

Biogenic Zinc oxide nanoparticles from *Celosia argentea* : Toward improved antioxidant, antibacterial and anticancer activities

Rana Abdullah Alghamdi

King Abdulaziz University

Maryam Hassan Al-Zahrani

King Abdulaziz University

Lamaia R Altarjami

King Abdulaziz University

Waleed Al Abdulmonem

Qassim University

Nadia Samir

South Valley University

Alaa Said

South Valley University

W. S. Mohamed

Jouf University

Mohammed Ezzeldien (✉ Meabas@ju.edu.sa)

Jouf University

Article

Keywords: *Celosia argentea*, ZnO NPs, antioxidant, antibacterial, anticancer

Posted Date: June 9th, 2023

DOI: <https://doi.org/10.21203/rs.3.rs-3009513/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Additional Declarations: No competing interests reported.

Biogenic Zinc oxide nanoparticles from *Celosia argentea*: toward improved antioxidant, antibacterial and anticancer activities

Rana Abdullah Alghamdi¹, Maryam Hassan Al-Zahrani²

Lamaia R Altarjami¹, Waleed Al Abdulmonem³, Nadia Samir⁴, Alaa Said⁵, W. S.

Mohamed^{6,7}, Mohammed Ezzeldien^{6,8,*}

¹Department of Chemistry, College of Sciences & Arts, King Abdulaziz University, Rabigh, Saudi Arabia

²Biochemistry Department, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia.

³Department of Pathology, College of Medicine, Qassim University, P.O. Box 6655, Buraidah 51452, Kingdom of Saudi Arabia

⁴Zoology Department, Faculty of Science, South Valley University, Qena, Egypt

⁵Electronic and Nano Devices Lab, Faculty of Science, South Valley University, Qena, Egypt

⁶Physics Department, College of Science, Jouf University, Al-Jouf, Sakaka, P.O. Box 2014, Sakaka, Saudi Arabi

⁷Physics Department, Faculty of Science, Sohag University, 82524 Sohag, Egypt

⁸Metallurgy and Material Science Tests Lab., Physics Department, Faculty of Science, South Valley University, Qena, 83523, Egypt

*Corresponding author: Dr, Mohammed Ezzeldien, Meabas@ju.edu.sa

ABSTRACT

In this work, biogenic ZnO nanoparticles (NPs) were greenly synthesized from *Celosia argentea* plant extract. Structural analysis confirms the successful synthesis of biogenic Zinc Oxide NPs from *C. argentea* extract and the produced NPs have average particle size 21.55 ± 4.73 nm with a semispherical shape and specific surface area about 50 m²/g. The biogenic synthesized ZnO NPs showed a powerful radical scavenging activity (Ic₅₀=91.24 µg/ml) comparing with ascorbic acid (ASC) as standard (Ic₅₀=14.37 µg/ml). Using an agar diffusion disk, the antibacterial efficacy was tested against both +1 gm and -1 gm bacteria. In gram positive strains, biogenic ZnO NPs had a greater bactericidal impact than gram negative strains in a concentration dependent manner. Anticancer activity against HepG2 was evaluated using MTT assay, the results reflect the concentration dependent cytotoxic effect of biogenic ZnO NPs against HepG2 cells even at low concentrations with Ic₅₀=49.45 µg/ml. Using RT-PCR and flowcytometry, the cell cycle and gene expression were analyzed to determine the potential anticancer mechanism. The results showed that biogenic ZnO NPs activate the apoptotic genes NF-κB, CY-C and P53, which start the apoptotic process. These results suggested that *C. argentea* improved the antioxidant, antibacterial and anticancer activities of ZnO NPs without altering its structural properties, which make it a potential candidate in many of the biomedical applications.

Keywords: *Celosia argentea*, ZnO NPs , antioxidant, antibacterial, anticancer.

Introduction

Zinc oxide NPs is a very distinguished semiconductor in the biomedical field. The antibacterial, anti-inflammatory antifungal antiviral and anticancer activities of ZnO NPs had been reported by many researchers ¹⁻³ . According to the literature, the cytotoxicity of ZnO NPs is mostly determined by the formation of reactive oxygen species (ROS). One of the most amazing properties of ZnO NPs is their selectivity towards cancer cells. ZnO NPs were discovered to be more selective towards proliferative cells than non-proliferative cells ⁴⁻⁶. Particle size, shape, and surface area are all physiochemical features of NPs that can affect their therapeutic efficacy. The manufacturing technique of NPs, which may be broken down into physical, chemical, and biological categories, has a significant impact on tuning these characteristics ^{7,8}. Biological methods as an eco-friendly methods attract the attention researchers due to its advantages compared with the other methods including low cost, less toxicity and design flexibility ⁹. Biosynthesis of NPs include the use of biomolecules such as microorganism and plant extracts as reducing and capping agent that facilities the formation of NPs ¹⁰. These biomolecules control particle size, shape and functionalized the surface of NPs and enhance their biocompatibility and bioactivity ¹¹.

The future of green synthesis of NPs is expanded nowadays to include several microorganisms including viruses , bacteria, algae and yeast biosynthesis is available also form and plant extracts ^{12,13}. Plant extract is the easy, scalable, flexible and ecofriendly method ¹⁴. This technique provide NPs functionalized with different phytochemicals, which have can enhance the biocompatibility and bioactivity of NPs to serve efficiently in many of biomedical applications ¹².

Because it contains biomolecules such carbohydrates, lipids, amino acids, peptides, phenols, flavonoids, terpenes, and alkaloids, the *C. argentea* plant, also known as cockscomb, has hepatoprotective, antioxidant, anticancer, and antidiabetic effects ^{15,16}.

Several ailments, including jaundice, inflammation, wound healing, eye diseases, and glandular swellings, had long been treated with *C. argentea* in traditional medicine. Numerous studies on *C. argentea*'s anticancer mechanism have shown evidence that it causes cells to commit suicide through apoptosis ¹⁷⁻¹⁹.

The presented study devoted to highlight the influence of green synthesis of ZnO NPs from *C. argentea* extract on the physiochemical, antioxidant, antibacterial and anticancer activities of ZnO NPs .

1. Materials and methods

2.1 Synthesis of *C. argentea* leaves extract

The leaves of *C. argentea* were obtained from the South Valley University farm and rinsed with deionized water. The washed and dried leaves were cut into little pieces. 20 g of *C. argentea* were combined with 200 mL of deionized water and heated for 30 minutes at 80 °C. After cooling to ambient temperature, the mixture was filtered, collected, and stored in a refrigerator at 4°C for future experiments.

2.2 Green synthesis of ZnO NPs

In accordance with the coprecipitation approach, which was used for the synthesis of biogenic ZnO NPs, two solutions were initially produced. The precursor solution and the reduction solution. In a typical synthesis, 5 g of Zinc Acetate [$\text{Zn}(\text{CH}_3\text{CO}_2)_2$] is dissolved in 100 ml deionized water and stirred for 30 minutes. 10 ml of fresh *C. argentea* extract was added to the precursor solution and stirred for 1 hour. Sodium hydroxide and hydrochloric acid were used to adjust the pH of the mixture to 10.4 and the mixture was left under stirring for another 1 h. After precipitation overnight in room temperature NPs were filtered and washed 10 times with distilled water and one more time with ethanol and dried at 100 °C, **fig. 1**.

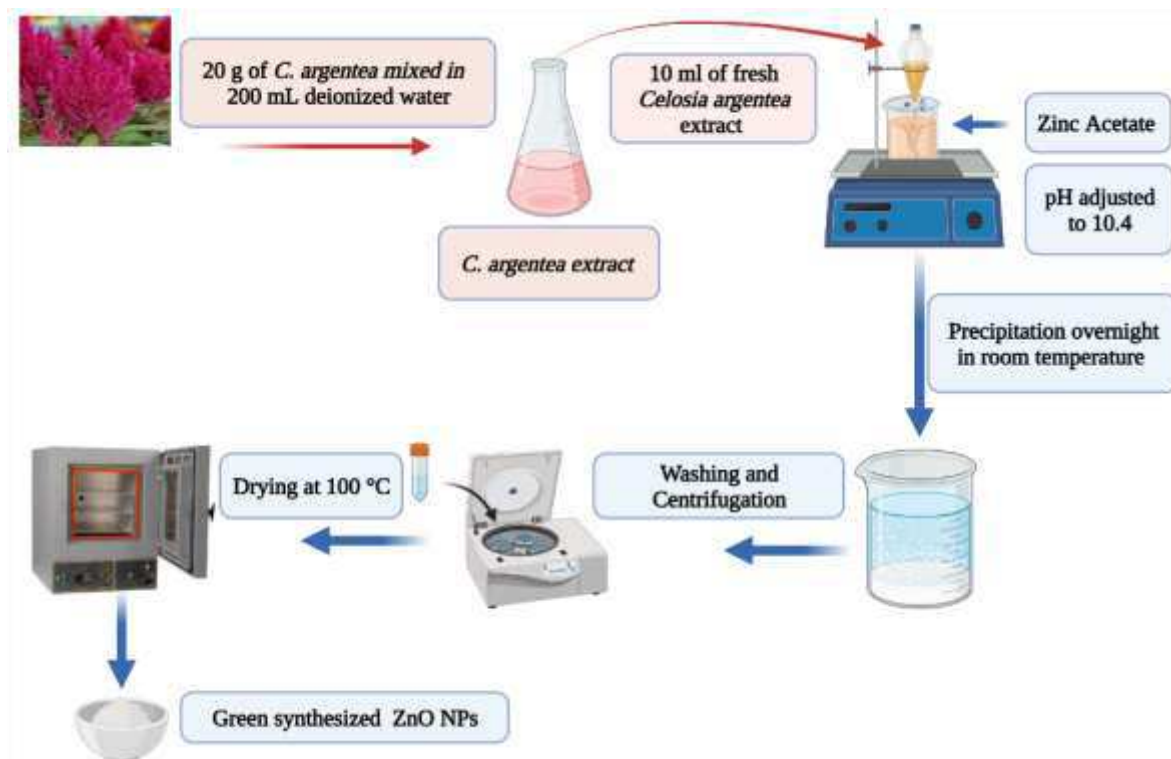


Figure 1. Green synthesis of ZnO NPs

2.3 Characterization of biogenic ZnO NPs

X-ray diffraction (X'Pert PRO-PAN,Cu-K = 1.54056 Å) at 40 kV and 30 mA and transmission electron microscope (JEOL, JEM 2100, Japan) were used to examine the structural properties of biogenic ZnO NPs. Infrared spectroscopy (FTIR Model 6100, Jasco-Japan) and UV-visible spectrophotometry (SPECORD 200 PLUS, Analytik Jena, Germany) were used to measure optical characteristics.

