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Synthesis and Characterization of Biogenic Silver Nanoparticles using Medicinally important *Nigella sativa* L. (black Cumin) and their Antioxidant, Anti-inflammatory, and DNA Cleavage Potentials

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ABSTRACT

Introduction: The application of nanotechnology in herbal medicine offers promising prospects for drug delivery by enabling targeted, controlled, and efficient delivery of medicinal ingredients, potentially minimizing side effects and improving treatment outcomes. *Nigella sativa* L. (black Cumin) seed infusions are useful in Asian and African ethnomedicines in remedying stress and inflammatory-related ailments. **Objective:** On this premise, black Cumin-based silver nanoparticles (BC-Ag NP) were developed and evaluated for their biological potential. **Materials and Methods:** Silver nanoparticles (AgNPs) were green synthesized using the seed aqueous extract of black cumin (BC). The BC-AgNPs were characterized using scanning electron microscopy (SEM), field emission scanning electron microscopy (FESEM), high-resolution transmission electron microscopy analysis (HRTEM), energy-dispersive X-ray spectroscopy (EDX), and X-ray diffraction (XRD). The biological potential of the NPs was based on NO, H_2O_2, $^{\bullet}OH$, and $O_2^{\bullet-}$ radical scavenging antioxidant, egg albumin denaturation (anti-inflammatory), and DNA cleavage assay methods. **Results:** The SEM and FESEM revealed spherical-to-cubical-shaped ultrafine BC-Ag NPs with a size of less than 100 nm. The HR-TEM micrograph confirmed each NP to be spherical in shape and within the 10-50 nm range. The X-ray diffractogram showed the crystallinity of the NPs with a sharp peak at 38.12° [reflection index (111)] at an average size of 47 nm. The transformation of metallic silver into elemental silver was validated by EDX analysis, with 97.58% elemental Ag at ~3 keV acute curve. The BC-Ag NPs showed dose-dependent antioxidant activity, with IC_{50} of 87.56 ± 1.54 and 110.5 ± 2.27 µg/mL against H_2O_2 and O_2^- free radicals, respectively. The anti-inflammatory activity of the NPs was one-third the potency of Diclofenac sodium (standard drug) at IC_{50} of 103.44 ± 5.35 µg/mL. Finally, the BC-Ag NPs acted as chemical nucleases to cleave DNA at a 20 mg/mL concentration for 120 minutes. **Conclusion:** This study has shown that AgNPs biosynthesized with black Cumin seed extract possess notable antioxidant, anti-inflammatory, and DNA cleavage properties and, thus, may be a useful nanomaterial for efficient pharmaceutical delivery.

Keywords: *Nigella sativa*, silver nanoparticles, antioxidant, anti-inflammatory, DNA cleavage.

INTRODUCTION

Nanotechnology is one of the most active areas of material science research. In the last few decades, material scientists have studied the various applications of nanoparticles (NPs) and nanostructured materials in the biological and healthcare industries.[1] The nanoparticles' new or enhanced properties can be influenced by their size, shape, and distribution. Nanotechnology has made significant strides in recent years, and several methods have been developed to produce precisely sized and structured nanoparticles that meet certain requirements.[2] Nanomaterials and nanoparticles are finding more and more uses every day. Because of their small size, nanoparticles differ greatly from the bulk form of the same substance, opening new possibilities for biosensors, biomedicine, and bionanotechnology.[3] Numerous physical and chemical techniques, including thermal breakdown, chemical reduction, laser ablation, ultrasonication, electrochemically aided synthesis, and others, can yield NPs.[4] Nevertheless, many of these operations are expensive and employ dangerous chemicals that could seriously endanger the environment and human health. Due to their relative safety, affordability, and environmental friendliness, biological approaches have emerged as the synthesis method for creating NPs in recent years.[5] Silver nanoparticles (AgNPs) are the most promising nanomaterials for biological applications in recent years due to their novel antibacterial, antioxidant, anticancer, larvicidal, catalytic, and wound-healing properties.[6] When creating nanoparticles, secondary metabolites from medicinal plants, including sugars, polysaccharides, alkaloids, and flavonoids, have been demonstrated to be quite beneficial.[7] Black Cumin, or black seeds, *Nigella sativa* L. (Family: Ranunculaceae), are also known to be essential oil-bearing plants. In Asian ethnomedicines, the herbal infusions from this plant are used as natural remedies for respiratory ailments, hypertension, diabetes, arthritis, wounds, cancer, fever, headaches, and memory loss.[8] Furthermore, they are used in African ethnomedicine to treat digestive, circulatory, respiratory, reproductive, and dermatology-related ailments.[8,9] A sustainable method for the biosynthesis of silver nanoparticles (AgNPs) using black cumin seed extract (BC) is being developed, considering the plant's immense medicinal value. The BC-Ag NPs were characterized using scanning electron microscopy (SEM), field

