

***In vitro* anti-inflammatory, anti-arthritic and anti-proliferative activity of green synthesized silver nanoparticles - *Phoenix dactylifera* (Rothan dates)**

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Traditionally dates is consumed as a rich source of iron supplement and the current research discuss the synthesis of silver nanoparticles (AgNPs) using methanolic seed extract of Rothan date and its application over *in vitro* anti-arthritic, anti-inflammatory and antiproliferative activity against lung cancer cell line (A549). FTIR result of synthesised AgNPs reveals the presence of functional group OH as capping agent. XRD pattern confirms the crystalline nature of the AgNPs with peaks at 38°, 44°, 64° and 81°, indexed by (111), (200), (220) and (222) in the 2θ range of 10-90, indicating the face centered cubic (fcc) structure of metallic Ag. HR-TEM results confirm the morphology of AgNPs as almost spherical with high surface areas and average size of 42 ± 9nm. EDX spectra confirmed that Ag is only the major element present and the Dynamic light scattering (DLS) assisted that the Z-average size was 203nm and 1.0 of Pdl value. Zeta potential showed -26.5mv with a single peak. The results of the biological activities of AgNPs exhibited dose dependent activity with 68.44% for arthritic, antiinflammatory with 63.32% inhibition and anti-proliferative activity illustrated IC₅₀ value of 59.66 µg/mL expressing the potential of AgNPs to combat cancer.

Keywords: Nanoparticles. Proliferation. Inflammatory. Arthritic. Green synthesis. Anticancer.

INTRODUCTION

Nano-biotechnology is a developing area of research that inculcates the application of nano-materials in the field of biomedical research as their improved properties are leading to the rapid development and discoveries in the medical, optics and electronic industries. The recent advancements of nano-materials are based on explicit characteristics such as size, distribution and morphology (Packialakshmi, Suganya, Guru, 2014; Rafi *et al.*, 2015). Green synthesis is a process of nanomaterial fabrication

following eco-friendly and sustainable approaches without usage of any toxic chemicals. Various biological entities like plant extracts, bacteria, algae, fungi, lichens and enzymes are used for green synthesis of nanoparticles (Dhand *et al.*, 2016).

Green synthesis of nanoparticles are specific due to their ability in shape confinement due to the diversified chemical, optical and electrical properties (Jayaseelana *et al.*, 2013). Silver nanoparticle synthesis is notably interesting due to their application in the field of drug delivery, energy systems and optics because of their surface properties. These silver nanoparticles are clinically used in cancer treatment and diagnosis. Generally, nanoparticles preparation involves chemical and physical methods that are expensive and emits hazardous chemicals to the environment causing pollution

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and various biological risks. The recent development of biotechnological processes for nanoparticle synthesis is evolving as the plant phytoconstituents can act as both reducing and capping agents (Ahmed *et al.*, 2016).

Green synthesized nanoparticles are predominantly accepted in pharmaceutical industry, due to its safe and zero side effect property. Since there is a rising interest in discovering the novel phytochemical correlating to its pharmacological activity as this study focus on understanding the bioactive functions of the dates seed extract. The antioxidant activity is studied to evaluate the potential of the AgNPs in reducing the oxidative stress and disease progression which is further compared with its anti-proliferative effect against cancer cells. The inter relation between arthritis and inflammation is that both are tissue destruction caused by immune complexes, however synthesised AgNPs are capable of controlling it effectively when compared with the standard drugs.

Phoenix dactylifera (Rothan variety), commonly known as “date palm”, is cultivated in various parts of Asia for its delicious fruits as well as medicinal importance. The date fruit contains phytochemicals like carbohydrates, proteins, sterols, flavonoids, anthocyanins and phenolics, mainly cinnamic acid derivatives (Farhadi *et al.*, 2017) thus resulting in the reducing property of the extract. Present work deals with the synthesis of AgNPs from the seed extract of *Phoenix dactylifera* without any extra stabilizing or capping agents (Shib *et al.*, 2013) and to investigate the *in-vitro* biological activities.

MATERIAL AND METHODS

Material

All chemicals, reagents, medium components are procured from Merck, India. *Phoenix dactylifera* (Rothan) fruits were procured from local market at Al Majmaah, Kingdom of Saudi Arabia, seeds were removed from flesh, shade dried then rinsed with water three times and then cleansed with Milli-Q water to remove the fine dust particles. Finally the seeds were air dried for one week to remove all moisture completely, coarsely powdered, packed in air tight container and stored under normal temperature for further use (Syed Zameer Ahmed *et al.*, 2019a).

Preparation of seed extract from *Phoenix dactylifera* (Rothan)

The powdered *Phoenix dactylifera* (Rothan) seeds were taken and methanolic extract was prepared using soxhlet's apparatus by adding 10g of seed powder and refluxed with 200mL methanol for 6 hours. The extract was then cooled at room temperature and filtered with Whatman No.1 filter paper to yield a deep brown coloured filtrate (Shib *et al.*, 2013).

