

Selective Anticancer Activity of Zinc Nanoparticles Synthesized from Goat's Milk Processed *Berberis aristata* against Oral Squamous Cell Carcinoma

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Abstract: Oral squamous cell carcinoma (OSCC) remains a major global health challenge due to its high incidence and limited improvement in survival rates despite advances in conventional therapies. The present study aimed to synthesize zinc acetate nanoparticles (ZnO-NPs) using *BaLac*, a traditional Ayurvedic formulation of *Berberis aristata* processed in goat's milk, and to evaluate their anticancer efficacy against OSCC cells. Biosynthesis of ZnO-NPs was achieved using *BaLac* extract (BLZnO-NPs) as a reducing and stabilizing agent. The synthesized nanoparticles were characterized using UV-Visible spectroscopy, FTIR, X-ray diffraction (XRD), scanning electron microscopy (SEM), and transmission electron microscopy (TEM). UV-Vis analysis revealed a characteristic absorption peak at ~430 nm, confirming nanoparticle formation. XRD patterns demonstrated a crystalline hexagonal wurtzite structure with an average crystallite size of 17.7 nm. SEM and TEM analyses indicated predominantly near-spherical morphology with nanoscale dimensions, along with minor nanorod structures. We evaluated the anticancer activity of BLZnO-NPs using the MTT assay in KB oral carcinoma cells and compared it with cisplatin. BLZnO-NPs exhibited dose-dependent cytotoxicity, achieving significant inhibition at higher concentrations ($p < 0.001$). IC_{50} values of BLZnO-NPs was found to be 50.64 $\mu\text{g/mL}$, and that for cisplatin was 34.81 $\mu\text{g/mL}$. Importantly, BLZnO-NPs demonstrated good cytocompatibility towards L929 fibroblast cells, in contrast to cisplatin, which showed marked toxicity toward normal cells. Acridine orange/ethidium bromide dual staining confirmed that BLZnO-NPs induced apoptosis in KB cells, primarily at the early stage. In conclusion, *BaLac*-mediated biosynthesis yielded stable, crystalline ZnO-NPs with selective anticancer activity against OSCC cells and minimal toxicity to normal fibroblasts, underscoring the promise of integrating Ayurvedic formulations with nanotechnology for targeted, biocompatible cancer therapy.

Keywords: *Berberis aristata*; biosynthesis; oral squamous cell carcinoma; ayurveda; zinc nanoparticles;

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1. Introduction

Oral cancer remains a major global health concern, posing a persistent challenge to the biomedical community due to its high incidence. Although significant progress has been made in research and therapeutic strategies, overall survival rates have shown little improvement [1]. Approximately 90% of oral cancer cases are oral squamous cell carcinoma. Along with pharyngeal cancers and malignancies arising in various regions of the oral cavity, it ranks as the sixth most common cancer worldwide. Recent literature estimates indicate that 377,713 cases of oral squamous cell carcinoma were reported globally in 2020, with Asia accounting for nearly 60% of the total disease burden [2]. Oral cancer is commonly managed through surgical resection of the affected tissue, often followed by chemotherapy and radiotherapy; however, these costly interventions can impose substantial physical, psychological, and economic burdens on patients. Therefore, there is a continual demand for novel therapeutic strategies. A major focus of contemporary cancer research is developing drugs and technologies that selectively target cancer cells while sparing healthy tissues.

Nanotechnology has emerged as a transformative approach in modern medicine, particularly in cancer therapy, by enabling the design of nanoscale systems with improved targeting efficiency, enhanced bioavailability, and superior biocompatibility compared with conventional treatments such as surgery, chemotherapy, and radiotherapy [3]. These advantages have positioned nanomaterials as promising tools for selective anticancer interventions. Among nanomaterials, zinc nanoparticles have gained considerable attention because of their favorable physicochemical properties, ease of synthesis, and broad applicability in drug delivery, biosensing, and therapeutic applications. Their inherent biocompatibility, solubility, and ability to interact selectively with cancer cells make them attractive candidates for oncological research.

Recently, nanotechnology research has shifted toward the green synthesis of ZnO-NPs using traditional medicinal sources rich in bioactive phytochemicals. Plant-derived phytoconstituents act as natural reducing, stabilizing, and capping agents, enabling nanoparticle production in a sustainable, cost-effective, and environmentally benign manner [4-6]. This phytochemical-mediated synthesis not only minimizes the use of toxic solvents and hazardous chemicals but also enhances the stability and biological activity of the resulting zinc nanomaterials. ZnO-NPs. Consequently, ZnO-NPs synthesized using traditional medicinal formulations known for their efficacy in managing chronic disorders may offer a promising, safer therapeutic alternative, particularly in conditions where effective treatment options are limited.

