

Biosynthesis of Gelatin-coated Zinc oxide nanocomposite from *Coccinia indica* extract and its antibacterial, antioxidant, anticancer and wound-healing properties

Kottaisamy Senthikumar

Periyar University

Manickam Rajkumar

Periyar University

Karuppaiya Vimala

Periyar University

Ramasundaram Thangaraj

National Institute for Research in Tuberculosis/Indian Council for Medical Research, Government Rajaji Hospital Madurai

Soundarapandian Kannan

skperiyaruniv@gmail.com

Periyar University

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Abstract

This present study deals with the synthesis of gelatin-loaded zinc oxide nanocomposites (GN/ZnONCs) using *Coccinia indica* seed extract using the green preparative method. Synthesized GN/ZnONCs were confirmed by using various characterization techniques for chemical and physical properties. The results of the UV-Vis spectral absorption peak showed 182 nm, and the FTIR study has confirmed the presence of various functional groups. The XRD study illustrated the crystalline structure of GN/ZnONCs. The DLS measurement indicated a stable size of 189.18 nm, with a zeta potential value demonstrating high stability at -30 mV. SEM images displayed the morphology of cauliflower-like spherical shapes and revealed their average particle size, respectively. The 100 µg/mL concentration of GN/ZnONCs exhibited higher antibacterial efficiencies against gram-positive and gram-negative bacteria. Subsequently, the GN/ZnONCs also exhibited excellent antioxidant properties in the DPPH assay ($92.61 \pm 2.45\%$), the ABTS assay ($76.35 \pm 1.54\%$), and the hydroxyl scavenging assay ($88.42 \pm 1.73\%$) at 100 µg/ml concentration. In addition, GN/ZnONCs treatment on the cancer cell line HT-29 showed good anticancer activity with an IC_{50} value of 45.4 µg/mL. Furthermore, wound healing experiments with enriched GN/ZnONCs with the highest contraction of 98.37 ± 1.45 confirmed faster wound healing and a greater role in the formation of new tissues and cells. The study indicates that green synthesized GN/ZnONCs had anti-bacterial, anti-cancer, and wound healing properties that were also eco-friendly and cost-effective, and the possible mechanisms were illustrated for biomedical applications.

1. Introduction

Nanotechnology is an advancing field of science and technology that focuses on metals and metal oxides at the nanoscale. It can be described as the synthesis and design of nanoscale structures, specifically nanoparticles, which have sizes ranging from 1 to 100 nanometers [1, 2]. These nanoparticles are produced using a variety of methods, predominantly categorized as chemical, physical, and biological approaches. Nanoparticles are renowned for their exceptional physical and mechanical properties. Their small size grants them distinct properties that can differ from those of bulk materials [3]. These characteristics render nanoparticles highly sought-after for diverse applications across various fields. Among the numerous types of nanoparticles synthesized for zinc, silver, gold, and copper nanoparticles. These nanoparticles have garnered significant attention due to their potential applications and the intriguing properties they possess [4].

Zinc oxide nanoparticles (ZnONPs) have found widespread application in the field of biomedicine, owing to their diverse physical, chemical, and biological characteristics. ZnO nanoparticles possess various characteristics that make them suitable for incorporation in biosensors [5]. By utilizing these nanoparticles, researchers can visualize cellular structures and processes, providing valuable insights into cellular behaviour and function. ZnONPs have found applications in various industries and possess significant health benefits. These nanoparticles have the potential to be an alternative to non-steroidal anti-inflammatory drugs due to their ability to elicit anti-inflammatory responses [6]. Moreover, these nanoparticles have exhibited strong antioxidant properties, indicating their potential for a wide range of therapeutic uses. Bacterial infections represent a substantial public health challenge [7]. Recent research suggests that nanoparticles, particularly ZnONPs, possess antimicrobial properties, photocatalytic activity, increased biocompatibility, and selectivity [8]. This makes them suitable for antimicrobial therapy, as they are durable and heat-resistant. ZnONPs are an essential trace element and micronutrient that plays a crucial role in insulin metabolism, including synthesis, secretion, and integrity [9]. It is important to note that the information provided is based on current research findings and observations regarding the applications and effects of ZnONPs. Ongoing studies may lead to further discoveries and advancements in the field [10].

The synthesis of nanoparticles offers a green, simple, flexible, and sustainable method that has gained significant attention in recent years. This approach allows for the improvement of the physicochemical properties and compatibility of nanoparticles when the synthetic processes are well-controlled [11]. One of the key advantages of using plant extracts is the ease and cost-effectiveness of extracting biomolecules compared to traditional synthesis methods. Most plant extracts contain a diverse array of biomolecules rich in various functional groups, including flavonoids, polyphenols, anthocyanins, terpenoids, and more [12, 13]. The use of plant extracts in nanoparticle synthesis allows for the incorporation of these beneficial biomolecules, potentially enhancing the therapeutic properties of the resulting nanoparticles. The green synthesis of nanoparticles using plant extracts aligns with the principles of sustainability and environmental protection [14]. By utilizing natural resources and avoiding the use of harsh chemicals, this approach minimizes the negative impact on the environment and reduces the generation of hazardous waste. Recently, ZnONPs have been widely used for the production of nanoparticles using plant extracts from different plant species [15]. Plants possess secondary metabolites and biomolecules that act as reducing, stabilizing, and capping agents in the synthesis of nanoparticles. The interaction of nanoparticles with biomolecules is affected by various factors, including size, flexibility, rigidity, core composition, shape, surface properties, purity, stability, biosensors, catalysis, and the production method [16]. These nanoparticles exhibit characteristics like antibacterial and

catalytic activity, making them useful in scientific domains and biomedical technology. Metal oxide nanoparticles, due to their substantial surface area, find applications in areas such as antimicrobial, anticancer, antineoplastic, wound healing, and antioxidant properties [10]. Zinc oxide nanoparticles have been synthesized using plant extracts from various sources, including *Aquilegia pubiflora* [17], *Cananga odorata* [18], *Elaeagnus angustifolia* [19] and *Salvia officinalis* [20]. These ZnO nanoparticles have been studied for their antimicrobial, anticancer, antineoplastic, wound healing, ultraviolet scattering and antioxidant activities. It is a straightforward, convenient, cost-effective, and environmentally friendly approach that minimizes the use of hazardous chemicals [21]. This approach is biologically safe, cost-effective, and environmentally friendly, making it suitable for various applications. Additionally, plants and microorganisms can accumulate and utilize inorganic metal ions, making them valuable in nanoparticle synthesis [22].

Gelatin (GN) hydrogel is an exciting and promising material in the realm of non-toxic biocompatible biopolymers. It is widely used as a surfactant in various nanoformulations due to its remarkable ability to significantly enhance their chemical and physical properties [23, 24]. Gelatin offers a convenient solution for surface stabilization through its ability to be easily applied as a thin coating, enlarging the steric barrier. While various organic coatings have been explored in the field of nanoscience, gelatin as a coating material has received limited attention [25]. Gelatin is widely utilized in biomedical applications because of its ability to biodegrade and its compatibility with living tissues. In previous research, the emphasis has been placed on metal oxides, such as ZnO, TiO₂, Ag, and MgO, with gelatin as the capping material [26, 27]. Among these metal oxides, gelatin-coated ZnO stands out due to its useful physical properties, including conductive coating efficacy. They possess essential trace metals, demonstrate consistent behaviour, possess a wide band gap, exhibit photoluminescence, and display predictable anti-oxidant properties. These features contribute to the potential clinical utility of ZnONPs. Reports also indicate that gelatin-bound ZnO nanoparticles are biodegradable, biocompatible, cost-effective, highly permeable, and have antimicrobial properties [28, 29].