Synthesis of silver nanoparticles (AgNPs)

For the synthesis of the AgNPs, 20mL of seed extract was added to 50mL of analytical grade AgNO₃ solution (10⁻³ M) and the volume was adjusted to 180 mL with de-ionized water. The flask was then incubated at room temperature for 24 hours and observed for change in colour intensity (Baghizadeh *et al.*, 2015).

Characterization of synthesized AgNPs

The AgNPs synthesized from Rothan date seeds were processed suitably for characterization studies. The reducing components of phytochemical extract was found by Fourier Transform-Infrared Spectroscopy (FTIR 8400) in solid state. The morphology of AgNPs were analysed using HR-TEM (High resolution-Transmission emission of microscopy) and the crystallinity of AgNPs was examined using Bruker AXS D8 Advance X-ray Diffractometer (XRD) and Energy-Dispersive X-Ray Spectroscopy Analyser (EDXA: Oxford Link ISIS-300), Zeta potential and hydrodynamic diameter of nanoparticles were determined by dynamic light scattering using Malvern Zeta-sizer Nanoseries (UK).

In vitro free radical scavenging activity on synthesized AgNPs DPPH radicals scavenging assay

DPPH assay is commonly used to determine the free radical scavenging ability. Shimada *et al.*, (1992) method with minor modifications was used to screen the free radical scavenging activity of AgNPs. The

reaction mixture consisted of 1.0 mL AgNPs varying concentration (2, 5, 10 mg/mL) mixed with 1.0 mL of 0.8 mM/L DPPH solution and incubated at room temperature for 30 min under shaking condition. Then absorbance was recorded at 517 nm against gallic acid as control. The inhibition percentage for scavenging of DPPH radical was calculated by the following formula.

Ferric ions (Fe³⁺) reducing antioxidant power assay (FRAP)

The ferric ion reducing power of AgNPs was analysed by the method of Oyaizu. (1986) with minor modification. The reaction mixture consisted of 2, 5, 10 mg/mL of AgNPs was diluted with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) followed by 2.5 mL of 1% potassium ferricyanide [$K_3Fe(CN)_6$] and incubated at 50°C for 20 min. 2.5 mL of 10% trichloroacetic acid was added and centrifuge at 3000 rpm for 10 min. Finally 2.5 mL of supernatant was mixed with 2.5 mL of deionized water and 0.5 mL of 0.1% $FeCl_3$. The absorbance at 700 nm was measured against ascorbic acid as positive control.

Hydroxyl radical scavenging assay

The hydroxyl radical scavenging activity was performed by Halliwell *et al.*, (1987) method with minor modification. Prepare stock solutions of EDTA (1mM), $FeCl_3$ (10mM), ascorbic acid (1mM), H_2O_2 (10mM) and deoxyribose (10mM) in distilled deionized water. The reaction mixture was prepared with 0.1mL of EDTA, 0.01mL of $FeCl_3$, 0.1mL of H_2O_2 , 0.36mL of deoxyribose, 1.0mL of AgNPs extract (2, 5, 10 mg/mL), 0.33mL of phosphate buffer (50 mM, pH 7.4), 0.1mL of ascorbic acid in sequence and then incubated at 37°C for 1hr. Finally 1.0mL of the mixture was taken separately mixed with 1.0mL of 10% TCA and 1.0mL of 0.5% TBA. The final mixture was measure at 532nm.

Hydroxyl scavenging activity (%) = $(A_0 - A_1/A_0) \times 100$

(A1 is the absorbance of standards or reaction mixture, A0 is the absorbance of the negative control).

Anti-arthritis activity (protein denaturation assay) of synthesized AgNPs

Anti-arthritis activity of the AgNPs was determined using the method of Deshpande *et al.*, (2009) with minor modifications. The reaction mixture consisted of the 100 μ l AgNPs extracts (final concentrations 100, 250, 500 μ g/mL) and 100 μ l of 5% aqueous solution of bovine serum albumin (BSA); pH was adjusted using 1N HCl. The test samples were kept for incubation at 37 °C for 20 min and heated to 57 °C for 3 min. The mixture was allowed to cool for 10 min after which absorbance was measured at 416 nm. The blank comprise of the sample and distilled water was used as the negative control. The positive control was diclofenac sodium (final concentration 100, 250, 500 μ g/mL). Percentage inhibition was calculated using the formula:

Anti-inflammatory activity of synthesized AgNPs

The anti-inflammatory method of Mizushima and Kobayashi (1968) was used for inhibition of albumin denaturation. The reaction mixture consisting of (100 μ g/mL, 250 μ g/mL, and 500 μ g/mL) of AgNPs extract is taken with 1% aqueous solution of bovine albumin fraction and the pH was maintained at 6.3 using 1N HCl. The reaction mixture was incubated at 37°C for 20 min and then temperature was increased to 51°C for 20 min further allowed to cool. The absorbance of the mixture was measured spectrophotometrically at 660 nm and their results were compared with standard drug Diclofenac-sodium (100, 250, 500 μ g/mL). Percentage inhibition of protein denaturation was calculated by

% Stabilization = $100 - [A_1 (\text{sample} / \text{standard})/A_0 (\text{control}) \times 100]$

Where A1 was the absorbance in the presence of the sample/standard (Diclofenac sodium) and A0 was the absorbance of the control reaction

In vitro anticancer activity for synthesised AgNPs

Cell lines and culturing

Human lung adeno carcinoma cell lines (A549) was obtained from National Centre for Cell Sciences (NCCS), Pune, India. A549 cell line were cultured in DMEM (Dulbecco's modifications of Eagle's medium with L-glutamine & 4.5G/L glucose) supplemented with fetal bovine serum, 100 units/mL of penicillin G and (0.1 mg/mL) of streptomycin sulphate in a humidified atmosphere of a 5% CO₂ at 37°C.