Berberis aristata (*B. aristata*), a shrub belonging to the family *Berberidaceae*, is extensively used in Ayurveda for the management of a wide range of disease conditions. Berberine, the principal isoquinoline alkaloid present in its root bark, has been extensively studied for its diverse pharmacological activities, including anticancer, anti-inflammatory, and antioxidant properties [7]. Notably, several studies have shown tumor regression, chemoprotective effects, and downregulation of inflammatory mediators after treatment with different *B. aristata* extracts, particularly in OSCC models [8,9]. This study also focused on the whole-drug concept, as mentioned in Ayurveda literature, rather than an isolated compound from the formulation. Single phytochemical compounds from plants may be selected because

of their strong ability to treat human illnesses, but they pose certain drawbacks, one of them being the phenomenon explained as the extract's lack of interacting chemical compounds [10,11]. However, the majority of these biologically active phytochemical components in herbal formulations have certain drawbacks, such as limited distribution, low absorption rates, and generally low target specificity, which leads to reduced bioavailability and biological activity. To compensate this, high dosages are necessary to generate the action of these phytochemical substances [12]. Drug delivery systems based on nanotechnology can increase the bioavailability and bioactivity of phytochemicals, among other constraints that herbal medicines encounter [13]. Considering all these aspects, *BaLac*, an Ayurvedic formulation of *B. aristata* processed in goat's milk, which is known for its traditional use in the management of various oral cavity disorders, including tumors [14], was employed for the Biosynthesis of ZnO-NPs, and subsequently, its anticancer efficacy was also evaluated. KB cells were originally derived from an oral epidermoid carcinoma and continue to be widely used in oral cancer-related research. We have used KB cells in screening the anticancer activity of BLZnO-NPs on OSCC cells, as several studies have utilized these cells in investigations pertaining to oral epithelial carcinoma biology, drug screening, and nanoparticle toxicity. Despite HeLa cross-contamination, KB cells retain the major characteristics of epithelial carcinoma.

2. Materials and Methods

2.1. Plant material and cell line.

The *B. aristata* root bark used in this study was collected from Jammu and Kashmir's Poonch area (Latitude: 33° 46' 12.00" N, Longitude: 74° 05' 60.00" E), India. Oral squamous carcinoma cells (KB cells) were procured from the National Center for Cell Science (NCCS), Pune, India. This is a certified repository, wherein regular quality control procedures, including authentication and mycoplasma testing, are performed before supply. The cells were used within limited passage numbers after receipt to minimize phenotypic drift. They were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% antibiotic-antimycotic solution, and 1% L-Glutamine. Cells were maintained in a humidified atmosphere at 37°C and 5% CO₂. After attaining 70% confluence, they were subcultured using trypsinization and used for the experiments after three consecutive passages.

2.2. Preparation of *BaLac*.

B. aristata roots, which were free from any diseases and fully grown, were collected. The specimen was authenticated, and a voucher specimen (No: YENDG01) was deposited at our institution's pharmacognosy department. To obtain *BaLac*, classical Ayurvedic preparation was followed [15]. 1 part of the dried root of *B. aristata* was crushed and boiled with 16 parts of water. The resulting solution was evaporated until barely one-eighth of its original volume remained and subsequently filtered. The mixture was combined with an equal quantity of goat's milk and heated until it reached an approximate solid consistency. The end product was subsequently dehydrated in a high-temperature oven at 40-50°C for a duration of 72 hours and transformed into a powdered form. Then, 10 g of powdered *BaLac* was heated in 100 mL of distilled water for 15 minutes in a conical flask. After cooling, the extract was filtered and centrifuged at 3000 rpm for 5 min. The supernatant was used as a fresh extract for the synthesis of nanoparticles.

2.3. Biosynthesis of zinc nanoparticles.

Zinc acetate (1 mM), sodium hydroxide (1M), and an ethanol–water mixture (1:1) were combined to prepare a 1 mM nano solution. Subsequently, *BaLac* extract (1 mg/mL) was added dropwise to 100 mL of zinc acetate solution. The solution was kept stirring until it turned yellow. The solution was centrifuged at 6000 rpm for 20 minutes at 28 °C. Then, the sediment was separated and dried at 45 °C for 24 hours.

