salvia rosmarinus,* silver nanoparticles (AgNPs), phytopathogens, anticancer

**Introduction**

Plant pathogens in general, and fungi cause immense loss to plants by drastically lowering their yield, which affects global economy. As such, the quality of edible parts (fruits and vegetables) of crops is affected, which raises serious concerns regarding human health. Crop protection has attracted various agricultural scientists from all parts of the world. In the past few years, most agricultural research aimed to increase the crop yield to meet the growing demands of the rising global population (Savary et al., 2012; Nellemann et al., 2009). However, this struggle of the 21st century remains, as the need for food is still growing and natural resources are shrinking (Brown 2011).

Fungal pathogens, such as *Fusarium spp., Colletotrichum spp., Penicillium spp., Botrytis cinerea, Alternaria* *alternata, Rhizopus stolonifer, Lasiodiplodia  sp,* and *Aspergillus sp* cause various devastating postharvest diseases on fruits and vegetables and incur a 25 to 60% loss of total production (Gonzalez -Estrada et al., 2018). Nowadays, postharvest spoilage due to fungi is predominantly controlled by applying chemical fungicides (Shridhar et al., 2018). Although most fungicides in use are proven to be effective against some fungal pathogens, their use is still questionable due to harmful effects on human health, the environment, and the emergence of resistant strains that have a wider host range. Thus, this compels exploring new eco-friendly biological fungicides that are less harmful to the environment and human health.

Nanotechnology is a novel technology that opened new avenues and concepts that are applicable in various fields, such as medicine, pharmacology, chemistry, physics, and recently food sciences (Sinha et al., 2017; Balaure et al., 2017). However, its use in agriculture has received less attention in the past, but is currently being extensively explored. Nanotechnology involves synthesis, characterization, and application of nanomaterials or particles at the size range between 1 and 100 nm (Bajpai et al., 2018). Further, at this nano size, particles exhibit unique properties that are not present in their original form (Sandoval, 2009). Nanoparticles are now being used in plant protection, water management, seed germination, transfer of target genes, nano-barcoding, and controlled release of agrochemicals (Hayles et al., 2017; Duhana et al., 2017). A large number of materials are used to prepare nanoparticles, such as metals and their oxides, lipids, emulsions, and ceramic. Recently, biological synthesis of nanoparticles from plants and microorganisms have emerged as excellent antimicrobials.

Silver nanoparticles (AgNPs ) have drawn tremendous attention and increasing interest due to properties, such as high conductivity, catalytic activity, chemical stability, localized surface plasma resonance, and antimicrobial and anti-inflammatory activities (Ahmad et al., 2003; Ahmed et al., 2016).

Due to the previously listed properties, AgNPs are used in various fields— such as for drug delivery, diagnosis, and tissue regeneration (Naidu et al., 2015); as post-harvest/biological coatings (Balamurugan et al., 2017) and antimicrobials (Qasim et al., 2018); and in the textile (Gokarneshan and Velumani, 2017), cosmetic (Naidu et al., 2015), and food (Carbone et al., 2016) industries. The conventional method of forming silver nanoparticles is costly and poses harmful environmental effects. Thus, to overcome the toxic effects of silver nanoparticles, natural resources are now being employed. Green synthesis is when plant extracts and microorganisms are used for nanoparticle synthesis. The various bioactive compounds in plants serve as reducing agents that render them safe and eco-friendly.

*Salvia Rosmarinus,* commonlyknown as Rose Mary (synonym- *Rosmarinus officinalis* L), belongs to the Lamiaceae family and is a native of the Mediterranean region (Bakırel et al., 2008). It is an herb and its leaves are fragrant and used in culinary settings as flavouring agents that are consumed all over the world (Panda, 2009; Ibarra, 2010). Rosemary has shown antiangiogenic (Kayashima and Matsubara, 2012), antibacterial (Georgantelis et al., 2007), and hepatoprotective potential (Raskovic et al., 2014). Extracts have also shown anticancer properties against prostrate, colon, and skin cancer cell lines. (Mirghaed and Yadollahi, 2013). In addition, the European Union has approved rosemary extracts (E3920 in EU additive regulation (No. 1129/2011). The European Food Safety Authority has proposed rosemary extracts as feed additives in the antioxidant class. Nowadays, per the European Union, rosemary extracts are added to food and beverages at levels of up to 400 mg/kg (as the sum of carnosic acid and carnosol) (Aguilar et al., 2008).