Cancer, a leading cause of death worldwide, is characterized by the invasive growth of mutated cells in the body. The widespread prevalence of cancer highlights the urgent need for effective diagnosis and treatment methods [30]. While current treatment options such as surgery, chemotherapy, and radiation have their benefits, they also come with drawbacks such as tumour recurrence, hair loss, fatigue, and potential side effects leading to other diseases. Even after undergoing treatment, there is a risk of cancer spreading throughout the body. In light of this critical situation, there is a growing demand for non-toxic and traditional treatment techniques that offer hope for improved outcomes [31]. Although the application of nanoparticles in cancer research is still in its early stages, there is tremendous potential in emerging technologies to develop reliable anti-cancer treatments using green synthesized nanoparticles [32]. ZnONPs, known for their biocompatibility and biodegradability, have been proposed as a promising option for cancer treatment. ZnONPs have shown promising results in treating various cancer cell lines. Positive outcomes have been observed in PC3 and VCaP cell lines for prostate cancer [33], COLO205 cell lines for colon cancer, MCF7 cell line for breast cancer [34], PA1 cell line for ovarian cancer, and A431 cell line for epidermis carcinoma [35]. The use of green synthesized nanoparticles, including ZnO nanoparticles, holds great potential for cancer treatment. By utilizing the unique properties of nanoparticles, researchers are exploring innovative approaches to deliver targeted therapies, enhance drug delivery, and minimize the side effects associated with traditional treatment methods [36].

The wound-healing process is a complex phenomenon that involves a well-coordinated process with the secretion of many growth factors and hormones essential for the proliferation and migration of various cell types [37]. Impaired wound healing is a significant challenge for individuals, resulting in the development of chronic wounds and foot ulcers. This not only increases healthcare costs but also negatively affects the quality of life of these patients. The complications associated with diabetes and chronic wounds are multifactorial, including chronic inflammation, microbial infections, impaired growth factor secretion, and impaired angiogenesis [38]. Poor wound healing is influenced by a variety of factors, including impaired collagen production and inadequate migration, translocation, and proliferation of fibroblasts and keratinocytes, which are critical for epithelial growth. Recently, nanotechnology-mediated wound healing technology and optimism have received attention. More specifically, various studies have indicated that nanoparticle-loaded biomaterials promote faster wound healing due to their different biodegradability, swelling properties, and biocompatibility [39]. Despite the abundance of biomaterial reports, there is a dearth of hydrogel platforms specifically designed for tissue engineering applications. Several important aspects must be considered when developing biopolymer-based hydrogels for wound healing purposes. Advances in fluid leakage absorption, biocompatibility, scaffolding for cell adhesion, and, if possible, facilitation of mechanical transport for tissue regeneration are emerging [40].

The novelty of this present study, applied the green precipitation method to the synthesis of gelatin-coated zinc oxide nanoparticles (GN/ZnONCs) using *Coccinia indica* plant seed extract. The synthesized GN/ZnONCs were confirmed by their chemical and physical property characterization techniques. Furthermore, the antibacterial activity of GN/ZnONCs and their antioxidant activities were

evaluated. The cytotoxicity of GN/ZnONCs against HT-29 colon cancer cells was analysed. To evaluate the wound-healing activity of the GN/ZnONCs scaffold mechanism, it was evaluated using an *in vivo* wound healing in animal models.

2. Materials and methods

2.1. Synthesis of *Coccinia indica* plant seed extract

The *Coccinia indica* (*C. indica*) plant ripe fruit seeds were obtained from the botanical garden at Periyar University, Salem, Tamil Nadu, India. The ripe fruit seeds of *C. indica* were gathered and meticulously washed with distilled water to eliminate any contaminants or debris. Further, the seeds were left to air dry for approximately 10 days and then powdered using an electric blender. To prepare the extract, 5 g of seed powder was mixed with 100 ml of deionized water and boiled at 60 °C for 20 min. Then, the mixture was thoroughly filtered using Whatman No. 1 filter paper. The filtered extract was stored in the refrigerator for further experimentation.

2.2. Synthesis of GN/ZnONCs

The GN/ZnONCs were prepared using a previously described method with some minor modifications [41, 42, 43]. Initially, at a temperature of 50°C, gelatin (1% w/v) was completely dissolved in 100 mL of phosphate-buffered saline (PBS) solution by utilizing magnetic stirring. 20 mL of the aqueous seed extract of *C. indica* was mixed with 80 mL of a 5 mM zinc acetate ($\text{Zn}(\text{CH}_3\text{COO})_2 \cdot 2\text{H}_2\text{O}$) solution under continuous magnetic stirring. Further, pH 13 was adjusted by using 50 mL of 2 M NaOH. This resulted in the formation of a ZnONPs solution, which was then cooled and collected. In a conical flask, 80 mL of the ZnONPs solution mixture was taken, and 20 mL of gelatin solution was added. The mixture solution was then kept under magnetic stirring at 60°C for 2 hrs. Afterward, the mixture was incubated in a dark environment at a temperature of $26 \pm 2^\circ\text{C}$ for 24 hrs. During this incubation period, the color of the mixture changed from dark brown to light brown. To separate the composite, the sample solution was subjected to centrifugation at 10,000 rpm for 20 min. The resulting pellet was then subjected to deep drying using a lyophilizer at -80°C. Finally, the sample was stored at 4°C for future use.

2.3. Characterization of GN/ZnONCs

The synthesized GN/ZnONPs were confirmed using various spectroscopic techniques. UV-Vis spectrophotometry was employed to measure the absorbance values in the range of 200 to 500 nm, providing information about the optical properties of the synthesized nanoparticles. Fourier transform infrared (FT-IR) spectroscopy was utilized to identify the various functional groups present in the samples. This technique covers a range of 4000–400 cm^{-1} and helps in determining the chemical bonds and molecular structure. X-ray diffraction (XRD) analysis was conducted using a Rigaku D/Max III ultra-diffractometer to determine the crystalline structure of the GN/ZnONCs. The obtained data was aligned using dynamic light scattering (DLS) with zeta potential (ZP) and the Debye-Scherrer equation to calculate the particle size. To investigate the surface morphology and elemental composition of the GN/ZnONCs, scanning electron microscopy (SEM) coupled with energy-dispersive X-ray spectroscopy (EDX) was employed. SEM-EDX analysis provides high-resolution images and elemental mapping of the samples. Overall, these analytical techniques were used to confirm the synthesis of Ge/ZnONPs, characterize their optical properties, determine their crystalline structure, and analyze their surface morphology.

2.4. Antibacterial activity of GN/ZnONCs

The biosynthesized GN/ZnONCs were evaluated for their antibacterial activity against bacterial pathogens using the agar-well diffusion method [44]. The microbial cultures used in the study were maintained in the Department of Microbiology at Periyar University, Tamil Nadu. Both Gram-positive strains, for *Staphylococcus aureus* and *Bacillus subtilis*, and Gram-negative strains, for *Escherichia coli* and *Pseudomonas aeruginosa*, were selected for the study. To experiment, the pathogenic bacterial cultures were separately swabbed on Muller-Hinton agar plates. The GN/ZnONPs were dissolved in DMSO (dimethyl sulfoxide) at various concentrations ranging from 10 to 100 $\mu\text{g/mL}$. The GN/ZnONCs was administered to the wells created in the agar plate using a gel puncher. Streptomycin was utilized as a positive control in the study. Subsequently, the culture plates were placed in an incubator set at a temperature of 37°C and left undisturbed for a period of 24 hrs. After incubation, the Zone of Inhibition (ZOI), indicating the extent of bacterial growth inhibition around the wells, was measured in millimeters (mm).