2.4. Characterization of zinc nanoparticles.

2.4.1. Spectroscopy.

The synthesized BLZnO-NPs were initially characterized by UV–Visible spectroscopy. Spectral analysis was performed over a wavelength range of 200–800 nm to detect the characteristic absorption peak to confirm the formation of Zinc oxide (ZnO) nanoparticles. Fourier Transform Infrared Spectroscopy (FTIR) analysis (Shimadzu QATR-S) was used to detect the functional groups on the surface of BLZnO-NPs. The spectra were scanned between the 4000–450 cm⁻¹ for dried BLZnO-NPs.

2.4.2. X-ray diffraction.

X-ray diffraction (XRD) (Empyrean diffractometer (3rd-Gen), Malvern Panalytical, Netherlands) was used to analyze the crystal structure of dehydrated as-prepared BLZnO-NPs with an operating voltage and current of 40 KV and 30 mA, respectively. The test was conducted according to the ASTM E2926-13 standard with an angular range of 25° to 75°, and the data were analyzed in X'Pert HighScore Plus software according to the ASTM E2626-09 standard, referencing the previously reported articles [16,17]. The crystallite size was calculated using the Debye-Scherrer formula as presented in Equation 1.

$$D = K\lambda / \beta \cos\theta \quad (1)$$

Where D is the crystallite size, k is a dimensionless shape factor with a value close to unity (~ 0.94), λ is the wavelength of the radiation (0.15405 nm for Cu K α), θ is the Bragg's angle in radians (half of the 2θ value of the chosen peak), and β is the full width at half maximum (FWHM) in radians.

2.4.3. Microscopy.

Two complementary microscopy techniques were employed to characterize the BLZnO-NPs. The mean particle size and surface morphology were determined using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). For SEM analysis (Zeiss Evo, UK), freeze-dried BLZnO-NPs were dispersed in distilled water by sonication. A small aliquot of the suspension was drop-cast onto a glass slide and allowed to air-dry. The dried samples were then sputter-coated with a thin layer of platinum under a vacuum of approximately 10⁻⁵ torr to improve electrical conductivity before imaging. The microscope was operated at an accelerating voltage of 10–20 kV, and the obtained micrographs were analyzed using ImageJ software to determine particle size distribution.

BLZnO-NPs were further characterized using TEM (JEM-2100, JEOL, Japan) to confirm particle size and morphology and to correlate the observations with SEM findings. Sample preparation was performed in accordance with ASTM E2864-13 guidelines, with reference to previously reported methodologies [18-20]. A nanoparticle suspension was

prepared using deionized water as the solvent in a 1:20 (w/w) solute-to-solvent ratio, followed by ultrasonication to ensure uniform dispersion. A drop of the well-dispersed suspension was placed onto a 300-mesh carbon-coated copper grid and allowed to dry for 50 h to ensure complete solvent evaporation, leaving a uniform nanoparticle layer. TEM analysis was conducted at an operating voltage of 200 kV with a beam current ranging from 102 to 105 μ A. Selected area electron diffraction (SAED) patterns and high-resolution TEM (HR-TEM) images were acquired at multiple regions to verify the crystalline nature and characteristic lattice planes of ZnO. The resulting TEM micrographs were analyzed using ImageJ and OriginPro 2025 software to determine particle size distribution.

2.5. Assessment of anticancer activity.

2.5.1. MTT assay.

The cytotoxic potential of BLZnO-NPs was evaluated using the MTT assay, following a previously established protocol. Briefly, KB oral carcinoma cells were seeded in 96-well microtiter plates at a density of 5,000 cells per well and incubated at 37 °C in a humidified atmosphere containing 5% CO₂ to allow cell attachment. After adherence, the cells were treated with varying concentrations of BLZnO-NPs and incubated for 24 h. Following treatment, the spent medium was removed, and MTT reagent was added to each well. The plates were further incubated for 4 h to allow the formation of formazan crystals. The resulting crystals were dissolved in dimethyl sulfoxide (DMSO), and absorbance was measured at 570 nm using a multimode microplate reader (FluoSTAR Omega, BMG Labtech). The percentage cytotoxicity was calculated relative to the untreated control group.