2.4 Antioxidant properties of biogenic ZnO NPs

Using a standardized (DPPH) assay, the free radical scavenging activity of biogenic ZnO NPs, *C. argentea* extract, and ascorbic acid was determined ^{20,21}.

The stock solution of green ZnO NPs sample was generated first by dissolving 4 g in 4 ml methanol, followed by eight serial dilutions (1000, 500, 250, 125, 62.5, 31.25, 15.625, and 7.8125 µg/ml). Second, 3 ml of DPPH was added to each test tube and incubated in the dark for 30 minutes at room temperature. Finally, at 517 nm, the absorbance (A) of the control and samples was measured spectrophotometrically.

The scavenging activity against DPPH free radicals was determined for each sample and reported as a percentage relative to a standard of Ref. ²⁰:

$$\text{Free radical scavenging activity(\%)} = \left(\frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \right) \times 100$$

The IC₅₀ was plotted as a function of concentration from a graph of free radical scavenging activity.

2.5 Antibacterial activity of biogenic ZnO NPs

Agar diffusion disks described by Mounyr Balouiri *et.al* ²² were used to test for the activity of the antibacterial against different bacterial strains: +2 gm [*Staphylococcus aureus* (ATCC 29213), *Bacillus subtilis* (ATCC 6633)] and – 2 gm [*E. coli* (ATCC 25922) and *Salmonella typhimurium* (ATCC14028)]. Briefly, five samples were prepared in normal saline (0.9 % NaCl). Three samples from green ZnO NPs with (25, 50 and 100 µg/ml), one sample from *C. argentea* extract with concentration (20 µg/ml) and one positive control sample from Gentamycin with concentration (20 µg/ml). bacterial strains were cluttered in (Luria–Bertani) LB broth Overnight in order to obtain tested bacterial suspensions. Sterile Whatman filter paper discs were placed on the surface of the agar media, then proper volume containing the tested concentration of each sample was added. After incubating the plates for 24 h at 37 degrees

Celsius, the diameters of the inhibitory zones were measured in millimeters. Each experiment was carried out three times to ensure accuracy.

2.6 Anticancer activity of biogenic ZnO NPs

2.6.1 Cell viability measurement

Cell viability of Liver hepatocellular cells (HepG2) was screened against biogenic ZnO NPs at different concentrations using standardized MTT assay ²¹. Briefly, HepG2 (10.000 cells/well in 96 well plate) were grown in complete DMEM culture media containing 10 % heat inactivated fetal bovine serum (FBS) and 1 % penicillin- streptomycin and incubated for 24 h at 37° C, 5% CO₂. Next day, the culture medium was cleaned with phosphate buffer saline (PBS) to remove the detached cells. The culture media containing biogenic ZnO NPs (500, 250, 125, 62.5, 31.25, 15.8, 7.8, 3.91, 1.5 and 1, µg/ml) were added to cells and incubated for 24 h at 37° C, 5% CO₂. At the end of the exposure time, cells were washed with PBS followed by the addition of 80 µL of media without FBS and 20 µL of MTT reagent. The reaction time for MTT assay was 3h then 100 µL of MTT solvent Dimethyl sulfoxide (DMSO) were added into each well and left for 15 min in dark. the absorbance at 590 nm is recorded by Analytik Jena UV spectrophotometer. Cell viability % is given by ²³:

$$Cell\ viability\% = \frac{A_{Control} - A_{Sample}}{A_{Control}}$$

2.6.2 Cell cycle analysis

HepG2 Cells (control) and cells after treatment with IC₅₀ concentration of biogenic ZnO NPs were seed in DMEM medium overnight, then fixed in 70% (v/v) ethanol in PBS at 4°C for 12 h., and re-suspended in PBS containing 40 µg/ml propidium iodide (PI), 0.1% (v/v) Triton X-100 and 0.1 mg/ml RNase in a dark room according to *Kuo, et. al.* protocol ²⁴ Sub-G1 and cell cycle analysis were performed after a 30-minute incubation at 37 degrees Celsius using a flow cytometer with an argon ion laser (wavelength of 488 nm).

Note:

Experimental research and field studies in this work complies with relevant institutional, national, and international guidelines and legislation.

2. Results and discussion

3.1 Characterization of biogenic ZnO NPs

Green synthesis of NPs considered as the most cost effective, biocompatible and flexible method for NPs synthesis. Biomolecules in can serve as reducing agent of metal ions to produce biocompatible NPs with functionalized surface. **Fig. 2** showed XRD pattern of biogenic ZnO NPs with all the characteristic peaks, which confirms the successful formation of ZnO NPs. The diffraction peaks at 2θ (31.5, 32.23, 36, 47.32, 56.42, 62.65, 67.76 and 68.86) corresponding to the plane (100, 002, 101, 012, 110, 013, 112 and 201) respectively. This results in contact with JCPDS card No. 36-1451 for ZnO NPs²⁵, and with the reported results of green ZnO NPs synthesized from different plants^{26–28}. No additional peaks, which reflect the purity of the prepared sample. Also, the presence of the sharp and narrow peak at $2\theta \sim 36^\circ$ indicate the crystallinity.

XRD pattern analysis is a method that is frequently utilized for the purpose of analyzing the structure of materials, particularly nanomaterials. The Debye-Scherrer and Williamson-Hall techniques are two of the more common methodologies that are used in XRD investigation of metal oxide nanomaterials. The Debye-Scherrer method is used to calculate crystal size (D_L). The approach presupposes that X-rays enter the material and interact with all of its crystallites. Using the Debye-Scherrer equation, the main diffraction pattern (101) is then utilized to calculate the crystal size and microstrain :

$$D_L = k\lambda/\beta\cos\theta$$

$$\varepsilon = \beta \cos \theta/4$$

where k is a constant, λ wavelength, β diffraction peak's and θ is the Bragg angle.

In contrast, the Williamson-Hall method considers the impact of crystal size and lattice strain on the diffraction pattern. The approach posits that the X-rays interact with a finite number of crystallites in the sample, and that the broadening of the diffraction peaks is caused by both the crystallites' finite size and the presence of lattice strain. According to this model, the average crystallite size (D_L) and average micro-strain (ε) were found by taking the reciprocal of the intercept of ($\beta \cos \theta$) versus ($4 \sin \theta$) (**Fig. 3**). The main difference between the two approaches is that the Debye-Scherrer method assumes that the diffraction pattern is solely due to the finite size of the crystallites, whereas the Williamson-Hall method considers the effects of both crystal size and lattice strain on the diffraction pattern.

The synthesized biogenic ZnO NPs were imaged by High resolution transmission microscope (HRTEM), **Fig. 4**. The images showed that almost all the particles have semispherical shape with a little agglomeration. The electrostatic interaction between Zn^{+2} ions and biomolecules of *C. argentea* affects the final shape of the produced green ZnO NPs to be a semispherical with a sort of agglomeration as already shown in TEM images. Similar results obtained for the biosynthesis of green ZnO NPs from different plants extract^{26–28}. The particle size as calculated from HRTEM images (n= 100 particles) was found to be 21.55 ± 4.73 nm, which support the results of XRD. As listed in **Table 1**, the calculated average crystal size as calculated from Scherer's equation and (W-H) methods of green ZnO NPs were found to be 24.53 ± 1.35 nm and 20.4 ± 2.16 nm respectively.

The following expression can be used to calculate the specific surface area (SSA), which is defined as the total surface area per unit mass and is applicable for adsorption and reactions that take place on the surface of the material:

$$\text{SSA} = S_f / \beta \cdot \rho$$

where S_f represents the form factor of biogenic ZnO NPs, β is the mean particle size, and ρ is the density of ZnO (5.61 g. cm^{-3}). We calculate the SSA for biogenic ZnO NPs in Table 1 using the assumption that all particles are spherical ($S_f = 6$), where particle sizes were measured using different methodologies.