MTT Assay for cell viability

The MTT assay was used to measure cell viability. The MTT substrate is prepared in a physiologically balanced solution, added to cells in culture, usually at a final concentration of 0.2 - 0.5mg/mL, and incubated for 1 to 4 hours. The quantity of formazan (presumably directly proportional to the number of viable cells) is measured by recording changes in absorbance at 570 nm using a microtiter plate reader. Viable cell converts MTT into a purple coloured formazan product due to the active metabolism with an absorbance maxima near 570 nm. The cells lose the ability to convert MTT into formazan when they are dead, thus colour formation acts as a useful and suitable marker for the viable cells. Thus cell survival is calculated by the formula.

RESULTS AND DISCUSSION

Characterization of synthesised AgNPs

FTIR interpretation

FTIR spectroscopy studies were carried out to investigate the presence of biomolecules responsible for the reduction and capping of the AgNPs synthesized by Rothan date seed extract is shown in Figure 1. The peak at 3450.8 cm⁻¹ is attributed to stretching vibration of O-H / N-H functionality and broad area indicating the intermolecular hydrogen bonding. The peaks at 2924 cm⁻¹ is due to saturated C-H stretching vibration. The presence of peaks at 1619.3 and 1383.9 cm⁻¹ region might be due to aromatic C=C stretching vibration. These peaks mainly belongs to the flavonoids and terpenoids in the plant extracts and is responsible for reduction of silver ions to silver nanoparticles by the plant extracts (Banerjee *et al.*, 2014; Khader *et al.*, 2018a), hence Rothan date seed extracts act as capping agent to produce silver nanoparticles. According to Haghghi Pak *et al.* (2016) both FTIR spectra show a shift in peaks: 1401–1396 (due to C–C in-ring stretching of aromatic amines) and in this investigation the peak was observed at 1619.3 and 1383.9 cm⁻¹, it conforms the presence of aromatic amines.

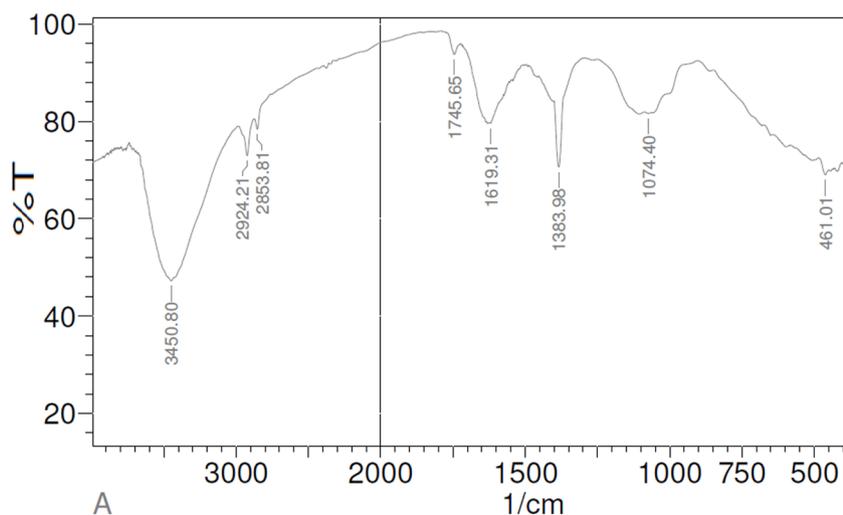


FIGURE 1 - Fourier transform infrared (FTIR) spectra of AgNPs synthesized from Rothan date seed extract.

X-ray diffraction measurements

XRD is a prompt investigative technique predominantly used for phase identification of a crystalline substance that can provide data on unit cell dimensions. The analysed material is finely ground, homogenised, and bulk composition is determined. The XRD pattern of silver nanoparticles of Rothan seed extract is illustrated in Figure 2. Three diffraction peaks as (111), (200), (220), and (222) were observed in the 2θ range of 10–90, indicated expressions of face centered cubic (fcc) structure metallic Ag, representing as a pure crystalline Ag (Khader *et al.*, 2018a). The AgNPs was formed by the reduction of Ag^+ ions by Rothan date seeds exudate are crystalline in nature which were confirmed by XRD pattern.