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emission scanning electron microscopy (FESEM), energy-dispersive X-ray spectroscopy (EDX), X-ray diffraction (XRD), and high-resolution transmission electron microscopy analysis (HRTEM). Furthermore, the biological potential was assessed based on in vitro antioxidant, anti-inflammatory, and DNA cleavage effects.

MATERIALS AND METHODS

Plant material

The seeds of black Cumin (*Nigella sativa*) were purchased from the traditional herbal market in Ile-Ife, Osun State, Nigeria (latitude 7°33′00″N and longitude 4°33′00″E) under the registered name of RC: 875523. The plant list catalog (http://www.theplantlist.org) confirmed the plant as kew-2381679. The dried seeds were powdered and kept in an airtight plastic bag for further use.

Synthesis of nanoparticles from silver nitrate ($AgNO_3$) using black Cumin seed extract

Silver nanoparticles (AgNPs) were synthesized by combining $AgNO_3$ [1.575 g (0.103 M) diluted in 90 mL of distilled water] with black cumin seed extract (30 mg dissolved in 10 mL of distilled water). The solution turned from colourless to greyish brown after boiling it at 80 °C for 6-8 h while being stirred magnetically. The solution was centrifuged at 6000 rpm for 20 min under room temperature, affording black cumin aqueous extract-based silver nanoparticles (BC-Ag NPs). The samples were stored at 4 °C after air drying.[10-12]

Scanning electron microscopy (SEM) and field emission scanning electron microscope (FESEM)

The powdered samples were placed on copper mesh for SEM and FESEM investigation, and a gold sputtering apparatus was applied with a 3 nm gold covering. SEM (JEOLJSM-IT100InTouchScopeTM, Tokyo, Japan) and FESEM (German manufacturer Carl Zeiss; model: SIGMA-0261) were used to record the surface morphology of the AgNPs at a 5 kV accelerating voltage at 12,000× and 100,000 × magnifications.

Energy-dispersive X-ray spectroscopy (EDX)

To determine the presence of elemental Ag, energy dispersive Xray spectroscopy (EDX) was done using a Scanning Electron Microscope (JEOLJSM-IT100InTouchScopeTM, Tokyo, Japan) equipped with Oxford-EDX software. The powdered dried nanoparticles were mounted on copper mesh, and a 3 nm gold coating was done using a gold sputtering unit. Eighty mm^2 SDD detectors that detect elements under high resolution were used.