In the present study, we aimed to synthesise silver nanoparticles using leaf extracts of *Salvia rosmarinus,* grown in an organic manner in Saudi Arabia. The synthesised nanoparticles will be characterised, screened further for their cytotoxicity and antifungal activity against some plant pathogens.

**Materials and Methods**

**Plant material and chemicals used**

*Salvia rosmarinus,* will be referred as RM throughout in this Paper. Fresh, disease free leaves of RM grown organically in Riyadh, Saudi Arabia, were provided by Dr Sara Al Rashid and identified by plant taxonomist Dr Najat Al Bukhari. All the reagents used in the experimental work were of analytical grade and were obtained from Sigma-Aldrich.

**Preparation of aqueous leaf extract**s

The leaves of RM were washed under running tap water to remove any adhering visible impurities and soil particles. 10 g of roughly chopped leaves that were dried at 25 0 C were added to a beaker that contains 100 ml of distilled water. This mixture was heated at 60 0C for 20 min. After cooling, the supernatant was filtered through Whatman filter paper (No.1) and centrifuged for 5 min at 5000 rpm. The supernatant was used to synthesise silver nanoparticles.

**Synthesis of green Ag nanoparticles**

Synthesis of the nanoparticles was implemented by following the method of Jain and Mehata (2017) with slight modifications. A fixed volume of AgNO3 powder (0.0085 gm) was dissolved in 25 ml of distilled water to prepare 1 mM AgNO3 solution. 1 ml of the RM extract was added to 5 ml of 2 mM AgNO3 solution and mixed thoroughly on a magnetic stirrer and observed for any colour changes. A change in colour from pale yellow to colloidal brown indicated the formation of silver nanoparticles (Ag-NPs).

**Characterization of the synthesised** **Ag-NPs**

**UV-Vis spectroscopy:** The synthesis of Ag-NPs was confirmed and further characterization was conducted using standard characterization techniques. UV-Visible (UV-Vis) spectroscopy (Thermo Scientific 1500, USA) was performed on the mixture to exhibit formation of Ag-NPs (colloidal brown solution). The absorbance of the reaction mixture was measured over the range of 200–700 nm.

**Zeta sizer:** The average size of the nanoparticles was measured by Zeta sizer (Nano–ZS-90 Malvern) after diluting the samples with pure water.

**Transmission electron microscopy (TEM):** The average size of the synthesised AgNPs was determined by observing the particles under TEM (JEOL JEM-1400 Plus). Preparation of the samples was done by carefully adding dropwise the synthesised silver nanoparticles on a grid that is coated with copper. Further, the particles (sample) were imaged under TEM.

**EDX analysis by scanning electron microscopy:** The elemental composition of nanoparticles was determined by EDX analysis. A thin film of AgNPs was prepared on a glass slide by adding the sample in a dropwise manner and allowing the solvent to evaporate. Then, it was coated with platinum and observed under FESEM (FESEM-JSM-7610F, JAPAN).

**Fourier transform infrared spectrophotometer:** The functional groups that are present in the extract and the synthesised nanoparticles were analysed by FTIR spectrometer (Thermo Scientific-Nicolet -6700, USA) at a scan range of 400-4000 cm‒1 with a KBr pellet.