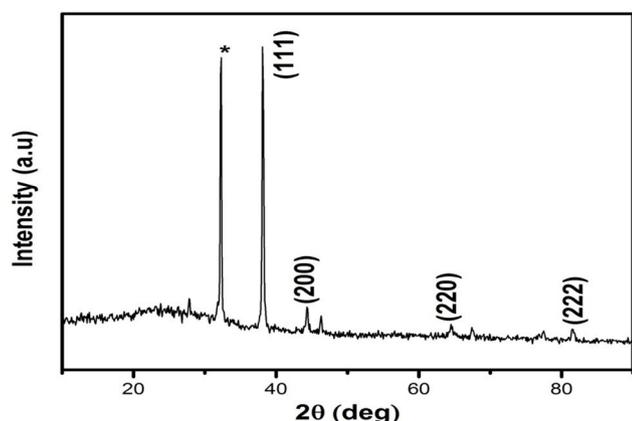


FIGURE 2 - XRD pattern of AgNPs synthesized from Rothan date seed extract.

High resolution-transmission electron microscopy interpretation

HR-TEM is an imaging mode of the transmission electron microscope that permits direct imaging of the atomic structure of the sample. HR-TEM is an instrument used to study the selected area electron diffraction (SAED) pattern (Figure 3e) which is in accordance with XRD studies. The resulted images were examined to provide insight on the morphological characteristics present in silver nanoparticles of

Rothan date seed extract. The size and morphology of synthesized AgNPs with Rothan date seeds were determined by TEM images, as shown in Figure 3(a-d). The morphology of AgNPs is almost spherical, and exhibited high surface areas with average size of $42 \pm 9 \text{ nm}$. The particles are arranged in poly dispersity based on particle size, functionalized with biomolecules having primary amine group, carbonyl group, hydroxyl groups and other stabilizing functional groups as shown by FTIR spectroscopic technique which is in line with Shankar *et al.*, (2004).

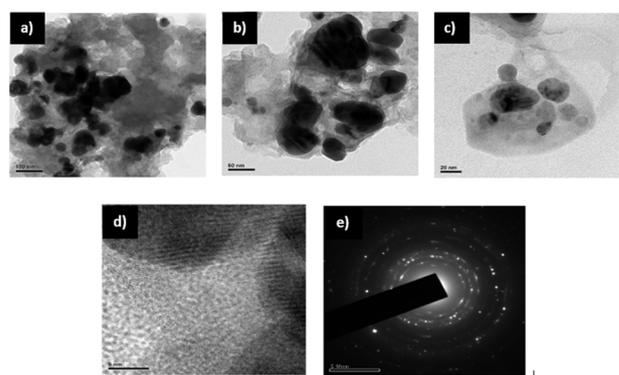


FIGURE 3 - (a-d). HRTEM images and (e) SAED pattern of synthesized AgNPs with Rothan date seed extract in different magnifications.

Energy dispersive X-ray spectroscopy results

Energy dispersive X-ray spectroscopy is an analytical technique used for the elemental analysis of a sample. Its characterisation abilities are due to the important principle that each element has a unique atomic structure allowing a unique set of peaks on its electromagnetic spectrum. The presence of elemental silver in Rothan date seed extract was confirmed by EDX analysis where optical absorption peak was observed approximately at 2.95kV. This is the typical value for the absorption of metallic silver nano-crystallites due to surface plasmon resonance and the amount of silver being 21.68%. As shown in Figure 4, the EDX results indicate that Ag is the only major element present in as-prepared sample. The insignificant amounts of observed Mg, Si, S and Ca are attributed to the plant biomass attached to the AgNPs.

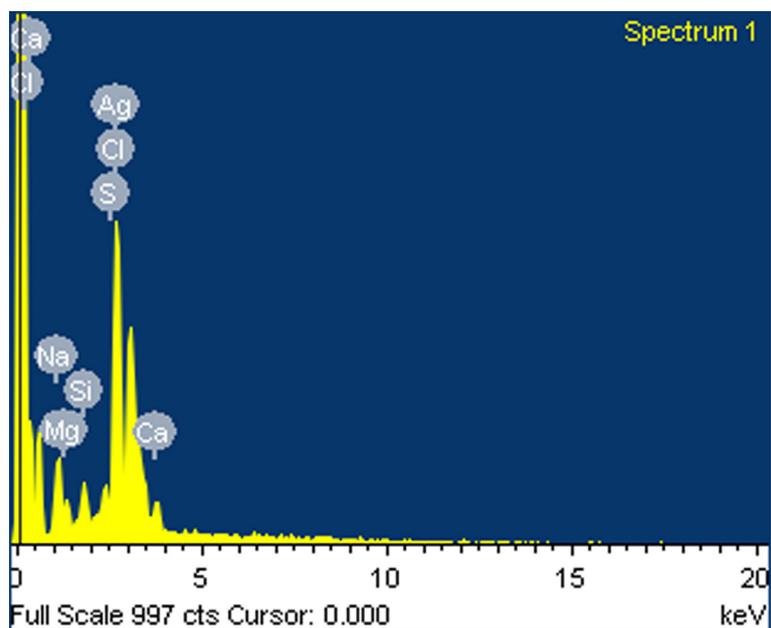


FIGURE 4 - EDX spectrum of AgNPs synthesized from Rothan date seed extract.