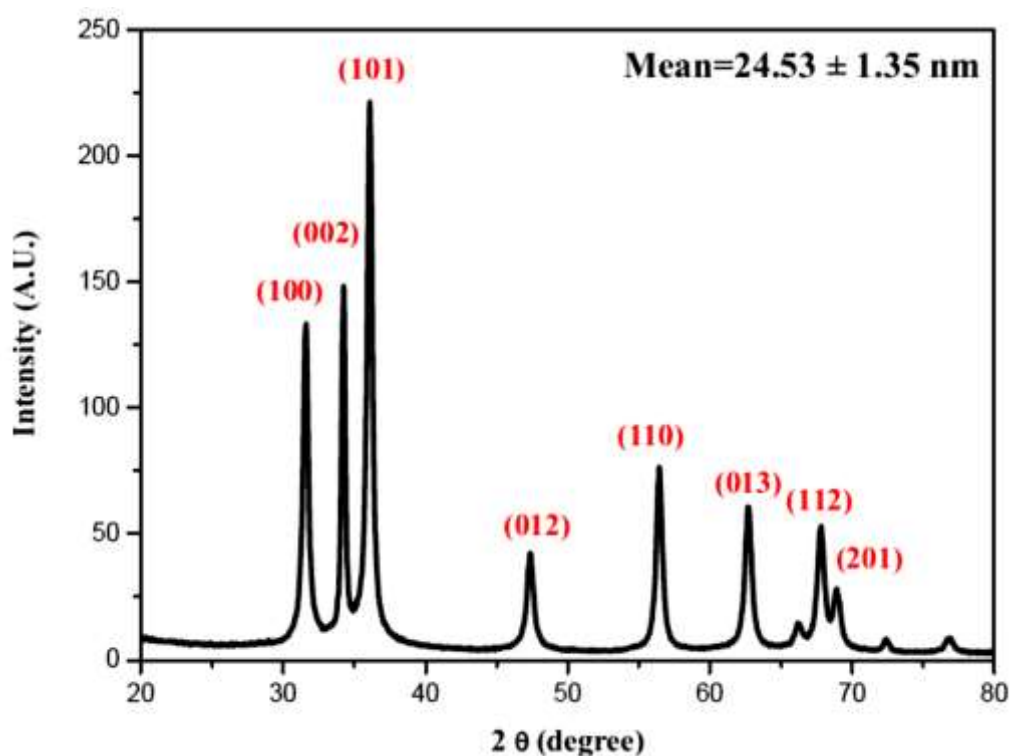


Figure 2. XRD pattern of biogenic ZnO NPs

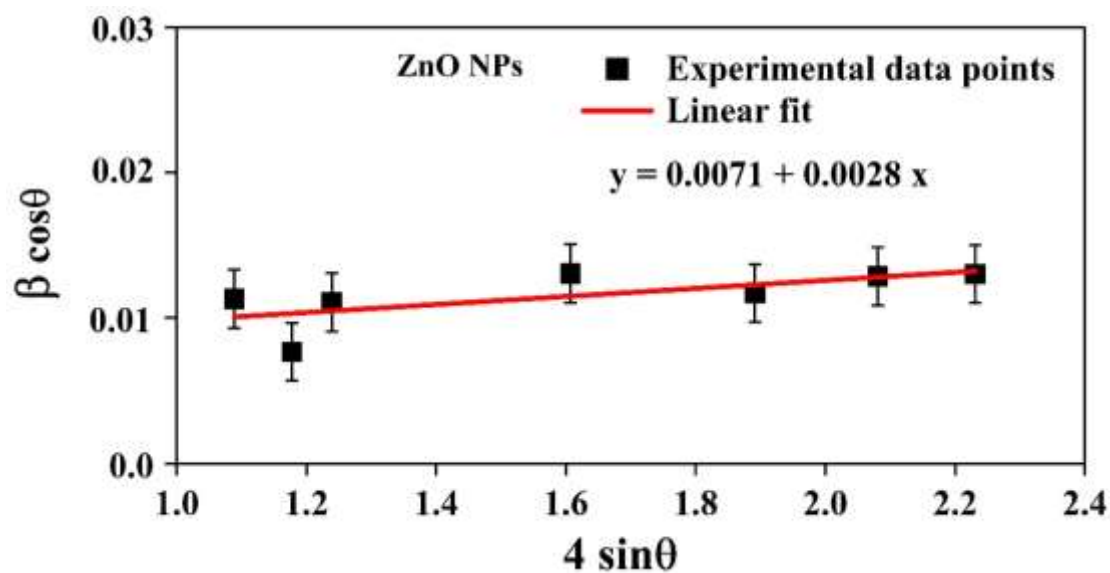


Figure 3. The W-H plot for biogenic ZnO NPs

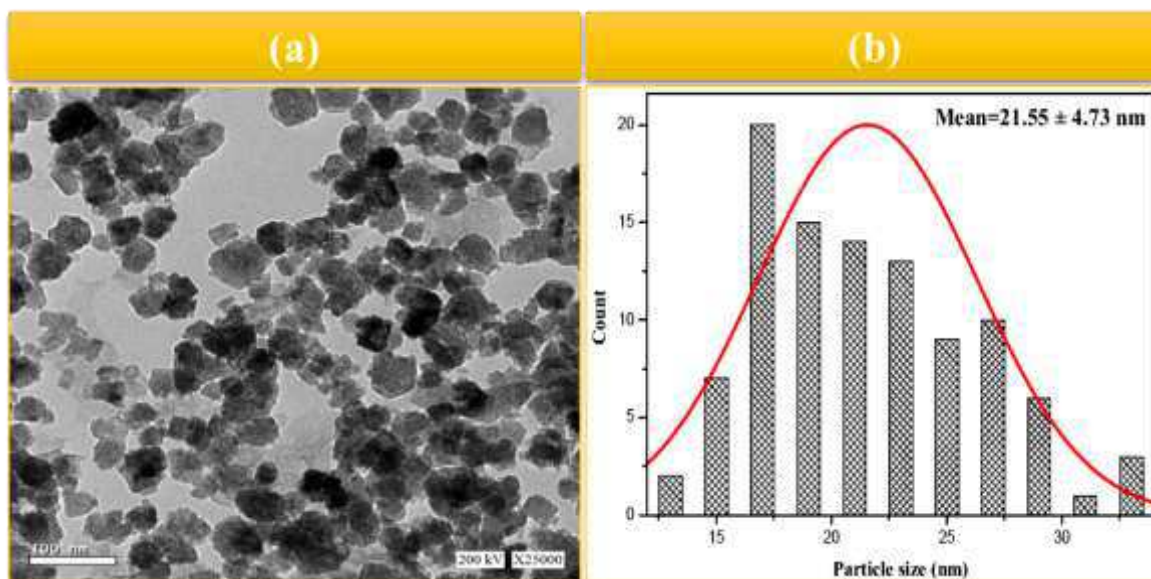


Figure 4. HR-TEM image (a) and particle size distribution of biogenic ZnO NPs as determined from HRTEM image (n=100) (b).

Table 1: Crystallite size (D_L), microstrain (ϵ) and specific surface area (SSA) of biogenic ZnO NPs calculated from XRD and HR-TEM analysis.

XRD analysis by Debye-Scherrer equation			XRD analysis by Williamson-Hall method			HR-TEM analysis	
D_L (nm)	ϵ (10^{-3})	SSA ($m^2 g^{-1}$)	D_L (nm)	ϵ (10^{-3})	SSA ($m^2 g^{-1}$)	D_L (nm)	SSA ($m^2 g^{-1}$)
24.53	2.7	43.6	20.4	2.8	52.4	21.55	49.6

The antioxidant, antibacterial, and anticancer capabilities of ZnO NPs can be improved by increasing their specific surface area and decreasing their particle size value. It has been found that increasing the specific surface area of the ZnO NPs and decreasing the particle size distribution both have a good impact on the aforementioned endeavors.

To begin, a larger number of reactive sites on the surface of ZnO NPs can enhance their ability to scavenge free radicals and their antioxidant activity. The increased surface area of the NPs prevents oxidative damage to cells and tissues by efficiently adsorbing free radicals. This has a promise for treating a wide range of medical issues, including cardiovascular illness, neurological disease, and even cancer. High specific surface area ZnO nanoparticles (NPs) have a greater surface area relative to their volume, which can increase the amount of interaction between the NPs and bacterial cells and lead to greater antibacterial activity. This improved connection has the potential to increase the NPs' antibacterial activity by facilitating their penetration of the bacterial cell wall and disruption of cellular processes. This has applications in the development of antibacterial coatings for use on medical equipment and surfaces and in the management of bacterial infections. To generate reactive oxygen species (ROS) and cause cellular damage in cancer cells, ZnO NPs with a high specific surface area can induce apoptosis (programmed cell death). The high surface area of the NPs allows for more efficient ROS generation, which improves their anticancer effect. Because of their larger surface area, NPs can more easily penetrate the tumor microenvironment and home in on cancer cells while avoiding collateral damage to healthy tissue. This may help in the development of more precise tumor-targeting cancer medicines.

Fourier transformation Infrared spectroscopy (FTIR) was employed in order to investigate the possibility of Zn ions reduction and formation of ZnO NPs. Several absorption bands were observed due to the presence of Zn O NPs and the organic molecules of *C. argentea* **Fig. 5-a**. Vibrational bands observed at 452 and 526 cm^{-1} corresponding to Zn-O stretching vibration. The absorption bands appeared at 698, 1410 and 2384 cm^{-1} are due to C-H bending vibrations, while the band at 890 cm^{-1} can be attributed to N-H stretching. C=C stretching vibration appeared at 1620 cm^{-1} , while C=O appeared at 2508 cm^{-1} . Finally, the broad band observed approximately between 3000 to 3500 cm^{-1} corresponding to OH vibration of hydroxyl group. The formation of ZnO NPs was confirmed by the presence of Zn-O vibrational bands at 452 and 526 cm^{-1} in the FTIR spectra, which considered as the fingerprint of inorganic NPs^{26,28}. Some functional groups were detected due to the interaction of Zn^{+2} ions with the biomolecules of *C. argentea* leaves and suggesting the successful reduction of ZnO and formation of ZnO NPs²⁹. **Fig. 5-b** showed the absorption spectrum of biogenic ZnO NPs with absorption peak at 342 nm, which is the characteristic peak for ZnO NPs³⁰.