X-ray diffraction analysis (XRD)

X-ray diffraction was analyzed using an EMPYREAN machine (Make PAN analytical, Netherlands), equipped with CuKα radiation (λ=1.54584Å), operated at 40kv voltage and 30 mA current. The data obtained were compared with the standard JCPDS library to determine the crystalline structure. The average size of the crystalline structure has been calculated using the Debye-Scherrer equation: $D = (0.9\lambda/\beta \cos\theta)$, where D is the diameter of crystallite size, λ is the wavelength for CuKα, β is the full width at half-maximum (FWHM), and θ is the Bragg diffraction.[10]

High-resolution transmission electron microscopy analysis (HRTEM)

A high-resolution transmission electron microscope (model name FEI TECNAI G2 F300) running at an accelerating voltage of 300KV was used to determine the size and shape of biogenically produced AgNPs. The carbon-coated copper stub was covered with a little drop of nanoparticle suspension, allowed to air dry, and then subjected to HR-TEM.[13]

Determination of *in vitro* antioxidant activity

Nitric oxide (NO) radical scavenging assay

The quantification technique of the Griess-Ilosvoy reaction at physiological pH was used to measure the nitric oxide radical scavenging activity with minor adjustments.[14] Briefly, phosphate-buffered saline (pH 7.4), sodium nitroprusside (SNP; 10 mM), and different concentrations of BC-Ag NPs (0-200µg/mL) were mixed and made the final volume of 3 mL. After the mixture was thoroughly vortexed and incubated for 150 min at 25 °C, 0.5 mL of the pre-incubated reaction mixture was mixed with 1 mL of sulfanilamide (0.33%), which was diluted in 20% glacial acetic acid and allowed to sit at room temperature for 5 min. To facilitate colour production, 1 mL of N-(1 -Naphthyl) ethylenediamine dihydrochloride (NEED; 0.1%) was added, and the mixture was kept at 25 °C for 30 min. The absorbance was measured at 540 nm using distilled water as a blank. Curcumin functioned as a standard reference. The percent of inhibition was calculated according to the following equation I:

The equation I: Percentage of scavenging= (A0-A1)/A0 ×100

Where, A0= absorbance of the control and A1= absorbance in the presence of samples and standard.

Hydrogen peroxide scavenging assay

The hydrogen peroxide (H_2O_2) scavenging capacity was calculated using a modified version of Long *et al.*[15]. H_2O_2 (50 mM) different concentrations of BC-Ag NPs (0-200 µg/mL) were combined in a screw-capped bottle, and the mixture was allowed to dark-incubate for 30 min at room temperature (≈25 °C). Next, 90 µL of H_2O_2, 10 µL of HPLC-grade methanol, and 0.9 ml of FOX reagent (made by combining 9 volumes of 4.4 mM BHT in HPLC-grade methanol with 1 volume of 1 mM xylenol orange and 2.56 mM ammonium ferrous sulfate in 0.25 M H_2SO_4) were added. After giving the mixture a gentle vortex and letting it sit for 30 min, the absorbance at 560 nm was determined. Ascorbic acid was utilized as a positive control. The percentage inhibition was calculated as before using equation I.

Hydroxyl radical scavenging assay

Hydroxyl radical scavenging activity was conducted using the Fenton reaction model with a small modification.[16] 2-deoxy-2-ribose (2.8 mM), monopotassium phosphate-potassium hydroxide buffer (KH_2PO_4-KOH; 20 mM; pH 7.4), $FeCl_3$ (100 µM), ethylene diamine tetraacetic acid (EDTA; 100 µM), hydrogen peroxide (H_2O_2; 1.0 mM), ascorbic acid (100 µM), and varying concentrations of BC-Ag NPs (0–200µg/mL) were added to the reaction mixture until a final volume of 1 mL was achieved. After carefully mixing the reaction mixture, it was incubated for 60 min at 37 °C. Following the incubation period, the 0.5 ml mixture was carefully transferred into a fresh tube and mixed with 1 mL each of aqueous thiobarbituric acid (TBA; 1%) and TCA (2.8%). Once more, the finished mixture was incubated for 15 min at 90 °C. The absorbance at 532 nm was measured after the mixture had cooled to room temperature compared to an appropriate blank solution. A positive control was employed as ascorbic acid. The percentage inhibition was calculated as before using equation I.