Zeta potential measurements of synthesized AgNPs

The most important characterizations of the silver nanoparticles are the Particle size, size distribution and zeta potential because they oversee the other characterizations, such as saturation solubility and dissolution velocity, physical stability, or even biological performances. The zeta potential and hydrodynamic diameter of synthesized silver nanoparticles from Rothan date seed extract were

determined by DLS is shown in Figure 5 (a, b). The measured zeta value (ζ) was found to be -26.5mv and it showed long term stability with good colloidal nature and high dispersity index of the synthesized silver nanoparticles. The hydrodynamic diameter of the synthesized nanoparticles was 203nm and it is observed that the particles obtained are polydisperse mixture (Roy *et al.*, 2019).

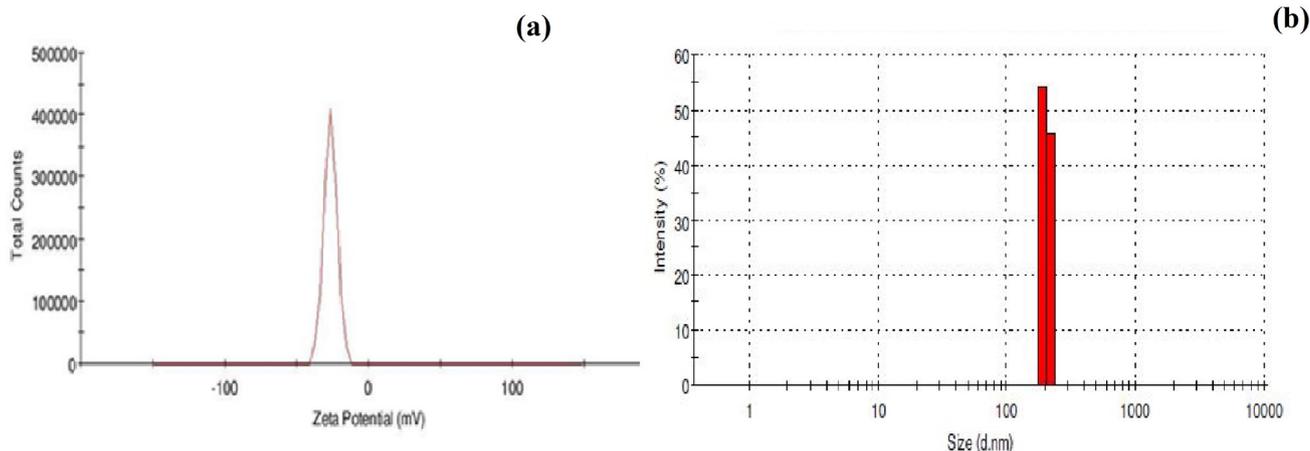


FIGURE 5 - (a, b). Zeta potential and hydrodynamic diameter of synthesized silver nanoparticles from Rothan date seed extract.

Effect of *in vitro* radical scavenging activity of AgNPs

Radical scavenging activity of AgNPs was assessed using DPPH, FRAP and OH radical scavenging activity (Figure 6) and the results are interpreted in % inhibition. From the performed DPPH assay it was found that AgNPs extract was capable of neutralizing the DPPH free radicals via hydrogen donating activity at different levels. The scavenging activity was dose dependent as the concentration of AgNPs extracts increases scavenging ability improves. Minimum scavenging activity was found at the concentration of 2mg with 22% inhibition and the maximum inhibition was observed at concentration 10mg with 64% inhibition. The reducing ability (FRAP)

of AgNPs is represented in Figure 6, demonstrating dose dependent activity at higher concentration with 79.2%. Similarly, the results of hydroxyl radical scavenging activity demonstrates that AgNPs synthesised from dates extract exhibited maximum activity (78.7%) at 10mg concentration (Figure 6). Many research has stated that various medicinal plants and their products has confirmed to be a potential to radical scavenger and has the ability to combat against various diseases (Khader *et al.*, 2018b; Syed *et al.*, 2019b; Sidhra *et al.*, 2017). DPPH, FRAP and OH radicals are frequently used in research to identify the scavenging potential, in the present study the radicals produced are scavenged by the synthesized AgNPs from Rothan date seeds by donating electrons and making it a more stable compound.