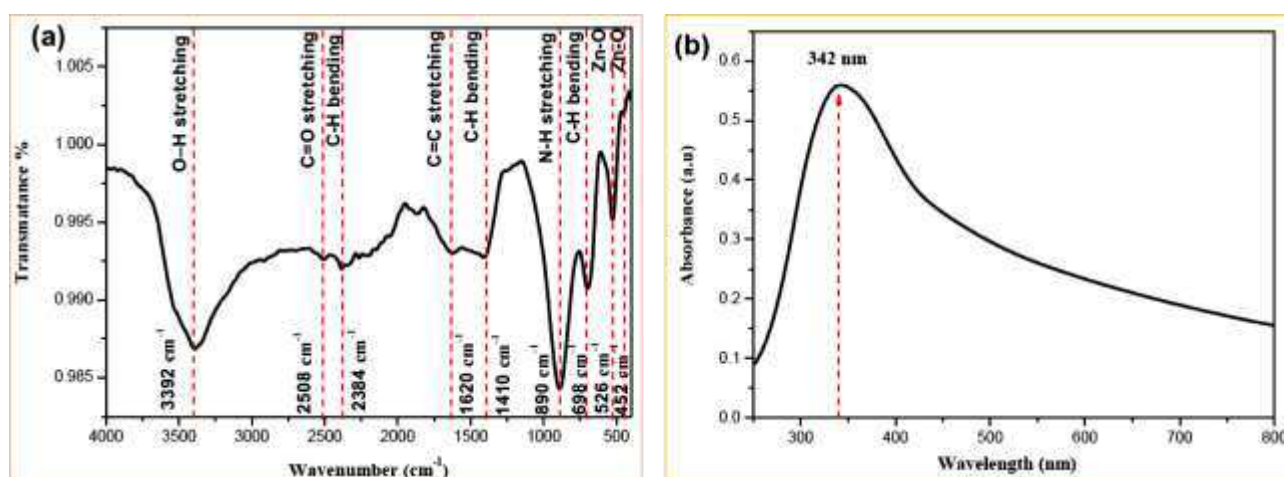


Figure 5: optical spectroscopy of biogenic ZnO NPs, FTIR spectrum (a) and Uv-vis spectrum (b).

3.2 Antioxidant activity of biogenic ZnO NPs

The antioxidant activity of biogenic ZnO NPs is attributed to the ability to donate hydrogen. The formation of electron-hole pair in the surface of ZnO NPs has a great potential on the reduction of H_2O molecules, which can work as scavenger of DPPH molecules¹. However, the biogenic synthesis of ZnO NPs can contribute to modulate the scavenging ability due to the presence of phytochemicals such as phenolics and polyphenolic compounds in the plant extract³¹. These phytochemicals have their antioxidant ability, which contribute to inhibition of free radical scavenging.

Figure 6 shows DPPH radical scavenging activity of green ZnO NPs in comparison with *C. argentea* and ascorbic acid (ASC) as standard. These results reflect a dose dependent behavior in the scavenge activity of green ZnO NPs with $I_{c50} = 91.24 \mu\text{g/ml}$ compared with $I_{c50} = 58.76 \mu\text{g/ml}$ and $14.37 \mu\text{g/ml}$ for *C. argentea* and ascorbic acid respectively. Similar results were reported for green ZnO NPs synthesized using DPPH assay represented in **Table 2**. The I_{c50} of aqueous extract of *C. argentea* was $30 \mu\text{g/ml}$, which attributed to its phenolic content as reported by *Rukhsana Abdul Rub et.al.*³² Hence, the biosynthesis of ZnO NPs using *C. argentea* extract enriched its surface with biomolecules and improved its scavenging activity.

Table 2. Maximum scavenging activity of biosynthesized ZnO NPs from different plants using DPPH assay.

Plant Name	Maximum activity	Reference
<i>Knoxia sumatrensis</i>	95.8 %	33
<i>Azadirachta indica</i>	92 %	31
<i>Rosa canina</i>	> 90 %	34
<i>L. pruinatum</i>	86.5 %	35
<i>Eucalyptus globulus</i>	82 %	36
<i>Trianthema portulacastrum</i>	75 %	37
<i>Costus igneus</i>	75 %	38
<i>Tecoma castanifolia</i>	67 %	39
<i>Andrographis paniculata</i>	61.32 %	40
<i>Ceropegia candelabrum</i>	55.43%	41

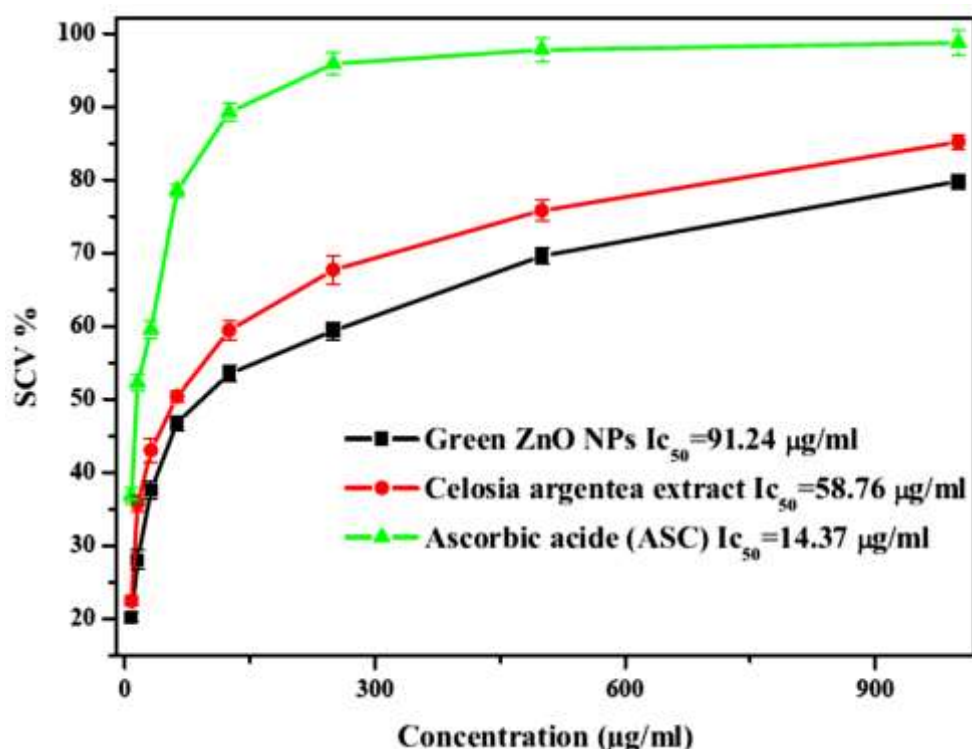


Figure 6. Scavaging activity of biosynthesized ZnO NPs from *C. argentea* , *C. argentea* aqueous extract of *C. argentea* and Ascorbic acid as evaluated from DPPH assay.

3.3 Antibacterial activity

Several mechanisms were reported for the antibacterial activity of green ZnO NPs, the main three possible mechanisms were illustrated in **Fig. 7**^{42–44}. The proposed mechanisms include:

- The release of Zn^{+2} due to the interaction of ZnO NPs with the bacterial cell. The negatively charged bacterial membrane attracted the released Zn^{+2} via Van der Waals forces leading to defragmentation of cell membrane, inhibition of cell growth and finally cell death.
- The generation of reactive oxygen species (ROS) including hydrogen peroxide, radical hydroxyl, and peroxide from the surface of ZnO NPs. The generated ROS causes oxidative stress by damaging the cell membrane, DNA and protein and subsequent cell death.
- Direct contact with the bacterial cell membrane is another mechanism for antibacterial action of ZnO NPs. according to Heinlaan et al. and Brayner et al. the contact of bacterial cell with ZnO NPs can cause changing in the contact area of cell membrane increasing the membrane permeability leading to subsequent internalization of ZnO NPs and cell damage.

The physiochemical properties such as size, shape and surface chemistry side by side with the high surface to volume ratio are the main responsible parameters for the antibacterial action of green ZnO NPs to different bacterial strains, **Table 3**.

Table 3. Antibacterial activity of green ZnO NPs from different plants against different types of bacterial strains.

Plant source	Bacterial strain	Antibacterial	
		activity (µg/mL)	Refrence
<i>Cassia fistula</i>	<i>Escherichia coli</i>	20	45
<i>Plectranthus amboinicus</i>	<i>Staphylococcus aureus</i>	8-10	46
<i>Vitex negunda</i>	<i>Staphylococcus aureus</i> , <i>Staphylococcus paratyphi</i> , <i>Escherichia coli</i>	15-17	43
<i>Ceropegia candelabrum</i>	<i>Bacillus subtilis</i> , <i>Escherichia coli</i> , <i>Salmonella typhi</i>	0.25	41
<i>Celosia argentea</i>	<i>Escherichia coli</i> , <i>Salmonella</i> , <i>Acetobacter</i>	3.1	47
<i>Couroupita Guianensis</i>	<i>Bacillus cereus</i> , <i>Klebsiella pneumonia</i> , <i>Escherichia coli</i> , <i>Mycobacterium luteus</i> , <i>V. cholera</i>	0.05-0.25	48
<i>Caulerpa peltata</i> , <i>Hypnea valencia</i> , <i>Sargassum myriocystum</i>	<i>Staphylococcus aureus</i> , <i>Streptococcus mutans</i> , <i>Vibrio cholerae</i> , <i>Neisseria gonorrhoeae</i> , <i>Klebsiella pneumonia</i>	0.10	49

In this study, the antibacterial activity of biogenic ZnO NPs from *C. argentea* extract against four types of bacterial strains; two gram positive [*Staphylococcus aureus* (ATCC 29213), *Bacillus subtilis* (ATCC 6633)] and two gram negative [*E. coli* (ATCC 25922) and *Salmonella typhimurium* (ATCC14028)]. The diameter of inhibition zone of green ZnO NPs, *C. argentea* extract and Gentamycin as a positive control was determined using agar diffusion disk and presented in **Fig. 8**. Biogenic ZnO NPs showed a significant antibacterial activity against the four bacterial strains compared with the positive control. Similar activity had been reported for

green synthesized ZnO NPs from different plants, table 1. Moreover, the inhibition zone of *C. argentea* extract against the four bacterial strains was significantly larger than that of Gentamycin, which due to the presence of the bioactive compounds such as Alkaloids, Flavonoids, Phenols, Terpenoids, Starch, Cellulose^{15,19}.