2.5. Antioxidant activity

2.5.1. DPPH (2, 2-diphenyl-1-picryl-hydrazyl-hydrate) (DPPH) assay

The DPPH scavenging activity of the biosynthesized GN/ZnONCs was evaluated following a previously reported protocol [45]. In the experiment, vitamin C was employed as a positive control to serve as a reference or standard for comparison. Absorbance was recorded

at 517 nm using the UV-Vis spectrum. The scavenging activity of the GN/ZnONCs at each concentration was calculated as a percentage using the standard formula. The formula involves comparing the absorbance values of the samples to the absorbance of the control to determine the extent of DPPH scavenging activity.

$$\text{Inhibition of denaturation (\%)} = \left[\frac{\text{Absorbance (Control)} - \text{Absorbance (Sample)}}{\text{Absorbance (Control)}} \right] \times 100$$

2.5.2. ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) assay

The ABTS-scavenging activity of the samples was determined following a previously described protocol [46]. In brief, 1 mL of diluted ABTS solution was mixed with 100 μL of the various GN/ZnONCs sample concentrations ranging from 10 to 100 $\mu\text{g/mL}$. The mixture sample was then incubated for 10 min in the dark. For the blank, ethanol was added instead of the sample. Following the designated incubation period, the absorbance of the samples was assessed at a wavelength of 734 nm. Using the standard formula, the percentage inhibition of the ABTS scavenging activity was calculated.

$$\text{Inhibition of denaturation (\%)} = \left[\frac{\text{Absorbance (Control)} - \text{Absorbance (Sample)}}{\text{Absorbance (Control)}} \right] \times 100$$

2.5.3. Hydroxyl scavenging (H_2O_2) assay

The hydroxyl-scavenging activity of the GN/ZnONCs was determined following a previously described protocol [47]. In this experiment, different concentrations of GN/ZnONCs (ranging from 20 to 100 $\mu\text{g/mL}$) were mixed with 1.0 mL FeSO_4 (1.5 mM), 20 mM of 0.3 mL sodium salicylate and 6 mM of 0.7 mL hydrogen peroxide in separate test tubes. The mixture was then incubated at 37°C for one hour. After incubation, the absorbance was measured at 562 nm using a spectrophotometer. The scavenging activity was calculated using the standard formula:

$$\text{Inhibition of denaturation (\%)} = \left[\frac{\text{Absorbance (Control)} - \text{Absorbance (Sample)}}{\text{Absorbance (Control)}} \right] \times 100$$

2.6. Anticancer activity of GN/ZnONCs

The anticancer activity of the GN/ZnONCs was determined following a previously described protocol [48]. The HT-29 colon cancer cell line was maintained in DMEM low glucose media supplemented with 10% FBS along with the 1% antibiotic-antimycotic solution in the atmosphere of 5% CO_2 , 18–20% O_2 at 37°C in the CO_2 incubator and sub-cultured every 3 days. The HT-29 colon cancer cell lines were treated with different concentrations (0, 6, 12, 25, 50, and 100 $\mu\text{g/mL}$) of GN/ZnONCs. Before treatment, the HT-29 cells were cultured in DMEM medium supplemented with non-essential amino acids and incubated at 37°C with 5% CO_2 until they reached 90% confluence. Subsequently, the cells were seeded in a 96-well plate at a density of 4×10^3 cells/well. To assess the cytotoxicity of GN/ZnONCs, the HT-29 colon cancer cells were incubated with GN/ZnONCs for 24 hrs. Following this incubation period, the MTT assay was performed. Specifically, 100 μL of MTT solution was added to the cells and incubated for 4 hrs. Subsequently, 100 μL of dimethyl sulfoxide (DMSO) was added to dissolve the purple-colored crystals formed by the MTT. To measure the optical density (OD) at 620 nm, the plates were read using a multi-well plate reader (Bio-Rad). The percentage of cell viability was then calculated using a specific formula:

$$\text{Percentage of inhibition} = \frac{\text{Mean OD of treated cells}}{\text{Mean OD of untreated cells}} \times 100$$

After the incubation period, both the control and treated HT-29 colon cancer cells were centrifuged at 4000 rpm. This centrifugation step resulted in the formation of a pellet containing a cell suspension. The pellet was then washed twice with PBS. The treated cells were visualized using a fluorescence microscope analysis.

2.8. Wound healing activity

2.8.1. Animals

This research investigation adhered to the established guidelines and protocols, as ensured by the Institutional Animal Ethical Committee (PU-IAEC/2023/R/07) of Periyar University, Tamil Nadu, India. In the study, male Wistar rats with a weight range of 200 ± 10 g ($n = 6$) were utilized. The rats were housed in polypropylene cages under controlled conditions, including a temperature of $25 \pm 2^\circ\text{C}$, humidity of 45–55%, and a 12-hour light/dark cycle. Throughout the experiment, the guidelines provided by the Animal Ethics Committee were strictly followed to ensure minimal animal handling and the use of the lowest possible number of animals for the study.

2.8.2. Wound creation and wound healing activity (WHA)

The rats were categorized into five groups: Group I (control, treated with normal saline), Group-II (positive control, treated with Povidone Iodine Ointment), Group-III (Gelatin-1 mg/mL), Group-IV (ZnONPs-1 mg/mL), and Group-V (GN/ZnONCs- 1 mg/mL). To initiate the experiment, each rat was anesthetized using ketamine hydrochloride at a dosage of 80 mg/kg/bt.w, followed by an intraperitoneal injection of xylazine at a dosage of 10 mg/kg/bt.w. The hair on the rats' spines was completely shaved and washed thoroughly. A 30 mm length measurement was taken on the right side of the dorsal midline at a distance of 1.5 cm. The induced wounds on the rats were then treated with GN, ZnONPs, and GN/ZnONCs, respectively. The effects of these treatments were compared with those of both the positive control group and the control group. To assess WHA, the length and surface area of the wounds were measured. For surface area measurement, each rat was anesthetized using ether inhalation. To measure the wound surface, a method was employed using tracing paper and millimeter-grid paper. The measurement of wound contraction percentage was conducted on day 0 (the day of surgery), as well as on days 3, 7, 10, and 14. The measurements were calculated as a percentage of the healed wound area. The wound healing values were determined using the following formula:

$$\text{Wound contraction (\%)} = \frac{\text{Wound surface area (0) days} - \text{Wound surface area (x) days}}{\text{Wound surface area (0) days}} \times 100$$

2.8.3. Histopathological analysis

The experimental rats were anesthetized, and a biopsy was taken of a wound area approximately 5 mm in circumference on the skin surface. Also, all the experimental animals were sacrificed on day 14, and their wound skin area and vital organs (brain, heart, liver, kidney, and lung) were dissected and examined. The skin and organ tissues were preserved in a solution of neutral buffered formalin (10%) for 2–3 days. Subsequently, the tissues underwent processing and were embedded in paraffin. Thin sections of the tissue, measuring 3 μm in thickness, were prepared using a microtome. This section was then stained using hematoxylin and eosin (H and E). The stained sections were examined under a light microscope equipped with a camera to capture images of neovascularization, epidermis, scars, and granulation tissues [49].

2.9. Statistical analysis

Characterization studies were carried out using Origin Pro software, and Statistical analyses were conducted utilizing the GraphPad Prism software (San Diego, USA). A one-way analysis of variance (ANOVA) was used to assess the significance of the treatments. Additionally, Dunnett's test for multiple comparisons was employed to compare the differences between the control group and the treatment groups. The results were reported as the mean $\pm$ standard deviation (mean $\pm$ SD). A significance level of $P < 0.05$ was used for all probability tests to determine if there were any significant differences between the groups.

3. Results and discussions

3.1. Characterization study

3.1.1. UV-Vis spectral analysis

The GN/ZnONCs were synthesized using the seed extract of the reducing agent plant, *Coccinia indica*. The biomolecules present in the leaf extract interacted with zinc acetate, leading to the formation of a compound that initiated the process of reverse micelle formation. When zinc acetate was dissolved in water, it produced $\text{Zn}(\text{CH}_3\text{COO})_2 \cdot 2\text{H}_2\text{O}$ ions, resulting in a colorless solution. The synthesis of ZnO-NPs was evident by the appearance of a white precipitate within the micelle upon the inclusion of NaOH. To examine the visual properties of the biosynthesized GN/ZnO-NPs, UV-Vis absorption spectroscopy was employed. The biosynthesized GN/ZnO-NPs

exhibited a change in color from dark brown to light brown, indicating the surface plasmon resonance effect. The presence of an absorption peak at 273 nm further confirms the stability of the synthesized ZnONPs (Fig. 1A). The green synthesis of GN/ZnO-NCs relied on the ability of plant metabolites to act as both reductases and stabilizers. The color changes observed during the synthesis process can be attributed to the reduction of Zn acetate into GN/ZnO-NCs. UV analysis confirmed the presence of strong absorption peaks, similar to the strong absorption peaks (280 nm) observed in the gelatin/ZnO in the previous study [50].