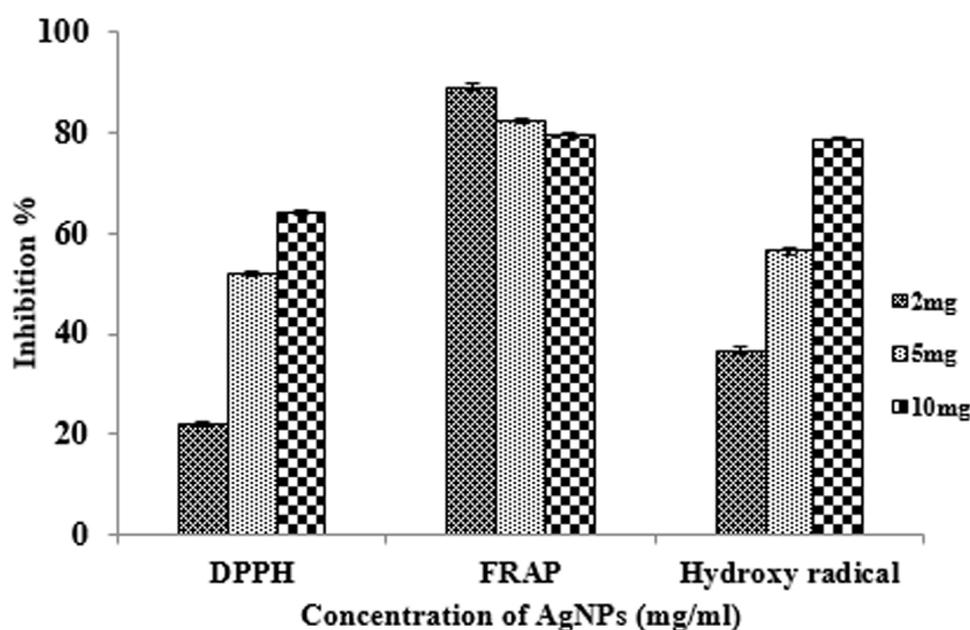


FIGURE 6 - Radical scavenging activity of synthesized silver nanoparticles using Rothan date seed extract.

Anti-inflammatory and antiarthritic activity on synthesized AgNPs

Research has proved that inflammation is the primary physiological defense mechanism against different stimuli and proteins denaturation is the primary cause for the onset of inflammation and arthritic diseases (Khader *et al.*, 2018b). The inflammatory response is due to the cellular activity caused by action of lysosomal

enzymes to an external stimuli. Figure 7 depicts the percentage inhibition of synthesized silver nanoparticles against membrane protein denaturation considerably reducing the effect of inflammation gradually above 250 $\mu\text{g/mL}$ concentration. At 500 $\mu\text{g/mL}$ concentration the percentage inhibition was found to be 63.32%, and the IC_{50} value was above 100 $\mu\text{g/mL}$ exhibiting good anti-inflammatory activity. The study of Kehili *et al.*, (2016) represented anti-inflammatory activity using date

fruits and seeds, our results prove AgNPs synthesised from Rothan date seeds is efficient against inflammation by membrane stabilisation and can be better cure after further exploration using animal model.

The results of the antiarthritic study reveals that percentage inhibition of synthesized silver nanoparticles increases with increase in concentration and the higher percentage inhibition was found to be 68.44% at 500 µg/mL concentration (Figure 8). Hence, proving that the

synthesized AgNPs inhibits protein denaturation there by inhibiting auto antigen production the main cause for the onset of inflammation leading to arthritis. The IC₅₀ value was above 100 µg/mL and the silver nanoparticles exhibited good anti-arthritis activity. As in the previous study of Shrahan kumar *et al.* (2011) reported anti-arthritis activity of leaves of *Physalis angulata*. L. was 53.8%. Similarly, synthesized silver nanoparticles has higher potential to be a remedy against arthritis.

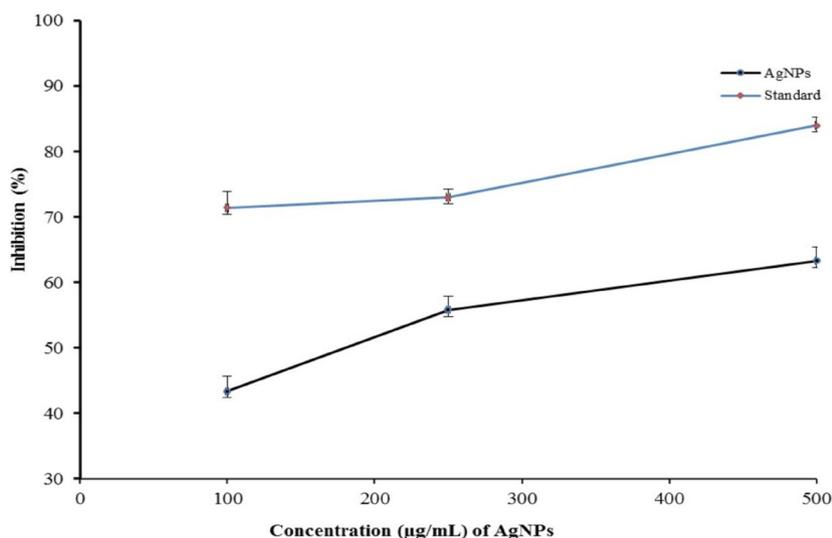


FIGURE 7 - Anti-inflammatory activity of synthesized silver nanoparticles using Rothan date seed extract.