**In vitro antifungal activity of the synthesised nanoparticles**

The inhibitory activity of synthesised RM Ag-NPs was tested against seven phytopathogenic fungal strains. All the strains were provided by the Department of Plant Protection at the College of Food and Agricultural Sciences at the King Saud University in Riyadh, KSA. The in vitro mycelial inhibition was tested using Potato dextrose agar medium. Roughly, 15 mL of sterilized PDA, which served as growth medium, were poured into a sterilized petri dish and allowed to solidify. Test fungi were separately grown in PDA plates for 5 days, and later used to remove a mycelial plug for an in vitro assay. To test the effects of nanoparticles on fungal growth, 1 ml of synthesised AgNPs was added to sterile petri dishes, followed by 15 ml of PDA. The mixture was then gently swirled and allowed to solidify. After solidification, a plug of a 6 mm mycelial disc was aseptically removed from the periphery of test plates. The removed mycelial disc was then placed in the centre of the petri plate, which received 1 ml of synthesised AgNPs and was incubated at 25 ± 2 °C for 7 days. The growth of the fungal colony was measured (mm) after 7 days. Petri plates with PDA medium and without AgNPs served as controls. All the treatment and control assays were conducted in triplicate.

The effects of AgNPs on percentage inhibition of mycelia growth was calculated using the following formula:

% inhibition= (Dfc− Dft) /Dfc × 100

where Dfc = average increase in mycelial growth in control; Dft = average increase at each treatment.

**Cytotoxicity by an MTT Assay**

The cytotoxicity of RM-AgNPs was examined using an MTT assay according to the method of Siddiqui et al. (2010). The human cervical cancer cell line was obtained from the American Type Culture Collection (USA). In brief, HeLA cells were plated in 96-well plates at a density of 1x104 cell/well, and allowed to settle for 24 h prior to treatment. Cells were treated with various concentrations of RM-AgNPs and prepared by a two-fold serial dilution. After incubating the plate for 24 h, the MTT was added into the wells and the plate was further incubated for 4 h.

To the reaction mixture, 200 µl/well DMSO was added and thoroughly mixed. All experiments were run in triplicate. Absorbance of the plates was measured at 550 nm, and the results were expressed as percentage cell viability. IC50 was assessed using the Graph Pad Prism Program Version 7 and a graph that shows the dose-dependent response was generated using regression analysis.

**Results**

**Formation of RM AgNPs nanoparticles: Visual examination and UV-Vis spectral analysis**

The formation of the RM AgNPs was visually observed when the colourless AgNO3 gradually transformed to a brown colour with an orange tint, upon addition of the rosemary aqueous extracts. This colour change indicated the formation of RM AgNPs, which is due to surface plasmons. Figure 1 shows the brown colour, which indicates the formation of AgNPs.

 The formation of the AgNPs was further confirmed and measured with a UV-Vis spectrophotometer to obtain a surface plasmon resonance band and for periodic monitoring until no further change in the band intensity occurred, which indicates the completion of the reaction. The surface plasmon resonance band was observed to be gradually increased in intensity for up to 50 min and then stopped after an hour. The absorption band at 450 nm shows the formation of silver nanoparticles (Figure 2).

**Dynamic light scattering (DLS)-particle size determination**

The particle size of the synthesised RM-NPs was measured with Zetasizer Nano Series (Malvern). The average size of the particles was 56 nm and the polydispersity index (PDI) was 0.285 (Figure 3).

**Transmission electron microscopy (TEM)**

To determine the size and morphology of the RM- AgNPs, the particles were observed under a transmission electron microscope (TEM Model –Jeol‒1011). The green synthesised AgNPs of the rosemary leaves showed uniform distribution and were nearly spherical in shape. The mean particle size was ranged between 7 nm to 58 nm (Figure 4). The microphotograph also demonstrates that the RM-AgNPs were separated in a uniform manner.

**Energy dispersive spectrum (EDX) of RM- AgNPs**

The EDX analysis of the synthesised RM-AgNPs showed the presence of Ag, which is evident in the spectrum (Figure 5). An absorption peak with an intense signal was observed at 3 KeV, which corresponds to the presence of RM- AgNPs that occur due to SPR. The presence of other elements were also observed in the spectrum. EDX analysis was conducted with a scanning electron microscope (FE-SEM-EDX, JSM-7610F).