Superoxide radical scavenging assay

The assay was conducted following Fontana *et al.*[17]. The nitro-blue tetrazolium (NBT) is reduced to purple formazan in the presence of the nonenzymatic PMS/NADH system, which produces superoxide radicals when exposed to oxygen. Phosphate buffer (20 mM, pH 7.4),

NBT (50 µM), PMS (15 µM), NADH (73 µM), and varying doses (0-200 µg/mL) of BC-Ag NPs were combined to create a reaction mixture (1 ml). After gently vortexing the mixtures, they were incubated for five min at room temperature. The amount of formazan produced was estimated by measuring the absorbance at 562 nm compared to the equivalent blank samples. Quercetin served as a positive control. The percentage inhibition was calculated as before using equation I.

In vitro anti-inflammatory study

The *in vitro* anti-inflammatory properties of BC-Ag NPs were determined by their inhibitory effect on protein denaturation using the egg albumin denaturation assay with slight modification.[18] A 0.2 ml of albumin from a fresh chicken egg, 2.8 mL of phosphate buffer saline at pH 6.4, and 2 mL of BC-Ag NPs at 12.5, 25, 50, 100, and 200 µg/mL concentrations were mixed in three replicates. The reaction mixture was incubated at 37 °C for 15 min away from direct light. Then, it was boiled at 70 °C for 5 min in a thermostatic water bath. The resulting mixture was allowed to cool before the absorbance was measured at 655 nm wavelength. Diclofenac was used as the reference anti-inflammatory drug. The percentage inhibitory effect of essential oils on egg albumin denaturation (EAD) was calculated according to the following equation I:

$$\% \text{ Inhibition of EAD} = (ABS_{control} - ABS_{sample})/ABS_{control} \times 100 \quad (I)$$

Where, EAD = egg albumin denaturation, ABS = absorbance

DNA cleavage assay

The ability of BC-Ag NPs to cleave DNA was investigated using agarose gel electrophoresis.[19,20] Plasmid DNA (pBR322) was the target DNA molecule. 20 µL of BC-Ag NPs at 10 mg/mL and 20 mg/mL were reacted with 3 µL of plasmid DNA for two hours at 37 °C. After incubation, 2 µL of loading buffer (pH 7.0) containing 25% bromophenol blue, 30% glycerol, and 0.25% xylene cyanol was added to each tube. Each sample solution was placed onto a 1% (w/v) agarose gel containing 0.5µg/mL of ethidium bromide. A double digest of lambda DNA/EcoRI/HindIII (2µL) was loaded as a molecular marker to determine the molecular size of the surrounding genomic DNA. An electrophoresis power supply unit and a mini-submarine gel electrophoresis apparatus were used to conduct the electrophoresis for two hours at 50 volts in 0.5X TBE buffer (Tris-Borate-EDTA). The gel was photographed and examined under a UV transilluminator after electrophoresis.

Statistical analysis

To compare the activity of BC-Ag NPs and references, Student's t-Test integrated with the KyPlot program (version 5.0) was used for statistical analysis.

RESULTS AND DISCUSSION

Scanning electron microscopy (SEM) and field emission scanning electron microscope (FESEM)

The SEM and FESEM images (Figures 1 A and B) were used to evaluate the size and surface morphology of the biosynthesized BC-Ag NPs, and the results showed a spherical to cubical shape. Most BC-Ag NPs are ultrafine particles with a size of less than 100 nm. Bulking of the NPs occurred due to solvent evaporation or crosslinking during sample preparation.[21]

Energy-dispersive X-ray spectroscopy (EDX)

The transformation of metallic silver into elemental silver was validated by EDX analysis, which revealed the presence of elemental Ag (97.58%) (Figure 2). Despite additional elements including N (0.00%) and P (2.42%), the EDX analysis only showed a substantial, acute curve at~3 keV, which is typical of biosynthesized BC-Ag NPs.[22]

X-ray diffraction analysis (XRD)