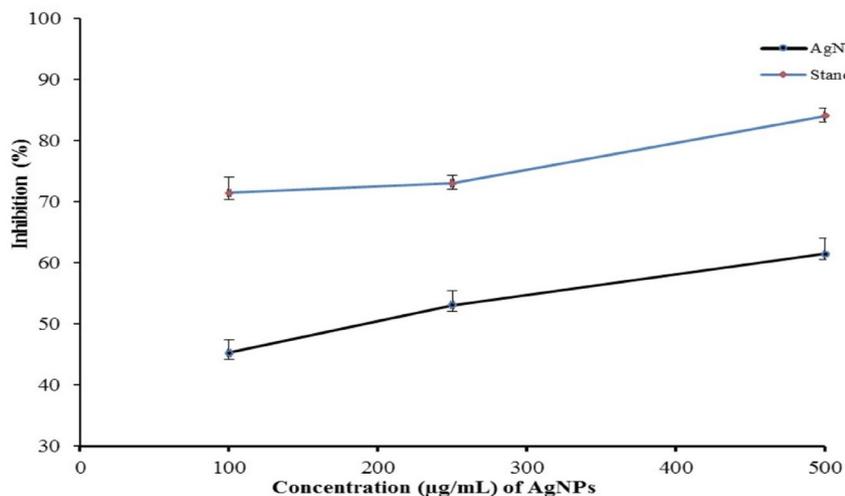


FIGURE 8 - Antiarthritic activity of synthesized silver nanoparticles using Rothan date seed extract.

Anticancer activity of synthesized AgNPs

Cancer causes mortality and morbidity with a major health problem worldwide (Arunachalam *et al.*, 2020; Khader *et al.*, 2020). Therapeutic demand is increasing for cancer and Nanomaterials is an expected hope for diagnosis of cancer and therapy (Rosarin *et al.*, 2013). Anticancer activity of green synthesized silver nanoparticles using Rothan date seed extract was analysed using lung (A549) adeno carcinoma cell lines. Cytotoxic analysis was performed from lower to higher concentration. Inhibiting concentration (IC_{50}) and morphological changes was observed at 100 $\mu\text{g}/\text{mL}$ and the results revealed that green synthesized silver nanoparticles has cytotoxicity effect in lung

cancer cell line Figure 9. At 50 $\mu\text{g}/\text{mL}$ concentration, synthesized AgNPs exhibited 77% of cell growth inhibition (Figure 10). The cell growth inhibition was 59% when treated with 100 $\mu\text{g}/\text{mL}$ concentration of synthesized silver nanoparticles with an IC_{50} value of 59.66 $\mu\text{g}/\text{mL}$. The results of the present study reveals that green synthesized silver nanoparticles with Rothan date seed extract produced significant cytotoxic effect against human lung cancer line and are in agreement with the previous studies with AgNPs used plant extracts tested against cancer cell lines (Venugopal *et al.*, 2017). The cytotoxicity effect of AgNPs might be due to the stimulation of reactive oxygen species leading to disruption of cellular components of cancer cell and cell death (Dhand *et al.*, 2016).