3.1.2. FTIR analysis

FTIR spectra were used to analyze and confirm the functional groups present in the synthesized GN/ZnONCs using the seed extract from *C. indica* (Fig. 2B). The absorption peak at 3442 cm^{-1} corresponds to the stretching of strong amine (N-H) groups. The presence of stretching alkenes ($=\text{C-H}$) groups is indicated by the absorption peak at 2078 cm^{-1} . The strong absorption peak at 1612 cm^{-1} indicates the presence of alkene ($\text{C}=\text{C}$) groups. The absorption peak observed at 1384 cm^{-1} is associated with the stretching of nitro compounds (NO_2). The absorption peak at 1259 cm^{-1} is associated with the stretching of alkyl and aryl halides (C-F) groups. The stretching of alkyl and aryl halides (C-F) is also observed at 1023 cm^{-1} , represented by a strong absorption peak. The intense absorption band at 525 cm^{-1} corresponds to the stretching of alkyl and aryl halides (C-I) groups. In a recent report, the analysis of pomegranate fruit peel extract revealed the presence of aryl halides, carboxylic acids, primary amines, and alcohol, which were used to preparation GN and ZnONPs [51]. Another report on Ciprofloxacin-conjugated GN and ZnONPs showed the presence of functional groups such as O-H, $\text{C}=\text{C}$, and N-H [52]. Additionally, strong absorptions of hydroxyl and carboxyl groups in the synthesized *Phyllanthus niruri* using ZnONPs suggest that the nanoparticles absorbed moisture from the nearby atmosphere [53].

3.1.3. XRD analysis

The biosynthesis of the products involved the use of *C. indica* seed extract, and the phase purity and composition of these products were evaluated using XRD. Figure 1C illustrates a typical XRD pattern of ZnO nanoparticles, which is characterized by multiple Bragg reflections with specific 2θ values. These values include 31.734 , 34.138 , 36.583 , 47.197 , 56.638 , 62.285 , 68.221 , and 69.471 , corresponding to the 100, 200, 101, 102, 110, 103, and 113 crystallographic planes, respectively. The XRD pattern indicates that the ZnONPs were synthesized using an environmentally friendly method. The diffraction peaks observed in the XRD pattern can be indexed to the hexagonal wurtzite phase of ZnO, indicating an excellent crystalline nature of the sample. The only impurity observed, apart from nitrate, is GN/ZnO, which exhibits characteristic peaks. The broadening of the peaks in the XRD patterns of solids can be attributed to particle size effects, which is a typical phenomenon. Previous research using similar structures, such as (100), (101), (102), (110), and (103), also supports nanoparticle synthesis using *Myristica fragrans* aqueous fruit extract [54]. Recent reports of gelatin-ZnO nanocomposite have also confirmed the crystalline and optical investigation of particle size [51]. The differences observed in the shape, crystallinity, particle size, and quantity of the synthesized ZnO nanoparticles can influence the variations observed between these particles [55].

3.2. DLS with ZP analysis

The DLS analysis, as depicted in Fig. 2A, revealed that the synthesized GN/ZnONCs exhibited broad-intensity peaks ranging from 86 to 278.60 nm, with an average size of 182.11 nm. A polydispersity index (PI) value of 0 indicates a uniform sample, while a PI value of 1 suggests a heterogeneous sample. In the case of the GN/ZnONCs, the DLS results revealed a PI value of 0.25, indicating a comparatively heterogeneous distribution with varying sizes. In a study by Karkhane et al. (2023) it was reported that ZnONPs synthesized using *Sargassum wightii* extract exhibited a DLS size of 189.18 nm and a PI value of 0.42 for monodispersed [56]. The hydrodynamic diameter value is influenced by the impact of the seaweed extract on phytoconstituents. Furthermore, the synthesized GN/ZnONCs were subjected to ZP analysis. As shown in Fig. 2B, the ZP value of the synthesized ZnONPs was measured at -6.36 mV . A ZP value greater than $+10\text{ mV}$ and less than -30 mV indicates good stability. A significant negative ZP charge for the synthesized ZnONPs indicates their high stability. Therefore, it is suggested that these ZnONPs are suitable for various biological applications [57].

3.3. SEM-EDX and mapping analysis of synthesized GN/ZnONCs

The SEM images (Fig. 3A) reveal the formation of cauliflower-like spherical shapes for the synthesized GN/ZnONPs. This morphology suggests the successful synthesis of the nanoparticles. The EDAX profile of the synthesized GN/ZnONPs confirms their high compositional purity. The major elemental peaks detected in the EDX profile correspond to carbon (C), oxygen (O), and zinc (Zn) (Fig. 3A). The atomic and weight percentages of the detected elements are as follows: C (atomic: 38.28%, weight: 18.33%), O (atomic: 40.25%, weight: 25.68%), and Zn (atomic: 21.47%, weight: 55.98%) (Fig. 3B). These results indicate that the synthesized GN/ZnONPs contain an elemental profile of 9% C, 31% O, and 60% Zn. Elemental mapping analysis was performed to examine the spatial distribution of elements within individual particles. The luminance images obtained from the mapping technique revealed distinct colors for different

elements (Fig. 3C), indicating their presence in the synthesized GN/ZnONPs. It is important to note that the EDX profile provides an average elemental composition of the entire sample, while elemental mapping provides information about the spatial distribution of elements at the nano level. Therefore, differences in the percentage of elements between the two techniques can be attributed to variations in spatial resolution, sensitivity, and the limitations of every method. Overall, the SEM analysis and EDX profile, along with the elemental mapping, provide valuable insights into the morphology, elemental composition, and spatial distribution of elements within the synthesized GN/ZnONPs [58, 59].

3.4. Antibacterial activity

In this study, different concentrations of GN/ZnONPs were used to investigate their effects on various bacterial strains using the disk diffusion method. The findings revealed that the antibacterial activity increased as the concentration of nanoparticles increased. These results strongly suggest that the antimicrobial effect of the nanoparticles is dose-dependent. Interestingly, a concentration of 100 µg/mL demonstrated the most potent antibacterial activity (Figs. 4A, B, C, and D). Specifically, the results showed that the *B. subtilis* bacterial strain had a small ZOI with a value of 17.9 ± 0.93 mm, while the *E. coli* ZOI had a value of 18.2 ± 0.93 mm. *P. aeruginosa* exhibited a significant ZOI of 19.3 ± 0.98 mm, and *S. aureus* displayed the most powerful antibacterial activity with a ZOI of 19.8 ± 0.97 mm (Table 1). It is worth noting that previous research has shown that GPBs are generally more resistant to the bactericidal effects of GN/ZnONPs compared to GNB. This is likely due to the differences in their cell wall structures. GPB has a single layer of peptidoglycan, while GPB has a thicker peptidoglycan layer. The bactericidal mechanism of ZnO nanoparticles involves direct physical contact with the bacterial cell wall, resulting in the release of Zn²⁺ ions when ROS are produced. To evaluate the effectiveness of the antibacterial properties of GN/ZnONCs, various analytical techniques and synthesis procedures were employed. In the previous study, we also considered several variables that can influence antibacterial activity through the underlying mechanism. These findings align with similar research conducted on ZnONPs derived from *Silybum marianum* plant extract, which also exhibited a ZOI of 18 mm for antibacterial activity [60].

Table 1

Antibacterial activity of synthesized GN/ZnONCs at different concentrations (10, 25, 50, and 100 µg/ml) against *E. coli*, *P. aeruginosa*, *B. subtilis*, and *S. aureus*. The data are presented as mean ± SD values, and the statistical significance was determined by comparing the treatments to the positive control group (P < 0.05).