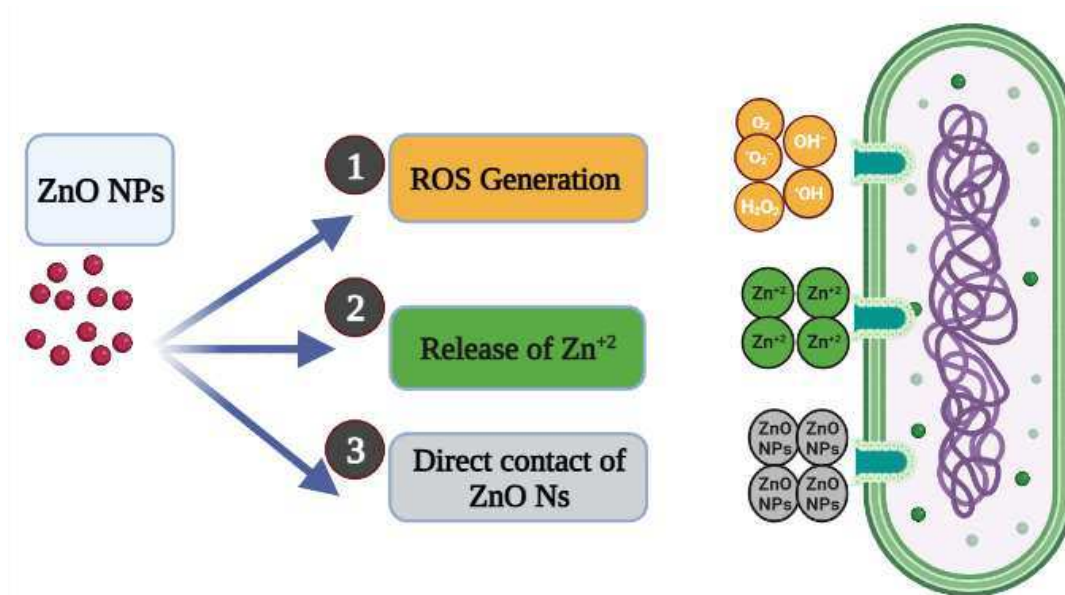


Figure 7. The possible antibacterial mechanism of ZnO NPs

Regarding the bacterial strain type, green ZnO NPs showed a higher bactericidal effect in gram positive (*S. aureus* and *B. subtilis*) compared with gram negative strains (*E. coli* and *Salmonella typhimurium*). The explanation of this result was investigated by Vijayakumar et al. and attributed to the different on the structure and components of gram-positive bacteria such as peptidoglycan layer, which facilitates the attachment and internalization of ZnO NPs to the cell wall.

Additionally, the inhibition zone of biogenic ZnO NPs increased dramatically with concentration increment from 25 to 100 µg/ml in the four bacterial strains. This behavior due to the increased number of ZnO NPs diffused in the agar medium, which in contact with reported results^{7,28,50}.

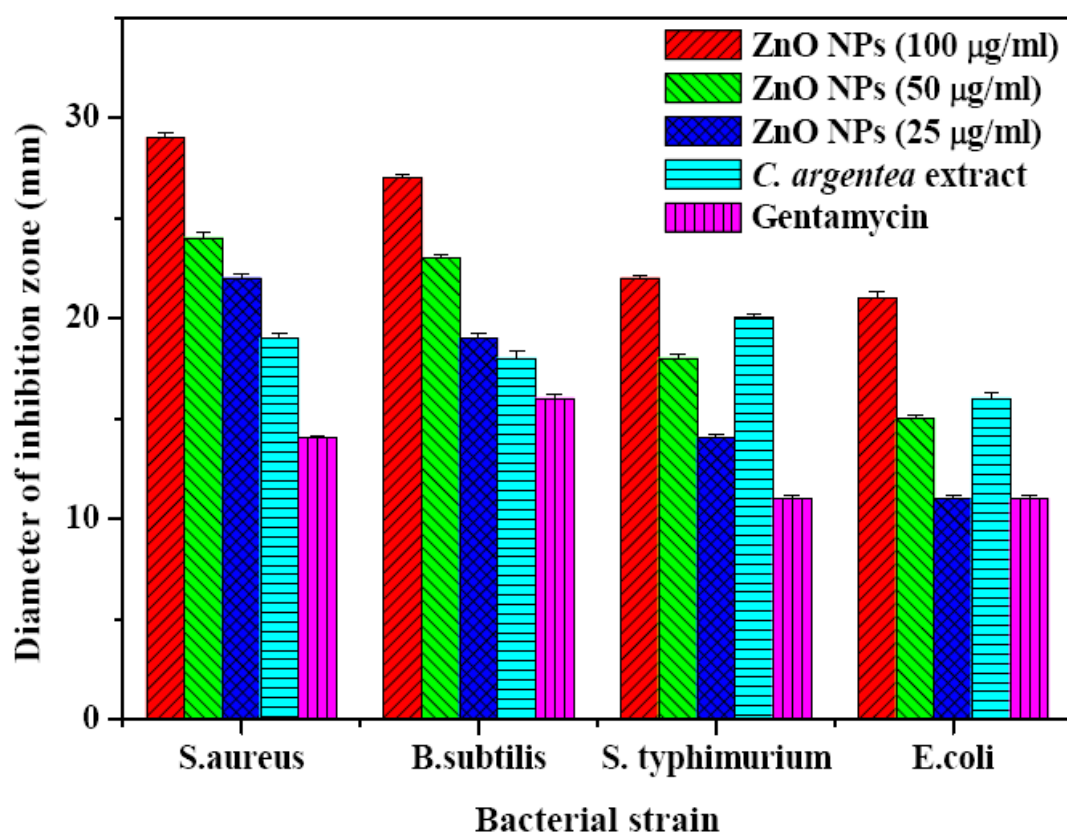


Figure 8. diameter of inhibition zone (mm) of green ZnO NPs with different concentrations (100, 50 and 25 µg/ml), *C. argentea* extract and Gentamycin against gram positive bacteria (*S. aureus* and *B. subtilis*) and gram-negative bacteria (*E. coli* and *Salmonella typhimurium*).

3.4 Anticancer activity

One of the main advantages of ZnO NPs as an anticancer agent is their selectivity to cancer cells. The ability of NPs to kill cancer cells varies depending on their intrinsic properties such as their ability to produce ROS. These ROS interact with the cellular components and initiate apoptosis. From the genetic point of view, apoptotic induction can occur through intrinsic or extrinsic pathways due activation of caspases. Despite the fact that a large number of genes can induce apoptosis, p53 is the master guardian of the cell, maintaining genomic stability through activated cell-cycle checkpoints, DNA repair, and apoptosis.^{51,52} Cell cycle arrest is induced by DNA damage and the p53 gene to allow for either self-mediated apoptosis or damage repair.^{53,54} The Bcl-2 level reduces resistance to apoptotic stimuli, resulting in apoptosis.⁵⁵ Intrinsic pathways of apoptosis are the source of the mitochondria integrate signals. The cell death resistance is motivated due to the activation of NF-κB that leads to downstream target gene expression⁵⁶. The NF-κB activation in the mitochondria leads to cytochrome c release, thus triggering caspase cascades and programmed cell death.

3.4.1 Cytotoxicity of green ZnO NPs against HepG2 cells

MTT assay was used to determine cytotoxicity of biogenic ZnO NPs with different concentrations (500, 250, 125, 62.5, 31.25, 15.8, 7.8, 3.91, 1.5 and 1, $\mu\text{g/ml}$) against HepG2 cells, **Fig. 9**. Cell viability were decreased in a concentration dependent reduction from 100% to 14.9 % with increasing the biogenic ZnO NPs concentrations from 0.5 to 500 $\mu\text{g/ml}$. However, cell viability reduction was slightly small in the small concentrations from 0.5 to 15.8 $\mu\text{g/ml}$, while it increased in the higher concentrations from 15.8 to 500 $\mu\text{g/ml}$. The calculated IC_{50} was found to be 49.45 $\mu\text{g/ml}$, which demonstrate the anticancer potential of biogenic ZnO NPs. These results supported the previous studies for anticancer activity of biogenic ZnO NPs and reveals that *C. argentea* extract can be used as a reducing agent for ZnO NPs synthesis with high selectivity to cancer cells and improved anticancer activity^{1,5,6,53}.

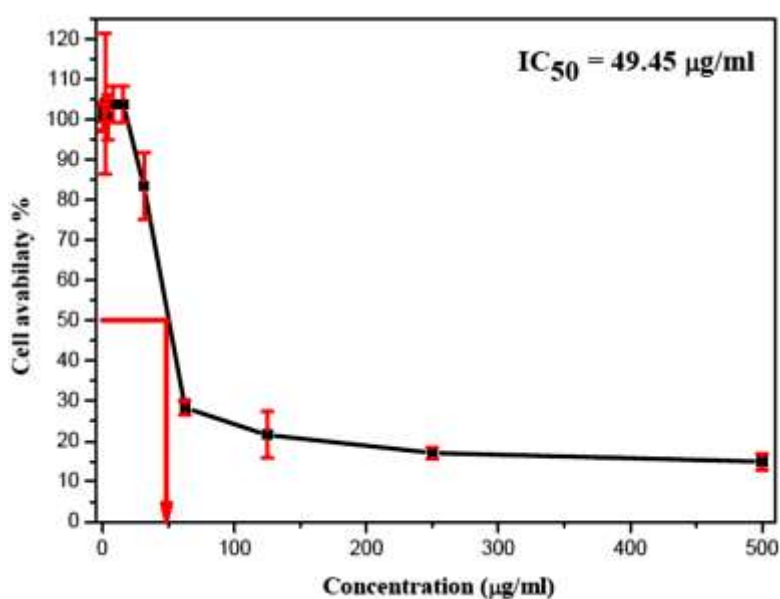


Figure 9. Cytotoxicity of biogenic ZnO NPs against HepG2 cells as screened with MTT assay.