**Fourier-transform infrared spectroscopy (FTIR) of the extract and synthesised nanoparticles**

The IR bands of both extracts and synthesised RM-nanoparticles are shown in Figure 6. Some common bands were observed in both the extracts and RM-AgNPs. The extract revealed bands at 3402 cm−1, which corresponds to the O-H stretching of phenols, and 1605 cm−1, which denotes the C=C stretching of conjugated alkene. Further, the two peaks at 1267 cm−1 and 1039 cm−1 are due to C-O stretching. However, the IR spectrum of the RM-AgNPs exhibited disappearance of certain bands at 1524 cm (N-O stretching), 1405 cm−1(N-H bending vibration), and 813 cm−1(C-H bending vibrations), which were quite evident in the IR spectrum of the RM extract. Appearance of new peaks at 2928 cm−1 and 1386 cm−1 were also observed in the spectrum RM-AgNPs. Further, peaks of the extracts shifted from 3402 cm−1, 1605 cm−1, and 1386 cm−1 to 3426 cm−1, 1620 cm−1, and 1068 cm−1 in the synthesised RM-AgNPs, and were tremendously narrower with weak transmittance (less intense).

**Antifungal activity**

The antifungal activity of the synthesised RM-AgNPs exhibited variable inhibition against examined phytopathogenic fungi. The highest antifungal activity was that of *Fusarium oxysporum*, followed by those of *Alternaria alternata* and *Fusarium graminearum* (61%, 50%, and 43%, respectively). However, the other fungal strains exhibited poor mycelial inhibition.

**Detection of cytotoxicity of RM- AgNPs by MTT assay**

To determine the cytotoxic activity (in vitro) of the synthesised RM-AgNPs, an MTT assay (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) was implemented. HeLA cell lines were treated with different concentrations that range from 3.125 to 100 µg/ml, as previously described, with synthesised RM-AgNPs. Figure 7 portrays the percent growth inhibition of cancer cell and their viability when treated with RM-AgNPs and control cells (untreated). The cancer cell inhibition was dose dependent, since the concentration of RM-AgNPs increased and the cell viability decreased. Noticeable inhibition began at 25 µg/ml with the cells at 19% viability and dropped to a maximum of 5% viability at 100 µg/ml. The IC50 value was 11.28 ± 0.33 µg/ml.

**Discussion**

In the present study, we demonstrated the synthesis of AgNPs with rosemary extracts (RM- AgNPs). The appearance of a brown colour, upon addition of silver nitrate to the extracts, is due to the bio-reduction of silver ions to silver nanoparticles. This was confirmed by a UV-Vis spectrophotometer, which monitors the bio-reduction process through the formation of a characteristic surface plasmon resonance (SPR) peak. The formation process of a brown colour was gradual and started within the first few minutes and took approximately an hour to complete. This was evident in the surface plasmon resonance band intensity as the band did not show any further change after 60 min. It is known that AgNPs display potent SPR activity in aqueous solutions (Shanker et al., 2004). In the present study, the synthesised RM- AgNPs showed a SPR peak at 450 nm, which was broad and pronounced and which indicates that the particles were poly-dispersed in nature. The characteristic silver nanoparticle peaks (λ max) were observed between 400-500 nm (Sastry et al., 1997). The absorption spectrum of silver nanoparticles is highly sensitive to several factors (Jain and Mehata, 2017). Thus, the SPR position, shape, and size are affected by the dielectric medium, the size and shape of the silver nanoparticles, and the surroundings medium, (Kelly et al., 2003; Zhao et al., 2008; Wani et al., 2010; Dada et al., 2017a). The rosemary extracts possess phenolic compounds, flavonoids, and amides, which are responsible for the bio-reduction of silver ions to silver nanoparticles. Similar findings that are related to the SPR peak from RM-AgNPs have also been previously reported (Ghaedi et al., 2015).