S. No.	Pathogenic microbes	Zone of Inhibition (mm) of GN/ZnONCs				
		Positive Control (<i>Streptomycin</i>)	Concentrations (µg/mL)			
			10	25	50	100
1.	<i>E. coli</i>	16.2 ± 0.82	5.2 ± 0.29	9.1 ± 0.51	14.1 ± 0.68	18.2 ± 0.93
2.	<i>P. aeruginosa</i>	12.4 ± 0.61	6.1 ± 0.37	9.8 ± 0.57	15.8 ± 0.75	19.3 ± 0.98
3.	<i>B. subtilis</i>	14.3 ± 0.73	6.7 ± 0.42	10.3 ± 0.64	14.6 ± 0.79	17.9 ± 0.94
4.	<i>S. aureus</i>	16.8 ± 0.87	7.2 ± 0.48	10.6 ± 0.71	14.9 ± 0.81	19.8 ± 0.97

3.5. Antioxidant activity

The demand for antioxidants has significantly increased in recent years due to their ability to prevent cellular damage caused by ROS and improve overall public health. These substances play a crucial role in scavenging free radicals and protecting cells from oxidative harm. In this study, biosynthesized GN/ZnONPs were evaluated for their ability to scavenge free radicals using the DPPH and ABTS assays, as well as their potential to inhibit H₂O₂ activity. The results showed that as the concentration of GN/ZnONPs increased, the DPPH cation radical scavenging activity also increased. At 100 µg/mL concentration, the radical scavenging activity was the highest, measuring 92.61 ± 2.45%, compared to vitamin C at 81.38 ± 2.02% (Fig. 5A). This finding aligns with previous research that observed a concentration-dependent increase in antioxidant activity with ZnONPs synthesized from *Tagetes Erecta* flower extract [61]. Similarly, the ABTS assay also demonstrated significant radical scavenging activity with GN/ZnONPs. At a concentration of 100 µg/mL, the ABTS cation radical scavenging activity was recorded at 76.35 ± 1.54%, while vitamin C showed an activity of 63.95 ± 1.17% (Fig. 5B). This result is consistent with previous findings using ZnONPs synthesized from *Strobilanthes cordifolia* plant extract [62]. Furthermore, the antioxidant potential of the GN/ZnONPs was assessed using an H₂O₂ assay, as H₂O₂ is known to be a potent ROS that can cause damage to biological systems. The study revealed that the GN/ZnONPs were capable of scavenging H₂O₂ in a concentration-dependent manner, as demonstrated in Fig. 5C. The highest inhibition of 88.42 ± 1.73% was observed at a concentration of 100 µg/mL, while vitamin C exhibited an inhibition of 79.51 ± 1.24%. These findings are consistent with a previous study using ZnONPs synthesized from

Hardwickia binata, which showed a scavenging efficiency of 55.86% for H₂O₂ activity [63]. Overall, the results of this study indicate that GN/ZnONPs possess significant antioxidant activity of 100 µg/mL. These findings contribute to the understanding of the potential applications of GN/ZnONPs as effective antioxidants in various fields.

3.6. Anticancer activity

In this study, we investigated the cytotoxic effect of biosynthesized GN/ZnONCs on the HT-29 cancer cell line. The study involved adding varying quantities of nanoparticles to the cells and incubating them for 24 hrs. It was observed that increasing concentrations (6.2, 12.5, 25, 50, and 100 µg/mL) of biosynthesized GN/ZnONCs resulted in a decrease in cell viability at a given dosage (Fig. 6A). Notably, the IC₅₀ value of GN/ZnONCs in the HT-29 cell line was determined to be 45.40 µg/mL, with a corresponding cell inhibition rate of 84.23% (Fig. 6B). In a research showed by Boskabadi et al. (2020), it was reported that treatment of HT-29 cells with green synthesized ZnONPs resulted in a dose-dependent decrease in cell viability (42 µg/mL) after 24 hrs, possibly interceded over the ROS generation [64]. Furthermore, recent reports highlighted that the sol-gel synthesis of ZnONPs exhibited an impressive 89% inhibition rate [65].

3.7. Wound healing activity

This study aimed to measure the WHA of control, PC, GN, ZnONPs, and GN/ZnONCs. The results showed that significant wound contraction occurred after 4 days of initial wound treatment. The faster wound healing areas were recorded for PC with a contraction of 93.76 ± 1.32, gelatin with a contraction of 95.36 ± 1.42, ZnONPs with a contraction of 92.63 ± 1.36, and GN/ZnONCs with a highest contraction of 98.37 ± 1.45, compared to 87.32 ± 1.28% in the control group on day 14 (Table 2). Interestingly, the wound healing potential of GN/ZnONCs, which incorporated ZnONPs, was even faster than the healing observed in the control and PC formulations. This trend was consistent on days 8 and 12, indicating that ZnONPs incorporated into the hydrogels significantly enhanced the wound healing capacity in the previous study. Additionally, the complete healing of wounds (re-epithelialization) was observed to be much faster in the group treated with GN/ZnONCs compared to other treatment groups. These findings were further supported by images captured using a camera, as shown in Fig. 7. Overall, the results demonstrate that GN/ZnONCs, with the incorporation of ZnONPs, exhibited superior wound healing potential compared to GN, ZnONPs, and control formulations. This study highlights the promising role of ZnONPs in accelerating wound healing when incorporated into gelatin, suggesting their potential application in wound care and regenerative medicine.

Table 2

Evaluation of the wound area (cm²) and wound contraction (%) of different experimental groups over a period of 14 days. The data are presented as mean ± SD values, and the statistical significance was determined by comparing the treatments to the positive control group (P < 0.05).

Observation Days	Wound Area (cm ²) (Mean ± SD) and % of Wound Contraction									
	Control		Positive Control		GN		ZnONPs		GN/ZnONCs	
	Area (cm ²)	Contraction (%)	Area (cm ²)	Contraction (%)	Area (cm ²)	Contraction (%)	Area (cm ²)	Contraction (%)	Area (cm ²)	Contraction (%)
0	6.36 ± 0.45	Nil	6.29 ± 0.52	Nil	6.83 ± 0.57	Nil	6.73 ± 0.55	Nil	6.58 ± 0.56	Nil
3	4.93 ± 0.34	42.19 ± 0.68	4.26 ± 0.45	46.38 ± 0.65	4.76 ± 0.42	49.35 ± 0.71	4.98 ± 0.41	47.25 ± 0.69	4.12 ± 0.34	58.39 ± 0.75
7	2.68 ± 0.29	57.43 ± 0.73	2.32 ± 0.39	65.19 ± 0.79	2.57 ± 0.34	68.43 ± 0.83	2.85 ± 0.36	66.39 ± 0.85	2.01 ± 0.28	74.26 ± 0.87
10	1.69 ± 0.22	79.14 ± 0.92	1.12 ± 0.25	88.61 ± 0.96	1.32 ± 0.20	90.29 ± 1.06	1.22 ± 0.24	88.48 ± 0.98	0.96 ± 0.19	89.52 ± 1.16
14	1.02 ± 0.15	87.32 ± 1.28	0.76 ± 0.18	93.76 ± 1.32	0.63 ± 0.14	95.36 ± 1.42	0.84 ± 0.13	92.63 ± 1.36	0.39 ± 0.11	98.37 ± 1.45

3.8. Histopathology

To observe the changes in skin tissue during wound healing, histological analysis was conducted using H and E staining. The results, presented in Fig. 8, revealed significant differences between the treatment groups and the control wounds. On days 7th and 14th, the group treated with ZnONPs incorporated into gelatin hydrogel showed scar formation with remarkable wound contraction, improved re-epithelialization, and significant migration of keratinocytes compared to the control wounds. The wound contraction was higher in the PC group at 93.76 ± 1.32 , the gelatin group at 95.36 ± 1.42 , the ZnONPs group at 92.63 ± 1.36 , and the GN/ZnONCs group at 98.37 ± 1.45 , compared to the control group at 87.32 ± 1.28 on day 14. In the GN/ZnONCs treatment group, moderate granulation tissues were observed, with a few inflammatory infiltrates and significant epidermal improvement. The plain gelatin-treated group also exhibited good cell proliferation in the wounded area and less scar formation. The positive control group showed a moderate collagen fiber pattern on the outer cell surface. In contrast, the control wounds showed no gain in the epidermal layer and displayed necrosis with mild inflammatory cells on the 7th day. By day 14th, the GN/ZnONCs-treated group displayed a well-formed dermal skin layer with a mixed collagen pattern, along with blood vessel and nuclei formation. The ZnONPs group exhibited a significant amount of granulation tissues, vertical collagen fiber orientation, and a high presence of inflammatory infiltrates. The ZnONPs group also demonstrated good formation of the dermal layer with a high rate of proliferating cell body maturation. These results support the hypothesis and provide substantial evidence for the synergistic potential of GN/ZnONCs treatment in accelerating wound healing in diabetes-induced animal models. The presence of more nuclei per field in the GN/ZnONCs group compared to other treatment groups indicates its effectiveness. Furthermore, according to a research by Rath et al. (2016), ZnONPs suspended in a polymeric matrix, such as gelatin, may be more suitable for treating skin wounds compared to ZnO nanoparticles [66]. Additionally, hydrogels are easy to apply and change, which enhances patient compliance. Previous researchers have also prepared topical formulations for delivering ZnONPs, further supporting the use of GN/ZnONCs in wound healing applications [67]. Also, as shown in Fig. 9, in the histological examination, the vital organ tissues and cells of various experimental mice were significantly improved. Nonetheless, the histopathological analysis of the experimental rats revealed no evidence of damage. Furthermore, the GN/ZnONCs treatment group promoted more healthy tissue and cell formation than the other groups. Thus, it further confirms that GN/ZnONCs are non-toxic and free from any side effects [49, 68].