3.4.2 Cell Cycle Analysis

In order to determine the possible mechanism for anticancer activity of biogenic ZnO NPs flowcytometry was used to analyze the cell cycle of HepG2 cells. Apoptotic rate of treated cells with biogenic ZnO NPs elevated for S phase 39.28 %, G1/G0 was 52.68 %, 8.04 % for the G2/M phase and 52.6 for Pre-G1 compared with cell control values 52.78 %, 42.58 %, 6.8 %, 0.58 %, respectively, **Fig. 10**. Also, there was a significantly elevated ($P < 0.05$) necrotic cells % compared with control value (11.61 % / 6.84 %).

An observed accrual of arrested cells in the G2/M phase with increase in the apoptotic cells as appeared in the higher number of cells in PreG1 phase, **Fig. 11**. Moreover, the apoptotic cells

slowed a significant elevation in the early and late apoptosis (22.45 %, 18.54 %) compared with control values (0.25 %, 0.158 %).

According to literature the cytotoxic effect of ZnO NPs can be attributed to two main mechanisms; necrosis and apoptosis. The exposure to ZnO NPs can induce the production of ROS or mediate the protein activity disequilibrium. The generated oxidative stress can lead to necrosis or DNA damage. As a result of DNA damage, mitochondrial apoptotic (p53 gene) pathway will be activated causing cell death by apoptosis^{6,30,57}.

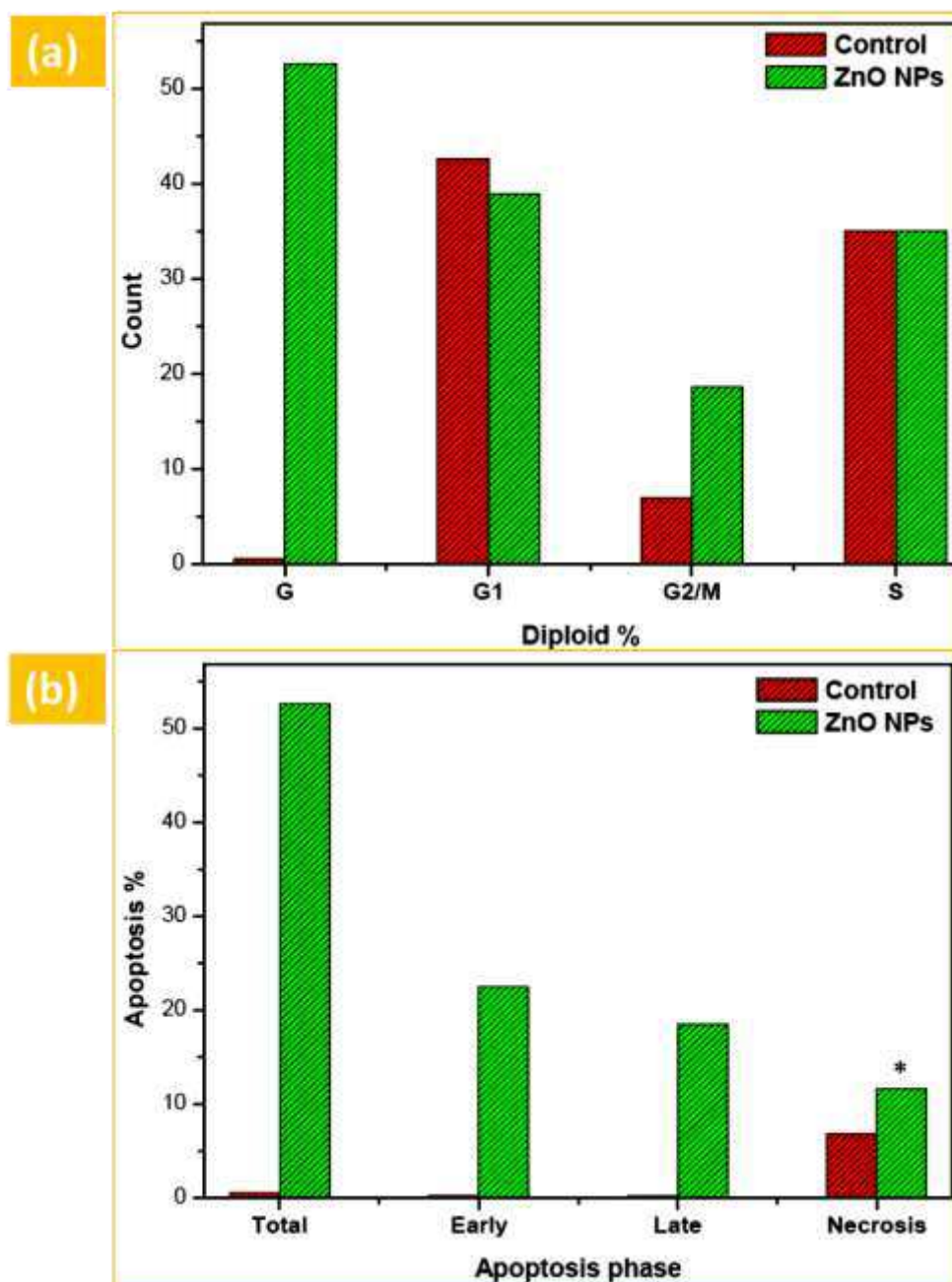


Figure 10. Flow cytometry. Cell cycle of HepG2 cells after treatment with biogenic ZnO NPs for 24 h. HepG2 cells in normal phases were represented by SubG1, S, and G2/M, while cells undergo apoptosis/necrosis represented in G phase. (a), Analysis of

necrosis and apoptosis percentage (Total, Early and Late) of HepG2 cell before and after treatment with biogenic ZnO NPs (b),

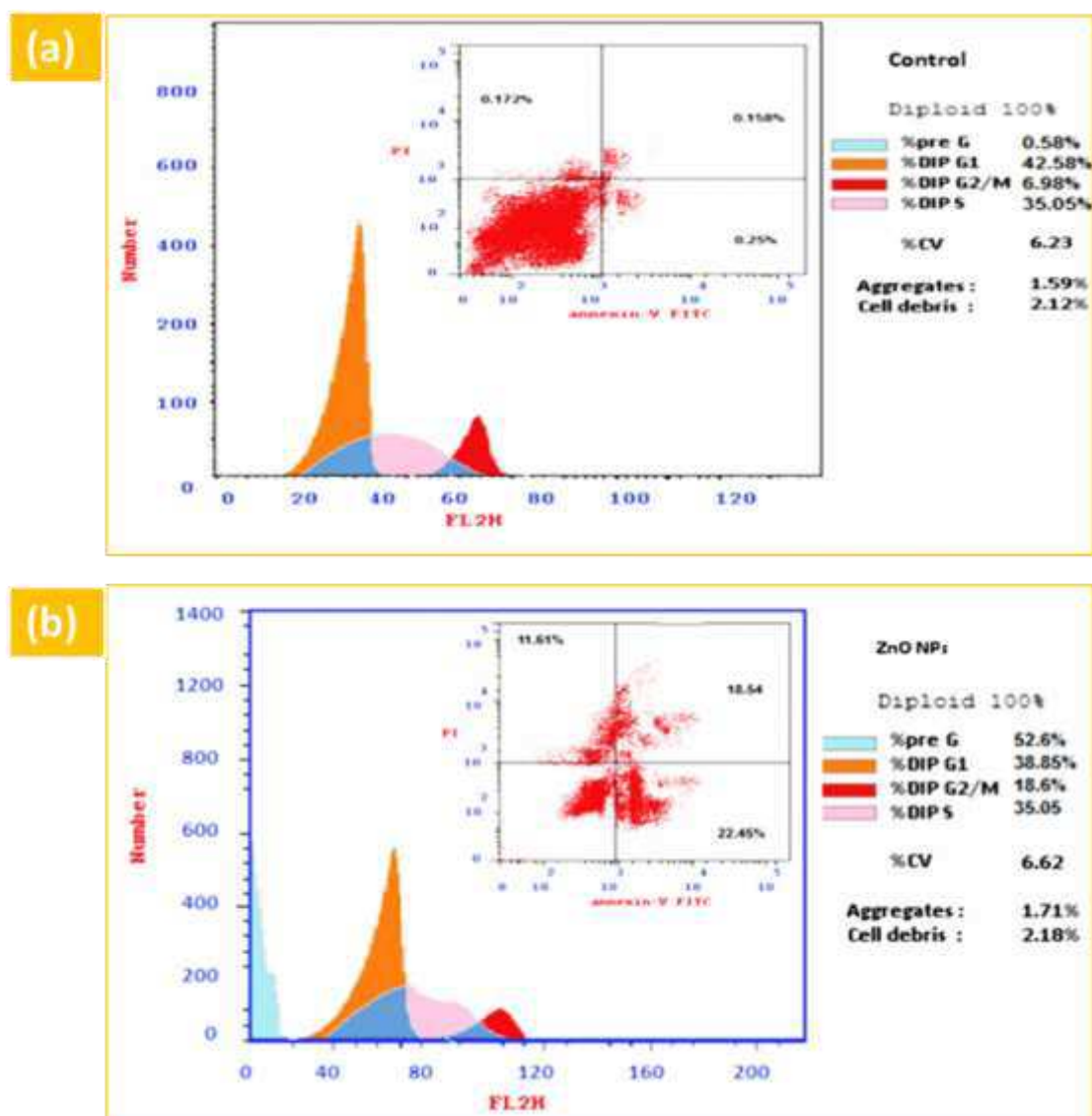


Figure 11: (a) Flow cytometry for evaluation of cell cycle profile using PI and annexin V. stains on HepG2 cells. (b) post-treatment with green ZnO NPs on HepG2 cells.

3.4.2 RT-qPCR analysis

The mRNA expression levels showed a significant increase in Cy-c, p53 and NF-KB genes, while the expression of antiapoptotic gene bcl-2 was significantly decreased compared with its values in untreated control HepG2 cells, **Fig. 12**.

Our study exhibited the expressions of the proapoptotic NF-KB –Cy-c genes, the master guardian of the cell p53 gene, and antiapoptotic Bcl2 gene; suggesting that biogenic ZnO NPs enter the cell by changing the mitochondrial membrane potential and activating the expression of important tumor suppressor gene p53 leadingg to increasing its value ^{30,57,58}.