The X-ray diffraction (XRD) analysis of the sample showed peaks at 38.10^0, 45.53^0, 64.33^0, and 77.25^0. The peaks (111), (200), (220), and (311), are representative of metallic silver reflections. By comparing its values with those provided by the Joint Committee on Powder Diffraction Standards (JCPDS pdf no: 89-3722), one can identify a sharp peak at 38.12° [reflection index (111)] that supports the crystalline form of AgNPs. Bragg's law indicates that the average size of the nanoparticle is 47 nm, and a comparison of this data with the Debye-Scherrer formula (Figure 3).[22]

High-resolution transmission electron microscopy analysis (HRTEM)

The majority of the biogenically produced AgNPs were spherical and ranged in size from 10 to 50 nm, according to HRTEM examination. Additionally, it was discovered from Figure 4 that the nanoparticles were well distributed and did not accumulate. This outcome agrees with Rajput *et al.*[13]. According to earlier research, bioactivity and nanoparticle size are inversely correlated. Our results confirm that AgNPs are tiny and highly bioactive.[21]

Determination of *in vitro* antioxidant activity

Superoxide anions ($O_2^{\cdot-}$) harm biological macromolecules such as DNA and proteins. The superoxide anion is a less reactive free radical that can produce more reactive species *in vivo* when oxidative stress is prevalent. The concentration-dependent increase in superoxide radical scavenging activity (71.27 ± 1.34 % at 200 µg/mL) was seen when manufactured AgNPs were added to black cumin seed extract (Figure 5A). Quercetin and AgNPs had relative IC_{50} values of 69.71 ± 2.98 and 110.5 ± 2.27 µg/mL, respectively. Due to the inclusion of many phytochemicals, including tannins, flavonoids, and phenolics, the optimized AgNPs produced using seed extract from black cumin demonstrated a strong antioxidant potential, as indicated by the results previously reported. By scavenging free radicals, these substances shield cells from oxidative harm. BC-Ag NPs (76.90 ± 0.86 % at 200 µg/mL) neutralize hydroxyl radicals, protecting cells and assisting in creating a stable cellular environment (Figure 5B).[10] Nitric oxide is an essential signaling molecule. Still, too much of it can damage cells and tissues by producing reactive oxygen species (ROS). Compared to standard, BC-Ag NPs demonstrated dose-dependent nitric oxide scavenging activity (58.55 ± 1.17 % at 200 µg/ml). In living things, nitric oxide and silver nanoparticles work together to prevent excessive nitric oxide accumulation that could damage membranes (Figure 5C). One form of oxygen that is not a radical is hydrogen peroxide. However, it occasionally turns toxic and releases cell-damaging hydroxyl (OH−) radicals.[23] Therefore, the antioxidant defense system depends on its removal. Comparing the synthesized AgNPs to the standard showed a strong dose-dependent H_2O_2 radical scavenging effect (Figure 5D). The IC_{50} values were 22.18 ± 2.68 for ascorbic acid and 87.56 ± 1.54 µg/mL for AgNPs.

Anti-inflammatory activity

Protein denaturation is the process by which proteins lose their biological function and structure due to inflammation caused by heat, stress, or certain chemicals; therefore, it is thought that protein denaturation is an indicator of inflammation.[24] The present study assessed the inhibitory effect of the biosynthesized AgNPs on albumin and protein denaturation and found that these AgNPs significantly prevented albumin denaturation, even at low doses, in comparison to the standard drug, diclofenac sodium salt (Figure 6). The IC_{50} values for AgNPs and Diclofenac sodium salt were 103.44 ± 5.35 and 36.84 ± 4.22 µg/mL, respectively. *A. belladonna*-produced AgNPs inhibited albumin denaturation with an IC_{50} value of 84 µM.[21] The previously reported