4. Conclusion

In this study, the eco-friendly synthesis of GN/ZnONCs was conducted using the aqueous seed plant extract of *Coccinia indica*. The presence of GN/ZnONCs was confirmed through UV results at 273 nm, and FTIR analysis revealed the presence of various functional groups such as carboxylic acids, primary amines, anhydrides, and alcohol. XRD analysis confirmed the crystalline structure of GN/ZnONCs, and SEM studies identified their cauliflower-like spherical shapes. EDAX analysis confirmed the presence of different elements like C, O, and Zn in synthesized GN/ZnONCs. The antibacterial activities of GN and ZnONCs were found to be excellent against both GPB and GNB. Additionally, ZnONPs exhibited remarkable antioxidant properties in DPPH ($92.61 \pm 2.45\%$), ABTS assay ($76.35 \pm 1.54\%$), and H_2O_2 assay ($88.42 \pm 1.73\%$) at a concentration of 100 $\mu\text{g/ml}$. The anticancer activity of GN/ZnONCs suggests positive results against HT29 cells. Furthermore, *in vivo* experiments with enriched GN/ZnONCs confirmed faster wound healing and a greater role in the formation of new tissues and cells. The exact mechanism behind the accelerated wound healing due to the application of GN/ZnONCs was elucidated. The synthesis of GN/ZnONCs using *Coccinia indica* plant seed extract offers an eco-friendly and cost-effective approach. The resulting GN/ZnONCs demonstrate excellent antibacterial, antioxidant, and anticancer properties, making them suitable for various biomedical applications.

Declarations

Author contributions: Kottaisamy Senthilkumar: Conceptualization, investigation, methodology, writing and original draft. Manickam Rajkumar: Supervision, writing – review and editing. Karuppaiya Vimala: Formal analysis and methodology. Ramasundaram Thangaraj: Corrections and software analysis, Soundarapandian Kannan: Supervision, corrections, writing – review and editing.

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Ethics approval: The Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, approved all treatment and experimental procedures of the current study. All research and animal care procedures were in accordance with the Institutional Animal Ethical Committee (PU-IAEC/2023/R/07) of Periyar University, Tamil Nadu, India.

Consent for publication: All author agree for publication

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Figures

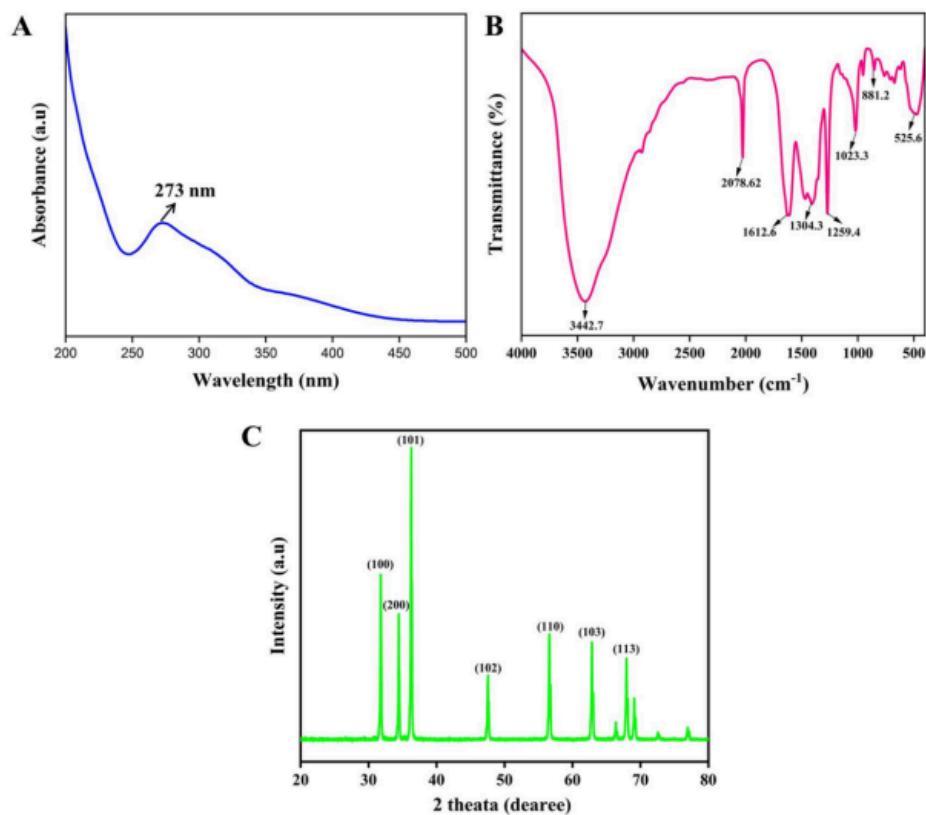


Figure 1

The synthesized GN/ZnONCs analysis of the UV-Vis spectrophotometer (A), FT-IR spectral analysis (B), and XRD pattern (C).

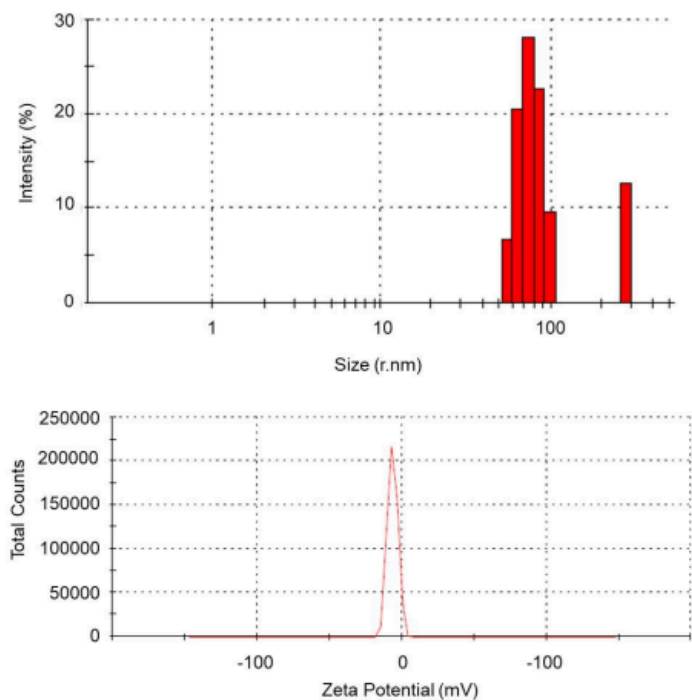


Figure 2

DLS analysis of synthesized GN/ZnONCs (A) and Zeta potential analysis (B).