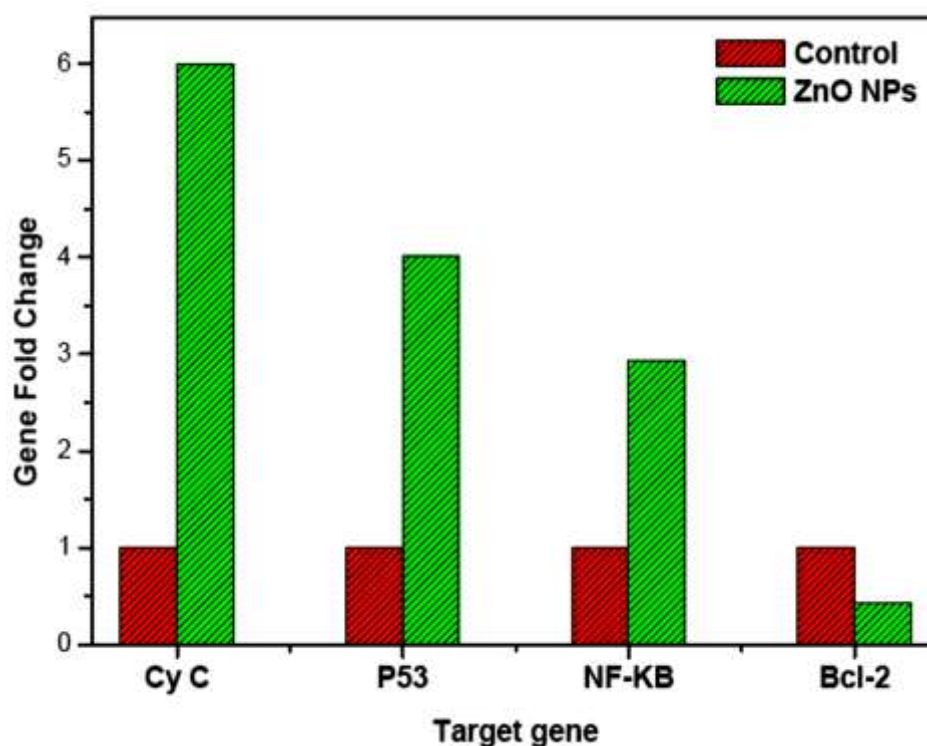


Figure 12. mRNA levels of pro-apoptotic and anti-apoptotic genes HepG2 cells treated with biogenic ZnO NPs.

Similar results were reported for apoptotic induction after treatment with biogenic ZnO NPs. *Line M. et al.*⁵⁸ investigated the apoptotic induction of biogenic ZnO NPs, they observed the downregulating of antiapoptotic genes of Bcl-2, AKT1, and JERK/2 and upregulating of proapoptotic genes of p21, p53, JNK, and Bax in *Pichia kudriavzevii* GY1 yeast after treatment with biogenic ZnO NPs. Moreover, *Alrokayan et al.*⁶ reported the upregulating of p53 and bax genes and downregulating of antiapoptotic gene Bcl-2 in HepG2 cells after treatment with ZnO NPs. In another study ZnO NPs synthesized from *E. prostrata* were found to promote autophagy, upregulate the expression of p53 and Caspase3, and trigger the apoptosis⁵⁹. While ZnO NPs synthesized from *Artemisia scoparia* extract caused upregulated pro-apoptotic genes while downregulating anti-apoptotic genes⁶⁰.

According to the obtained results, the anticancer activity of biogenic ZnO NPs caused by activation of apoptotic protease activator, decomposes DNA polymerase, and damages cell DNA, which in contact with the reported results^{25,61,62}.

Conclusion

Biogenic synthesis of NPs is a powerful technique to produce NPs with unique properties. The reduction of Zn ions was assessed by the biomolecules of *C. argentea* extract. The reduction process didn't affect the structure of the formed ZnO NPs, on the contrary a significant enhancement on its bioactivity was observed. The possible mechanisms of antioxidant,

antibacterial and anticancer activities were investigated in order to figure out the role of *C. argentea* extract on the bioactivity of ZnO NPs. The biogenic synthesis of ZnO NPs can contribute to modulate the scavenging ability due to the presence of phytochemicals such as phenolics and polyphenolic compounds in the plant extract. The physiochemical properties such as size, shape and surface chemistry side by side with the high surface to volume ratio are the main responsible parameters for the antibacterial action of biogenic ZnO NPs to different bacterial strains. The anticancer property of biogenic ZnO NPs due to the generation of ROS or mediate the protein activity disequilibrium. The generated oxidative stress can lead to necrosis or DNA damage. As a result of DNA damage, mitochondrial apoptotic (p53 gene) pathway will activated causing cell death by apoptosis.

Acknowledgement

This research work was funded by Institutional Fund Projects under grant no. (If PIP: 758-665- 1443). The authors gratefully acknowledge technical and financial support provided by the Ministry of Education and King Abdulaziz University, DSR, Jeddah, Saudi Arabia".

Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

References

1. Article, R., Bisht, G. & Rayamajhi, S. ZnO Nanoparticles : A Promising Anticancer Agent. (2016) doi:10.5772/63437.
2. Rajashekara, S. Biomedical Applications of Biogenic Zinc Oxide Nanoparticles Manufactured from Leaf Extracts of *Calotropis gigantea* (L .) Dryand . 654–671 (2020).
3. Kang, T. *et al.* In vitro toxicity of different-sized ZnO nanoparticles in Caco-2 cells. *Nanoscale Res. Lett.* 2013 81 **8**, 1–8 (2013).
4. Valdiglesias, V. *et al.* Neuronal cytotoxicity and genotoxicity induced by zinc oxide nanoparticles. *Environ. Int.* **55**, 92–100 (2013).
5. Raffa, V. *et al.* Zinc oxide nanoparticles as selective killers of proliferating cells. 1129–1140 (2011) doi:10.2147/IJN.S16581.
6. Alrokayan, S. A. Zinc oxide nanoparticles selectively induce apoptosis in human cancer cells through reactive oxygen species. 845–857 (2012).
7. Singh, J. *et al.* Green synthesis of metals and their oxide nanoparticles: applications for environmental remediation. *Journal of Nanobiotechnology* 2018 16:1 vol. 16 1–24

(BioMed Central, 2018).

8. Chan, S. Biosynthesis of Metal Oxide Nanoparticles. . *ChemBioEng Rev.* **3**, 55–67 (2016).
9. Das, M. & Chatterjee, S. Green synthesis of metal/metal oxide nanoparticles toward biomedical applications: Boon or bane. *Green Synth. Charact. Appl. Nanoparticles* 265–301 (2019) doi:10.1016/B978-0-08-102579-6.00011-3.
10. Sorbiun, M., Shayegan Mehr, E., Ramazani, A. & Mashhadi Malekzadeh, A. Biosynthesis of metallic nanoparticles using plant extracts and evaluation of their antibacterial properties. *Nanochemistry Res.* **3**, 1–16 (2018).
11. Khan, I., Saeed, K. & Khan, I. Nanoparticles: Properties, applications and toxicities. *Arab. J. Chem.* **12**, 908–931 (2019).
12. Makarov, V. V. *et al.* “Green” Nanotechnologies: Synthesis of Metal Nanoparticles Using Plants. *Acta Naturae* **6**, 35 (2014).
13. Patil, S. & Chandrasekaran, R. Biogenic nanoparticles: a comprehensive perspective in synthesis, characterization, application and its challenges. *J. Genet. Eng. Biotechnol.* **18**, 67 (2020).
14. Baker, S. *et al.* Plants: Emerging as nanofactories towards facile route in synthesis of nanoparticles. *BioImpacts* **3**, 111–117 (2013).
15. Malomo, S. O., Ore, A. & Yakubu, M. T. In vitro and in vivo antioxidant activities of the aqueous extract of *Celosia argentea* leaves. **43**, (2011).
16. Hamzah, R. U., Lawal, A. R., Madaki, F. M. & Erukainure, O. L. Methanolic extract of *Celosia argentea* var . *crista* leaves modulates glucose homeostasis and abates oxidative hepatic injury in diabetic rats. 1065–1071 (2018).
17. Rub, R. A., Pati, M. J., Siddiqui, A. A., Moghe, A. S. & Shaikh, N. N. Characterization of Anticancer Principles of *Celosia argentea* (*Amaranthaceae*). 97–104 (2016) doi:10.4103/0974-8490.172659.
18. Rub, R. A., Patil, M. J., Siddiqui, A. A., Ghorpade, R. & Moghe, A. S. Free Radical Scavenging and Cytotoxic Potential of *Celosia argentea*. **7**, 191–197 (2015).
19. Santana, E. J. De *et al.* Phytochemical analysis and antimicrobial and antioxidant activities of *Celosia argentea* leaves. **17**, 1–11 (2021).
20. Alsedfy, M. Y., Said, A. H., EbnaIwaled, A. A. & Moustafa, M. Effect of Monosodium Glutamate on the Digestibility of Different Nutrients Using Standardized Static In vitro Digestion Model. *J. Exp. Biol. Agric. Sci.* **10**, 1033–1043 (2022).
21. Kamal, Z. *et al.* Ameliorative effect of biosynthesized titanium dioxide nanoparticles