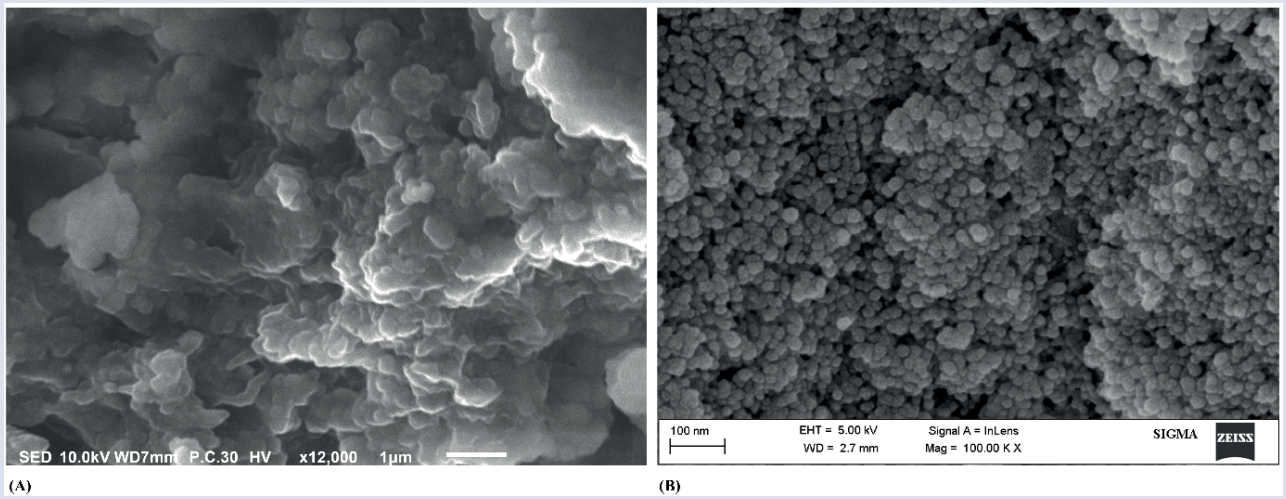

Figure 1. (A) SEM and **(B)** FESEM micrograph analysis of biosynthesized silver nanoparticles.

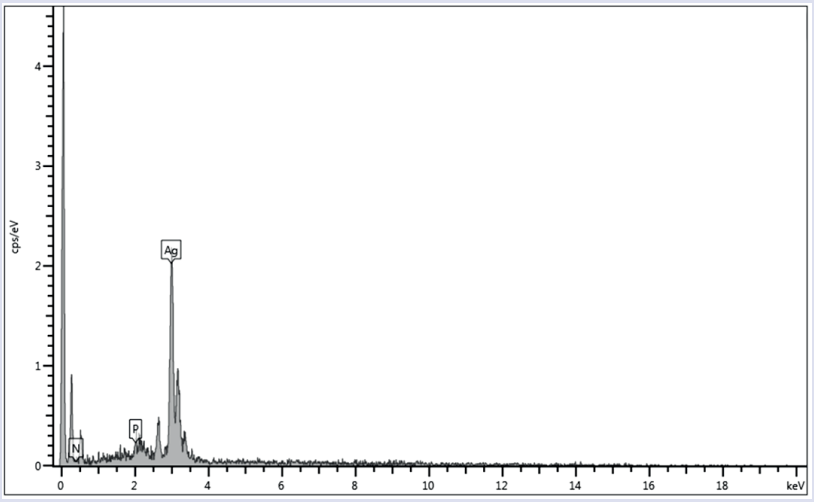

Figure 2. EDX spectra and elemental profile of biosynthesized nanoparticles.