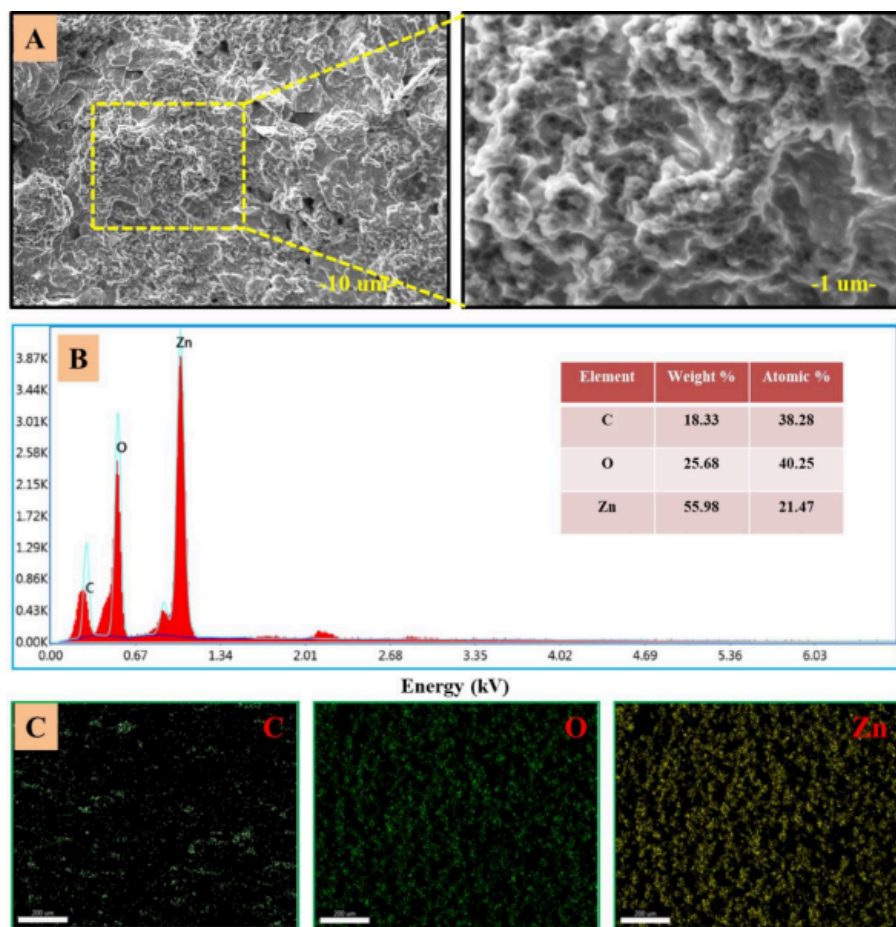


Figure 3

SEM morphological analysis of synthesized GN/ZnONCs (A), EDAX spectrum analysis (B), and elemental mapping analysis (C).

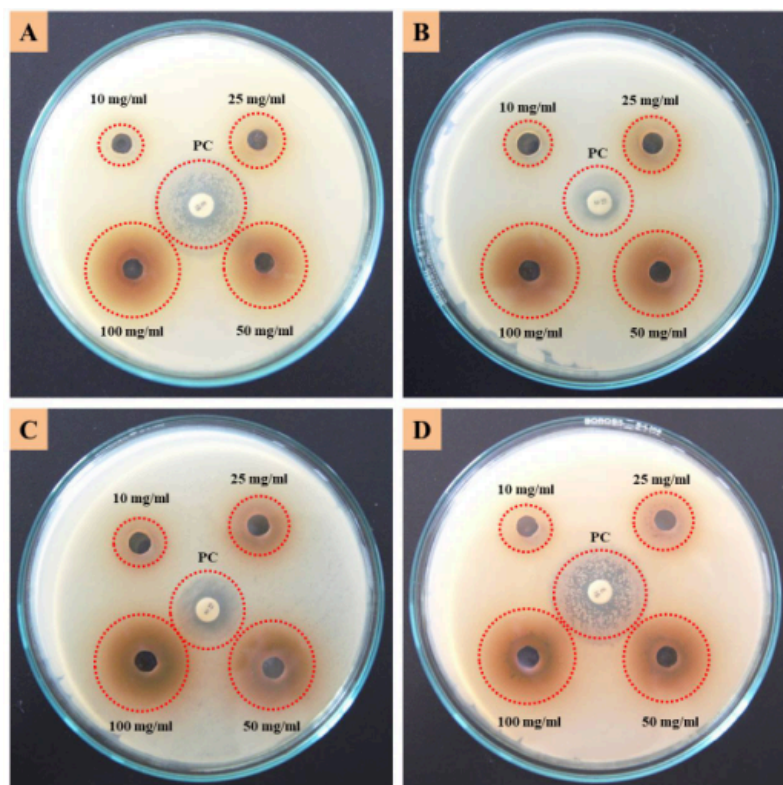


Figure 4

(A) Showing the results of the antimicrobial assay by disc diffusion method as the Zone of inhibition of GN/ZnONCs against *E. coli*, *P. aeruginosa*, *B. subtilis*, and *S. aureus*. All values are taken in triplicate and expressed as Mean $\pm$ SD. (B) % reduction in bacterial count of *E. coli*, *P. aeruginosa*, *B. subtilis*, and *S. aureus* against GN/ZnONCs.

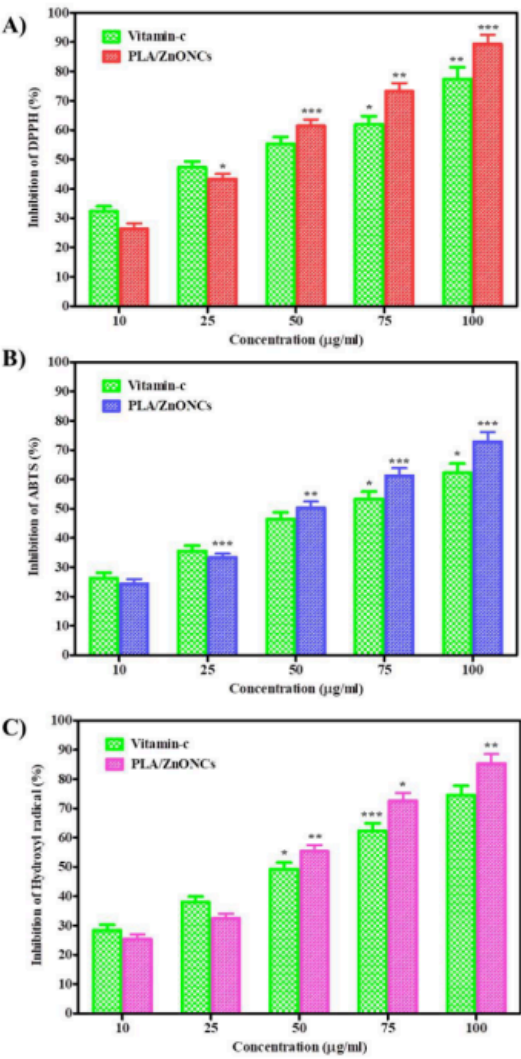


Figure 5

The synthesized GN/ZnONCs DPPH radical scavaging activity (A), ABTS activity (B), and H₂O₂ activity. Ascorbic acid was a standard. All values are expressed as mean $\pm$ SD, and the statistical significance was determined by comparing the treatments to the positive control group (P < 0.05).

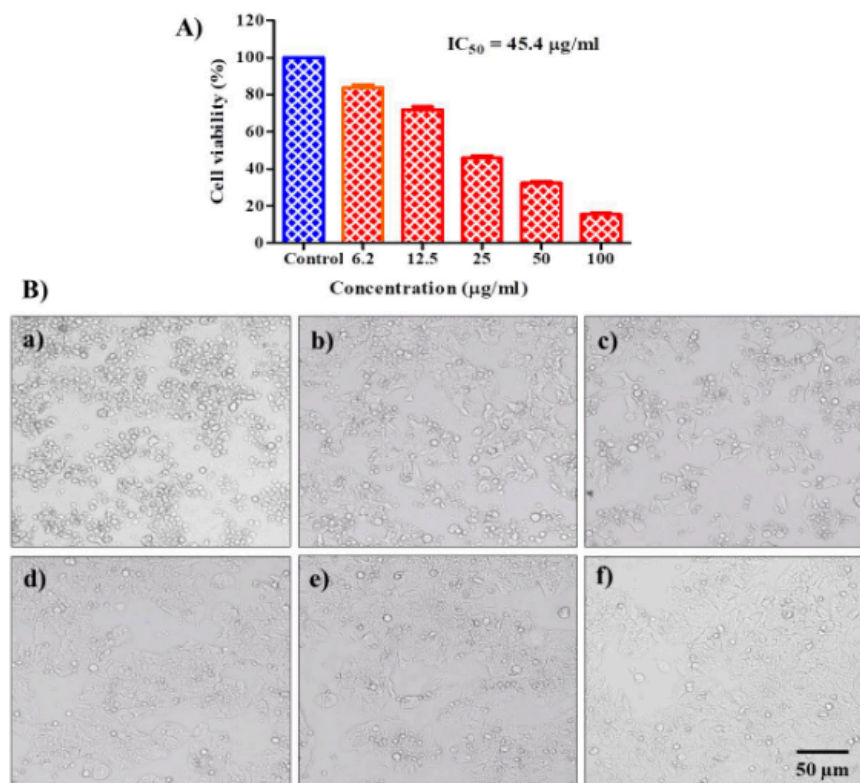


Figure 6

The anticancer effects of the synthesized GN/ZnONCs on the HT-29 colon cancer cell line were confirmed using the MTT assay (A). The anticancer activity of the GN/ZnONCs was evaluated at different concentrations: (a) control, (b) 6.2 µg/mL, (c) 12.5 µg/mL, (d) 25 µg/mL, (e) 50 µg/mL, and (f) 100 µg/mL. The cell morphology after treatment was visualized using a confocal microscope (B). The data are presented as mean $\pm$ SD values, and the statistical significance was determined by comparing the treatments to the control group ($P < 0.05$).