- using garlic extract on the body weight and developmental toxicity of liver in albino rats compared with chemically synthesized nanoparticles. *Front. Vet. Sci.* **9**, (2022).
22. Balouiri, M., Sadiki, M. & Ibensouda, S. K. Methods for in vitro evaluating antimicrobial activity: A review. *J. Pharm. Anal.* **6**, 71–79 (2016).
 23. Al-Harbi, L. M., Mohamed, W. S., Ebnalwaled, A. A., Said, A. H. & Ezzeldien, M. The effect of synthesis conditions on the photokilling activity of TiO₂ nanostructures. *Mater. Res. Express* **10**, 015004 (2023).
 24. Kuo, H. *et al.* Morin Inhibits the Growth of Human Leukemia HL-60 Cells via Cell Cycle Arrest and Induction of Apoptosis through Mitochondria Dependent Pathway. **406**, 395–405 (2007).
 25. Wahab, R. *et al.* Colloids and Surfaces B : Biointerfaces ZnO nanoparticles induced oxidative stress and apoptosis in HepG2 and MCF-7 cancer cells and their antibacterial activity. *Colloids Surfaces B Biointerfaces* **117**, 267–276 (2014).
 26. Yusof, H. M., Aini, N., Rahman, A. & Mohamad, R. Biosynthesis of zinc oxide nanoparticles by cell - biomass and supernatant of *Lactobacillus plantarum* TA4 and its antibacterial and biocompatibility properties. *Sci. Rep.* 1–13 (2020) doi:10.1038/s41598-020-76402-w.
 27. Manokari, M. & Shekhawat, M. S. Biosynthesis of Zinc Oxide Nanoparticles from the Aerial Parts of *Hibiscus rosa-sinensis* L . 1–6 (2017).
 28. Sadhasivam, S. & Shanmugam, M. Zinc Oxide Nanoparticles : Green Synthesis and Biomedical Applications. *J. Clust. Sci.* **32**, 1441–1455 (2021).
 29. Parvathy, K. V. G., Krishna, S. M. M. & Sudhahar, K. S. *Celosia argentea* leaf extract - mediated green synthesized iron oxide nanoparticles for bio - applications. *J. Nanostructure Chem.* (2021) doi:10.1007/s40097-021-00434-5.
 30. Press, D. Zinc oxide nanoparticles induce apoptosis and autophagy in human ovarian cancer cells. 6521–6535 (2017).
 31. Madan, H. R. *et al.* Facile green fabrication of nanostructure ZnO plates, bullets, flower, prismatic tip, closed pine cone: Their antibacterial, antioxidant, photoluminescent and photocatalytic properties. *Spectrochim. Acta Part A Mol. Biomol. Spectrosc.* **152**, 404–416 (2016).
 32. Rub, R. A., Patil, M. J., Ghorpade, P. & Siddiqui, A. Evaluation of antioxidant potential of *Celosia argentea* extracts. *Pharmacogn. J.* **5**, 140–141 (2013).
 33. Pasternak, H. & Shalev, B. A. The effect of a feature of regression disturbance on the efficiency of fitting growth curves. *Growth. Dev. Aging* **58**, 33–9 (1994).

34. Jafarirad, S., Mehrabi, M., Divband, B. & Kosari-Nasab, M. Biofabrication of zinc oxide nanoparticles using fruit extract of *Rosa canina* and their toxic potential against bacteria: A mechanistic approach. *Mater. Sci. Eng. C* **59**, 296–302 (2016).
35. Naiel, B., Fawzy, M., Halmy, M. W. A. & Mahmoud, A. E. D. Green synthesis of zinc oxide nanoparticles using Sea Lavender (*Limonium pruinsum* L. Chaz.) extract: characterization, evaluation of anti-skin cancer, antimicrobial and antioxidant potentials. *Sci. Rep.* **12**, 20370 (2022).
36. Siripireddy, B. & Mandal, B. K. Facile green synthesis of zinc oxide nanoparticles by *Eucalyptus globulus* and their photocatalytic and antioxidant activity. *Adv. Powder Technol.* **28**, 785–797 (2017).
37. Khan, Z. U. H. *et al.* Greener synthesis of zinc oxide nanoparticles using *Trianthema portulacastrum* extract and evaluation of its photocatalytic and biological applications. *J. Photochem. Photobiol. B Biol.* **192**, 147–157 (2019).
38. Vinotha, V. *et al.* Synthesis of ZnO nanoparticles using insulin-rich leaf extract: Anti-diabetic, antibiofilm and anti-oxidant properties. *J. Photochem. Photobiol. B Biol.* **197**, 111541 (2019).
39. Sharmila, G., Thirumarimurugan, M. & Muthukumaran, C. Green synthesis of ZnO nanoparticles using *Tecoma castanifolia* leaf extract: Characterization and evaluation of its antioxidant, bactericidal and anticancer activities. *Microchem. J.* **145**, 578–587 (2019).
40. Rajakumar, G., Thiruvengadam, M., Mydhili, G., Gomathi, T. & Chung, I.-M. Green approach for synthesis of zinc oxide nanoparticles from *Andrographis paniculata* leaf extract and evaluation of their antioxidant, anti-diabetic, and anti-inflammatory activities. *Bioprocess Biosyst. Eng.* **41**, 21–30 (2018).
41. Murali, M. *et al.* Antibacterial and antioxidant properties of biosynthesized zinc oxide nanoparticles from *Ceropegia candelabrum* L. – An endemic species. *Spectrochim. Acta Part A Mol. Biomol. Spectrosc.* **179**, 104–109 (2017).
42. Ahmadi Shadmehri, A. & Namvar, F. A Review on Green Synthesis, Cytotoxicity Mechanism and Antibacterial Activity of ZnO-NPs. *J. Res. Appl. Basic Med. Sci.* **6**, 23–31 (2020).
43. Ambika, S. & Sundrarajan, M. Antibacterial behaviour of *Vitex negundo* extract assisted ZnO nanoparticles against pathogenic bacteria. *J. Photochem. Photobiol. B Biol.* **146**, 52–57 (2015).
44. González, S. C. E. *et al.* Antibacterial and anticancer activity of ZnO with different

- morphologies: a comparative study. *3 Biotech* **11**, 1–12 (2021).
45. Suresh, D. *et al.* Green synthesis of multifunctional zinc oxide (ZnO) nanoparticles using *Cassia fistula* plant extract and their photodegradative, antioxidant and antibacterial activities. *Mater. Sci. Semicond. Process.* **31**, 446–454 (2015).
 46. Vijayakumar, S., Vinoj, G., Malaikozhundan, B., Shanthi, S. & Vaseeharan, B. *Plectranthus amboinicus* leaf extract mediated synthesis of zinc oxide nanoparticles and its control of methicillin resistant *Staphylococcus aureus* biofilm and blood sucking mosquito larvae. *Spectrochim. Acta Part A Mol. Biomol. Spectrosc.* **137**, 886–891 (2015).
 47. Vaishnav, J., Subha, V., Kirubanandan, S., Arulmozhi, M. & Renganathan, S. Green synthesis of zinc oxide nanoparticles by *Celosia argentea* and its characterization. *J. Optoelectron. Biomed. Mater.* **9**, 59–71 (2017).
 48. Sathishkumar, G. *et al.* Facile biosynthesis of antimicrobial zinc oxide (ZnO) nanoflakes using leaf extract of *Couroupita guianensis* Aubl. *Mater. Lett.* **188**, 383–386 (2017).
 49. Nagarajan, S. & Arumugam Kuppusamy, K. Extracellular synthesis of zinc oxide nanoparticle using seaweeds of gulf of Mannar, India. *J. Nanobiotechnology* **11**, 39 (2013).
 50. Publishers, S. Biomedical Potential of Zinc Oxide Nanoparticles Synthesized using Plant Extracts.
 51. Bisht, G. & Rayamajhi, S. ZnO Nanoparticles: A Promising Anticancer Agent. *Nanobiomedicine* **3**, (2016).
 52. Tabrez, S. *et al.* Biosynthesis of ZnO NPs from pumpkin seeds' extract and elucidation of its anticancer potential against breast cancer. *Nanotechnol. Rev.* **11**, 2714–2725 (2022).
 53. Król, A., Pomastowski, P., Ra, K. & Buszewski, B. Zinc oxide nanoparticles : Synthesis , antiseptic activity and toxicity mechanism. **249**, 37–52 (2017).
 54. Levy, J. M. M., Towers, C. G. & Thorburn, A. Targeting autophagy in cancer. *Nat. Publ. Gr.* **17**, 528–542 (2017).
 55. Tang, Z., Cao, W., Su, M., Chen, X. & Lu, J. Osimertinib induces autophagy and apoptosis via reactive oxygen species generation in non-small cell lung cancer cells. *Toxicol. Appl. Pharmacol.* **321**, 18–26 (2017).
 56. Albensi, B. C. & Braun, R. J. What Is Nuclear Factor Kappa B (NF- κ B) Doing in and to the Mitochondrion ? **7**, 1–7 (2019).

57. Wang, C., Hu, X., Gao, Y. & Ji, Y. ZnO Nanoparticles Treatment Induces Apoptosis by Increasing Intracellular ROS Levels in LTP-a-2 Cells. **2015**, (2015).
58. Line, M.-C. C., Ariff, A. Bin, Navaderi, M. & Mohamad, R. Eco-Friendly Formulated Zinc Oxide Nanoparticles : Induction of Cell Cycle Arrest and Apoptosis in the. (2017) doi:10.3390/genes8100281.
59. Chung, I. *et al.* An Investigation of the Cytotoxicity and Caspase-Mediated Apoptotic Effect of Green Synthesized Zinc Oxide Nanoparticles Using *Eclipta prostrata* on Human Liver Carcinoma Cells. 1317–1330 (2015) doi:10.3390/nano5031317.
60. Shiviyari, A. M., Tafvizi, F. & Noorbazargan, H. Anti-cancer effects of biosynthesized zinc oxide nanoparticles using *Artemisia scoparia* in Huh-7 liver cancer cells. *Inorg. Nano-Metal Chem.* **52**, 375–386 (2022).
61. Koopman, G. Annexin V for Flow Cytometric Detection of Phosphatidylserine Expression on B Cells Undergoing Apoptosis. 1415–1420 (1994).
62. Jolla, L. & Kingdom, U. Early Redistribution of Plasma Membrane Phosphatidylserine Is a General Feature of Apoptosis Regardless of the Initiating Stimulus : Inhibition by Overexpression of Bcl-2 and Abl. **182**, (1995).