 The dynamic light scattering (DLS) studies on synthesised RM-AgNPs showed that the average size of the particles and PDI as 56 and 0.28 nm, respectively, which indicates that the particles are stable. The PDI values that are greater than 0.7 indicate that the sample has a very broad size (Clayton et al., 2016; Roy et al., 2017). Since the PDI is lower than 0.7 in this study, the RM-AgNPs are of considerably good quality. Our findings agree with those of previous reports, in which PDI values of 0.398 and 0.7, respectively, were observed, since the plant extracts form a wide range of coatings around the nanoparticles (De Aragao et al., 2016; Roy et al., 2017).

The TEM microphotograph shows the shape and size of the synthesised RM- AgNPs, which appear to be roughly spherical. The average size ranged between 7 nm to 58 nm. It is also evident from the TEM micrograph that the particles were poly dispersed and well separated, and distributed in a uniform manner. The small size of the particles that is observed in the TEM microphotographs, in comparison to the DLS spectrum, is due to a physical state in which the sample is measured. As dry samples (nanoparticles) are used in TEM analysis and hydrated particles are measured in the DLS method, the hydrodynamic volume is larger in the hydrated state, which contributes to a large size of the nanoparticles (Gao et al., 2008). The biomolecules that are present in the RM leaf extract also caused capping of the AgNPs, which is evident in the TEM micrograph. Similar findings were previously reported, in which various organic bioactive compounds that are present in the plant extracts form a thin coating around the nanoparticles, facilitate the bio-reduction process, and stabilize the synthesised nanoparticles and sometimes may also result in few particles that agglomerate (Mallikarjuna et al., 2014; Henry et al., 2019).

The presence of elemental silver in the synthesised RM- AgNPs was analysed with an energy-dispersion X-ray (EDX) spectroscopy. The EDX spectrum showed a strong signal of silver at 3 KeV. The peak that arose in this region is attributed to SPR, which ascertains the formation of AgNPs (Das et al., 2003; Mallikarjuna et al., 2014). Besides a prominent peak that indicates the presence of silver, the peaks that are related to other elements can also be observed in the EDX spectrum. These peaks arise due to elements that are present in the extracts. The platinum peak is due to the coating that is used on the sample.

 An FTIR analysis was used in the present study to identify the potential biomolecules that are responsible in the bio-reduction and capping processes of the silver ions (Ag+ ions) during the synthesis of RM-AgNPs. The broad peaks in the extracts at 3402 cm−1 indicate that the RM extract are rich in phenols. However, after the synthesis of RM-AgNPs, the disappearance and narrowing of the bands in the spectrum indicate that the functional groups served a purpose in the bio-reduction and synthesis of RM-AgNPs. In addition, comparison of the IR spectrum of the RM extracts and RM- AgNPs show that several peaks shifted. Shifts from 1461 cm−1 to 1386 cm−1 and that from 3402 cm−1 to 3426 cm−1 suggest the involvement of N-H bending vibration of amines or alcoholic groups in the reduction of Ag. The peak at 1405 cm−1, which is due to N-H bending in amine group that is present in the extract, serves as a capping and stabilizing agent, as previously reported by Jyoti et al. (2016). Thus, the FTIR analysis of RM-AgNPs in the present study evidently indicates the presence of phenols, aliphatic amines, terpenoids, and flavonoids. These molecules seem to surround the AgNPs, and serve as strong binding sites for the nanoparticles during synthesis. Rosemary leaf extracts are rich in important secondary metabolites, such as carnosol derivatives, flavonoids, and phenolic compounds, which play a vital role in capping and with providing stability (Shah et al., 2014). Previous studies suggest that the presence of the functional groups on the surfaces of green synthesised silver nanoparticles are prepared from leaf extracts from rosemary and other plants (Fierascu et al., 2014; Prasannaraj and Venkatachalam, 2017; Femi-Adepoju et al., 2019)