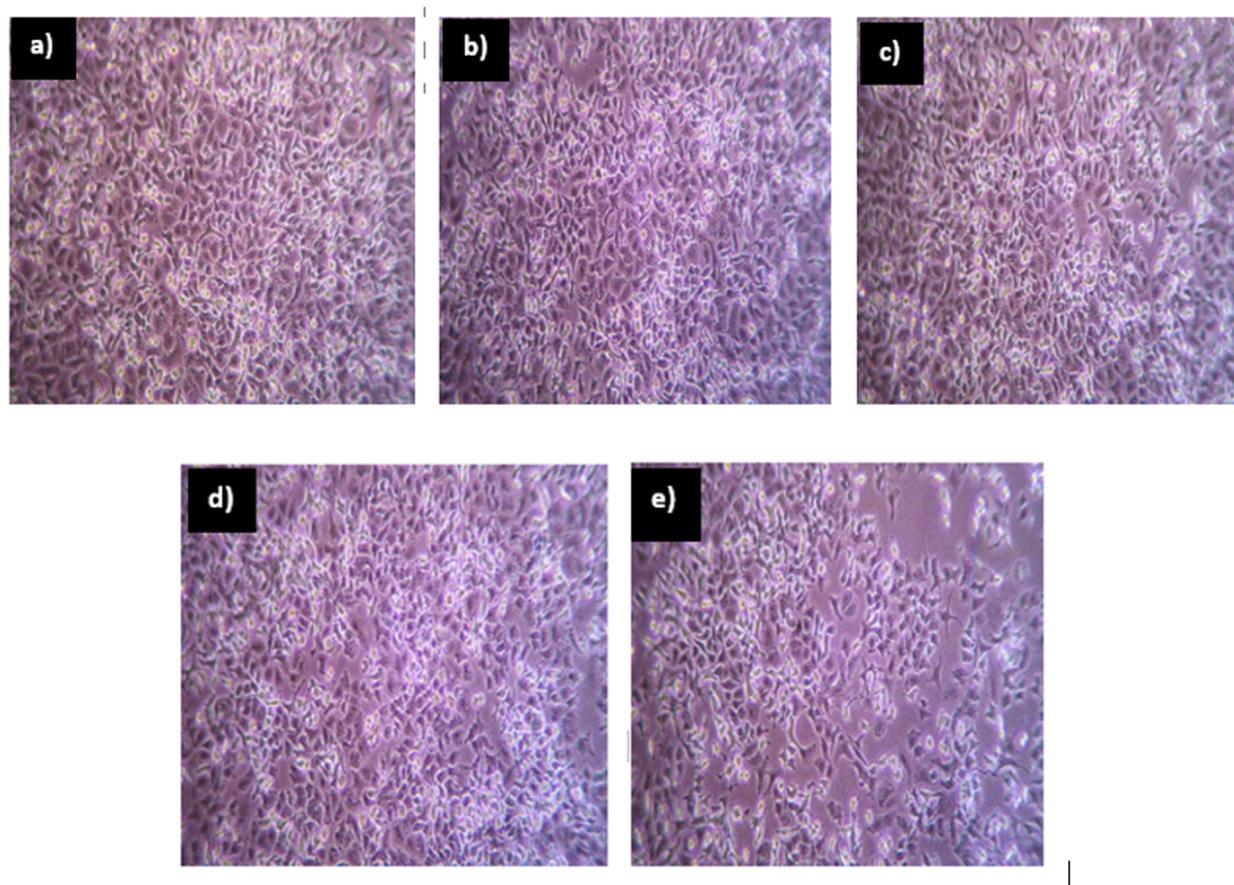


FIGURE 9 - The anticancer activity of lung cancer cell line images a) 6.25 $\mu\text{g}/\text{mL}$, b) 12.5 $\mu\text{g}/\text{mL}$, c) 25 $\mu\text{g}/\text{mL}$, d) 50 $\mu\text{g}/\text{mL}$ and e) 100 $\mu\text{g}/\text{mL}$ of synthesized silver nanoparticles from Rothan date seed extract.

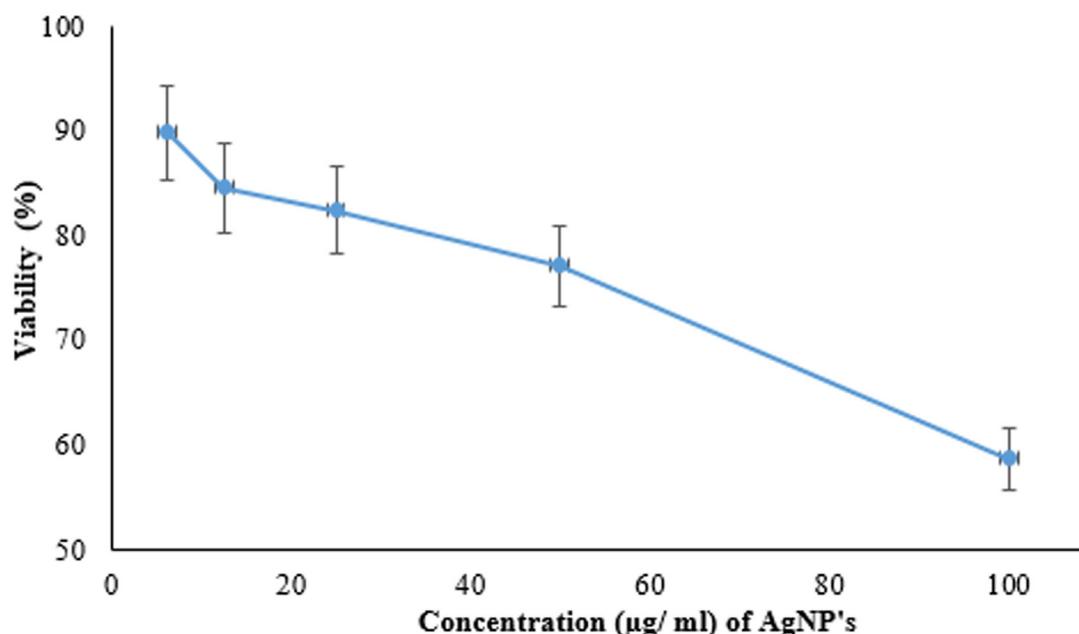


FIGURE 10 - The percentage of cell viability of synthesized silver nanoparticles from Rothan date seed extract.

CONCLUSION

The present study reveals the traditional usage of date fruits and its seeds as a better remedy for various ailment. A novel method is used to synthesise AgNPs from *Phoenix dactylifera* (Rothan) date seed extract and characterised using spectral data. The functional group present in the AgNPs were indentified using FTIR, HR-TEM and DLS represented the size, shape and distribution of AgNPs, crystallinity of AgNPs was examined using XRD. *In-vitro* analysis of silver nanoparticles over arthritis and inflammation represents a better cure by inhibiting protein denaturation the major cause for the onset of inflammation leading to arthritis. The cytotoxic studies using lung (A549) cancer cell line revealed the anti-proliferative property of AgNPs from Rothan date seed extract illustrated a potent cytotoxic effect. Hence proving the verdicts from Holy Quran that dates has a potential contribution in dietary supplement and as a medicine.

CONFLICT OF INTERESTS

The authors declare no conflict of interests.

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