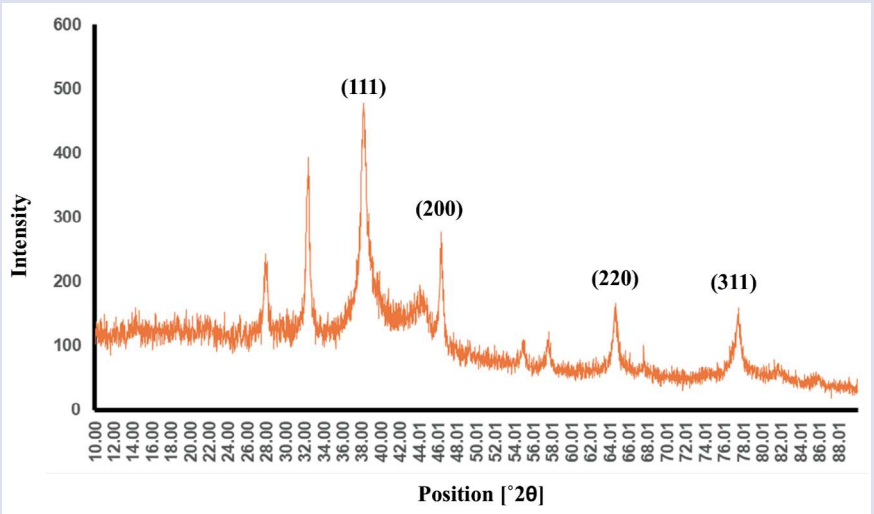

Figure 3. XRD pattern of biosynthesized silver nanoparticles.

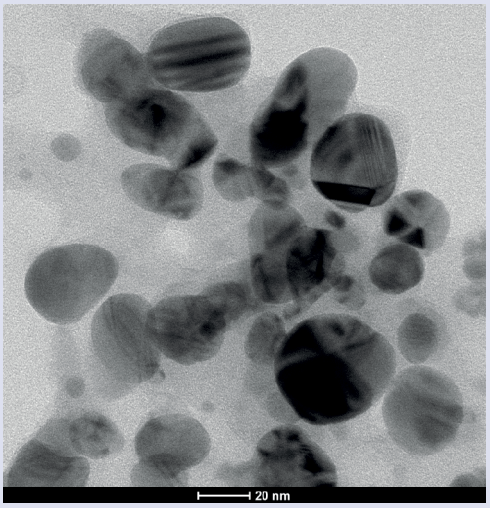

Figure 4. HRTEM micrograph analysis of biosynthesized silver nanoparticles.

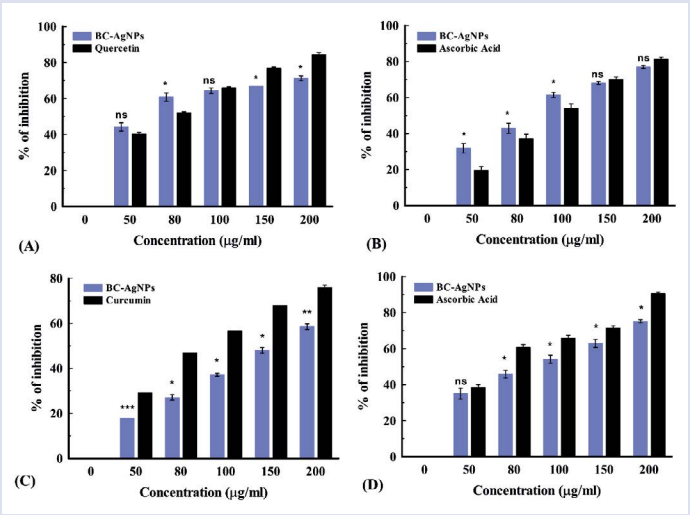

Figure 5. Antioxidant activity of synthesized BC-AgNPs **(A)** Superoxide radical. **(B)** Hydroxyl radical. **(C)** Nitric oxide. **(D)** Hydrogen peroxide scavenging activity. [Data expressed as mean ± S.D. *p<0.05; **p<0.01; ***p<0.001; NS-Nonsignificant when compared with standard].