 RM- AgNPs show significant antifungal activity against phytopathogenic fungi in the present study, but in a variable manner. The potent antifungal activity of certain green synthesised nanoparticles has been previously reported (Gupta et al., 2014; Bahrami-Teimoori et al., 2017; Al-Zubaidi et al., 2019). The exact mode of action of AgNPs as antifungals is not yet fully understood. However, certain studies show that AgNPs adhere to fungal hyphae and conidia, and potentially penetrate the cell membrane, which disrupts cell integrity (Srikar et al., 2016). Another perspective regarding significant antifungal activity involves interference with ergosterol synthesis, which directly affects the integrity of cell structures and which leads to cell death (Radhakrishnan et al., 2018; Roy et al., 2019). Yet another perspective is that the large surface area of AgNPs induces increased ROS production and free radicals and the leakage of DNA and proteins, which thus results in cellular damage (Ogar et al., 2015; Dakal et al., 2016; Ibrahim et al., 2020). Previous studies show strong in vitro inhibition of fungi, such as *Bipolaris sorokiniana*, *Magnaporthe grisea*, and *Rhizoctonia solani*, when treated with nanoparticles that ae synthesised from plants (Elgorban et al., 2016; Lopez et al., 2018). Recently, in vitro inhibition of *Aspergillus oryzae* and *C. albicans* by RM-AgNP was also reported (Ghaedi et al., 2015).

 Our findings show significant cytotoxic activity of RM -AgNP against the cervical cell line -HeLa, which may be due to the rich plant components that are attached to the AgNPs and the small size that enables effective cell penetration. AgNPs induce cytotoxicity by disturbing the cell cycle of cancer cells and inhibiting cell proliferation (Dziedzic et al., 2016). An MN assay recently revealed the genotoxic potential of AgNPs, as it induced chromosomal damage and abnormalities during mitosis (Sahu et al., 2016). Green synthesised nanoparticles caused HeLa cells to shrink, decrease in density, and lose its cell adhesion capability (Al Sheddi et al., 2018). Similar cytotoxic effects and cellular changes in a dose-dependent manner have been previously reported for RM-AgNPs and green AgNPs against various cancer cell lines (Vivek et al., 2012; Suman et al., 2013; Sulaiman et al., 2013; Al Sheddi et al., 2018). Thus, it can be inferred that the antiproliferative activity of RM -AgNPs could be due to their capability to induce ROS generation and apoptotic death (Stroh et al., 2004; Farah et al., 2016).

 Rosemary is a rich source of carnosol, carnosic acid, and rosmarinic acid. The two major components of rosemarinic acid and carnosic acid reportedly induce high cytotoxicity in breast cancer cell lines at an IC50 of 24.08–31.87 μg/ml and 12.50 μg/ml (Yesil-Celiktas et al., 2010). Rosemary extracts and carnosic acid were also shown to exert antitumorigenic effects and promote apoptosis (Huang et al., 1994; Petiwala et al., 2013). Our findings are consistent with all previous studies, as the extracts demonstrated significant antiproliferative activities against several human cancer cell lines.

**Conclusion**

The biosynthesised RM-AgNPs from organic leaves that are grown in Saudi Arabia have demonstrated significant antifungal and antiproliferative activities against plant pathogens and cervical cancer cells (HeLa). *Fusarium* and *Alternaria alternata* showed notable inhibition by biosynthesised AgNPs. The biosynthesised particles exhibited characteristic SPR peaks, and its synthesis was further confirmed and characterised by UV-vis spectroscopy, EDX, FTIR analyses, and TEM studies. The nanoparticle, due to its small size, induced dose-dependent cytotoxicity against HeLa cells with a low IC50 value. Based on our findings, RM- AgNPs can be applied in postharvest technology and for crop protection against harmful fungal pathogens. Further, these particles can be used in the development of a novel anticancer therapeutic formulation. However, future studies are needed to investigate the toxic effects of these particles on both normal human cells and beneficial microbial populations in soil and plant produce.

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