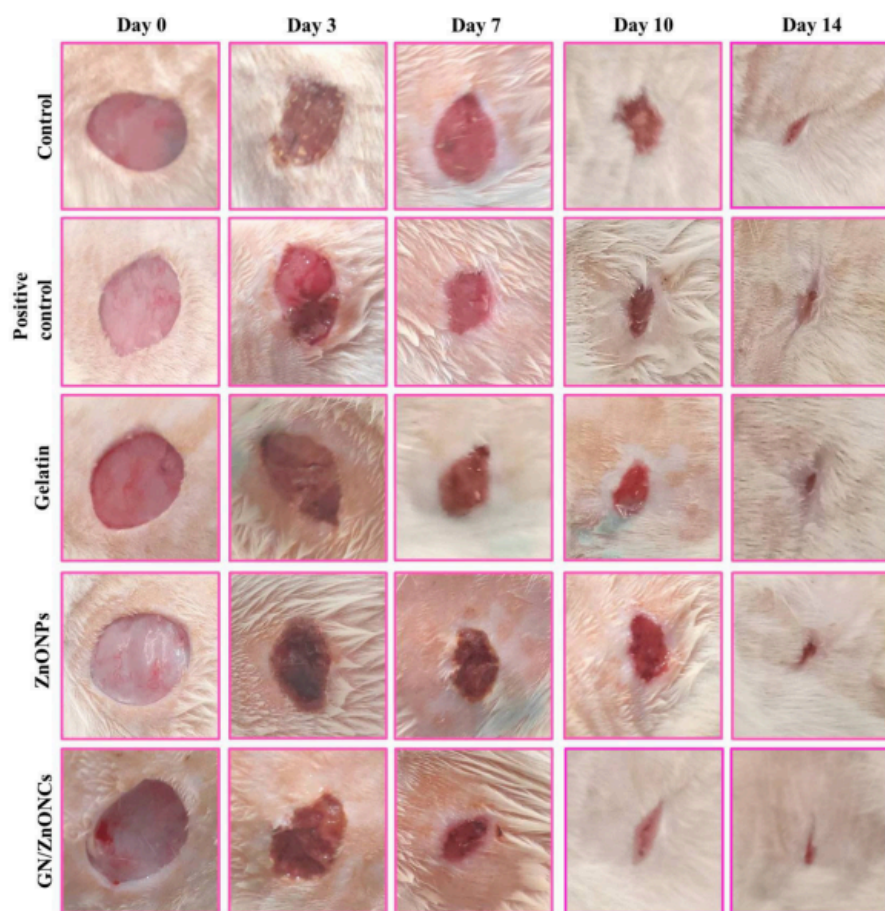


Figure 7

Macroscopic photographs of the wounds treated with the control, PC, and the gelatin, ZnONPs, and GN/ZnONCs specimens at different time periods.

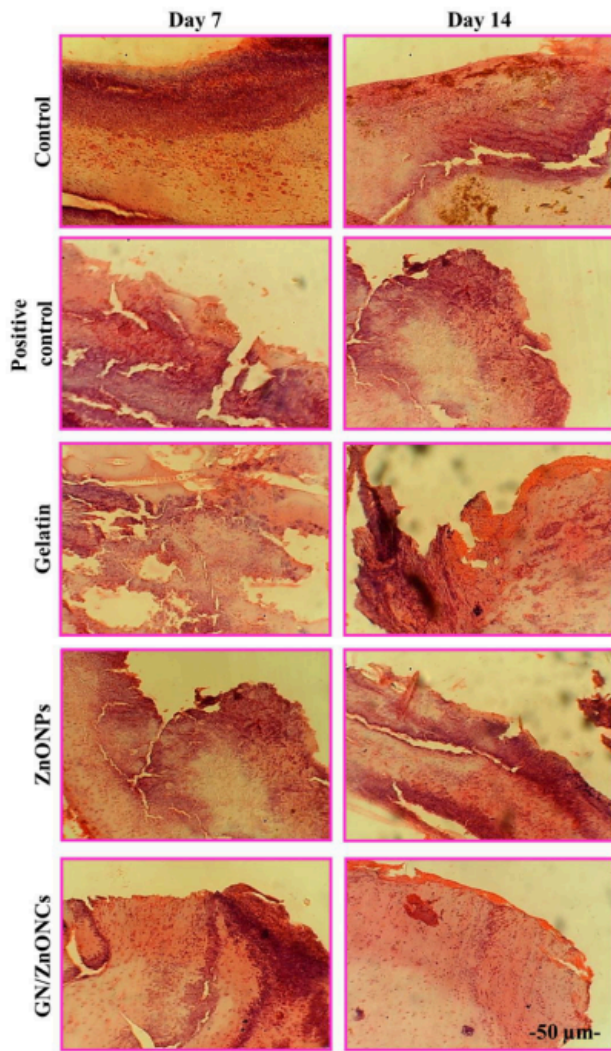


Figure 8

Histopathological studies of paraffin sections of biopsies of wounded areas on specific days were carried out through H and E staining. The microscopic images of all treatment groups on 7, and 14th days showed a significant difference between the control and experimental groups. Scale bar: 50 μm.

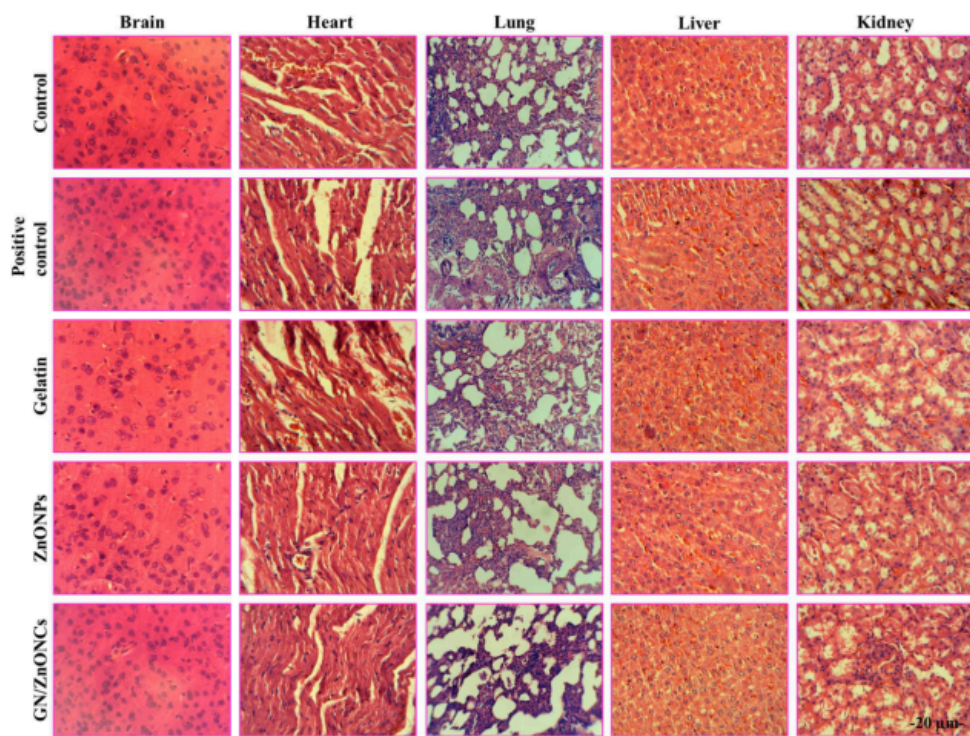


Figure 9

The H and E-stained images showed the vital organs (Brain, heart, lung, liver, and kidney) for different treatment groups. Scale bar: 20 μm .