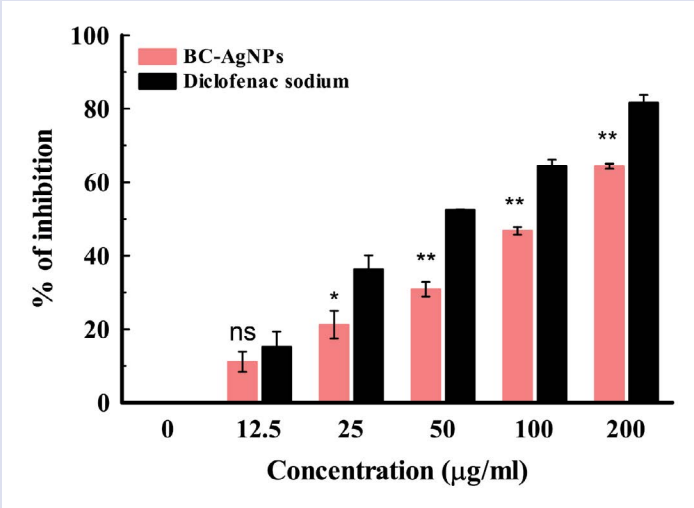

Figure 6. *In vitro* anti-inflammatory activity of the biosynthesized BC-Ag NPs. [Results are given as mean ± S.D. *p<0.05; **p<0.01; NS-Non-significant when compared with standard].

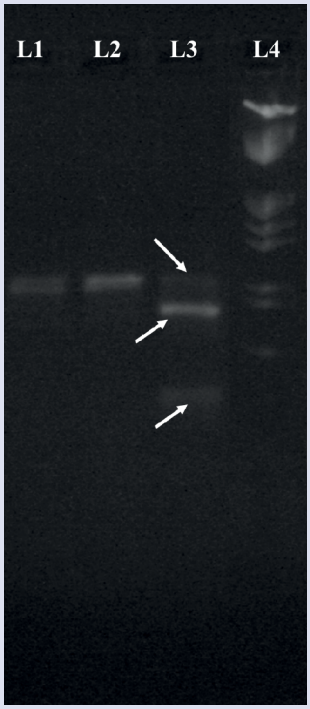

Figure 7: DNA cleavage activity of BC-Ag NPs. **Lane 1**, pBR 322 DNA; **Lane 2**, pBR 322 DNA + 10 mg/mL of BC-Ag NPs (120 mins incubation); **Lane 3,** pBR 322 DNA + 20 mg/mL of BC-Ag NPs (120 mins incubation); **Lane 4**, λ-DNA/EcoRI/HindIII double digest DNA ladder.

results indicate that synthetic AgNP can regulate the production of autoantigens, which denaturize proteins during inflammation.

DNA cleavage assay

The DNA cleavage activity was based on gel electrophoresis. The green-produced BC-Ag NPs cleave more efficiently than the control, which their efficient DNA cleavage capacity can explain.[25,26] The electrophoresis demonstrated how AgNPs interacted with plasmid DNA molecules. The differences between the bands in Lanes 3 and control DNA (Lane 1) are shown in Figure 7. The results showed that green-produced BC-Ag NPs acted as chemical nucleases to cleave DNA at a concentration of 20 mg/mL for 120 minutes. Newly manufactured green BC-Ag NPs may inhibit the growth of cancer cells and pathogenic organisms by cleaving the genome, as evidenced by the green creation of BC-Ag NPs that cleave DNA. Further, *in-vitro* and *in-vivo* studies are necessary to determine the toxicological effects of green-produced BC-Ag NPs.

CONCLUSION

The study obtained green synthesized AgNPs using an aqueous extract of black Cumin seed (BC). The BC-Ag NPs were characterized as spherical-shaped nano-sized (10-50 nm) particles with 97.58% elemental Ag. The biogenic NPs showed promising biological properties based on NO, H_2O_2, $^{\bullet}OH$, and $O_2^{\bullet-}$ free radicals' inhibition, ability to attenuate egg albumin denaturation, and DNA cleavage activity, thus may be a useful nanomaterial for efficient pharmaceutical delivery.

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

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