2.5.2. Evaluation of cytotoxicity on L929 fibroblasts.

L929 fibroblast cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic–antimycotic solution. Cells were subcultured at approximately 70% confluence using a standard trypsinization protocol. The cytotoxicity of the extract and synthesized nanoparticles was evaluated using the MTT assay. Briefly, L929 cells were seeded at a density of 5,000 cells per well in 96-well microtiter plates and incubated at 37 °C in a humidified atmosphere containing 5% CO₂ to facilitate attachment. After adherence, cells were treated with varying concentrations of the test compounds and incubated for 24 h. Following treatment, the spent medium was removed, and MTT reagent was added to each well, followed by an additional 4-hour incubation to allow formazan crystal formation. The crystals were dissolved in DMSO, and absorbance was measured at 570 nm using a multimode microplate reader. Percentage cell viability was calculated relative to the untreated control.

2.5.3. Apoptosis detection using acridine orange-ethidium bromide (AO/EB) dual fluorescence staining.

KB cells were seeded in 24-well plates at a density of 20,000 cells per well and incubated at 37 °C in a humidified atmosphere containing 5% CO₂ to allow cell attachment. Following adherence, the cells were treated with the IC₅₀ concentration of BLZnO-NPs and incubated for an additional 24 h. Subsequently, cells were stained with an AO/EB mixture (1:1; 2 μ g/mL) and incubated for 20 min in the dark at 37 °C. The staining solution was then

removed, and excess dye was washed off with 1× phosphate-buffered saline (PBS). Cells were overlaid with 1× PBS, and fluorescence images were captured using a fluorescence imager (ZOE, Bio-Rad) under green and red channels at 20× magnification.

2.6. Statistical analysis.

All the experiments were conducted as three independent biological replicates and technical triplicates, and the results were expressed as Mean ± SD (n=3). Two-way ANOVA was performed followed by sidak's multiple comparison test. A significance level of $p < 0.05$ was considered statistically significant. All analyses were performed using GraphPad Prism.

3. Results and Discussion.

3.1. UV-Vis spectroscopy.

The optical properties of BLZnO-NPs were evaluated using UV–Visible spectroscopy. The green synthesis of zinc nanoparticles was monitored over a wavelength range of 200–800 nm. The absorption spectrum of the synthesized BLZnO-NPs is presented in Figure 1. A distinct absorption peak was observed at approximately 430 nm, confirming nanoparticle formation. The position and intensity of the absorption band are indicative of the particle size, morphology, and dispersion characteristics of the synthesized zinc nanoparticles. Notably, the precursor zinc acetate solution did not exhibit any characteristic absorption peak within the scanned range, confirming that nanoparticle formation occurred only after biosynthesis.

3.2. FTIR Analysis.

The FTIR spectrum of BLZnO-NPs (Figure 1b) reveals characteristic absorption bands arising from molecular vibrations specific to functional groups present in the sample. Spectral analysis was performed in the range of 400–4000 cm^{-1} . An absorption peak at 2824 cm^{-1} corresponds to C–H stretching vibrations, while the band observed at 1709 cm^{-1} is attributed to C=O stretching of carbonyl functional groups. The peak at 1527 cm^{-1} is associated with aromatic C=C stretching vibrations. A distinct absorption band at 453 cm^{-1} , within the 400–600 cm^{-1} region, confirms Zn–O stretching vibrations, thereby validating the successful formation of ZnO (Zinc oxide) nanoparticles. The presence of these functional groups suggests that phytochemicals from *B. aristata* might play a crucial role in the biological reduction of Zn^{2+} ions and in the stabilization of the synthesized BLZnO-NPs [21–23].

3.3. X-ray diffraction.

The XRD data of the as-prepared sample is presented in Figure 1c. The corresponding crystallite size analysis is also presented in the XRD plot. Notably, nine distinct peaks at 2θ values of 31.9°, 34.5°, 36.4°, 47.6°, 56.7°, 62.9°, 66.5°, 68.1°, and 69.2° corresponds to (100), (002), (101), (102), (110), (103), (200), (112), and (201) planes, respectively are observed in the XRD plot. The indexing of the peaks is done by referencing the ICDD-00-036-1451 database and the previously reported articles [24,25]. Three planes with prominent intensities were observed at (100), (002), and (101). These planes are considered for obtaining the mean crystallite size value of the as-prepared sample. The mean crystallite size is observed to be 17.7 nm, as shown in Figure 1c. Interestingly, the XRD data show no impurity peaks, confirming successful synthesis of the desired phase without any secondary phase.

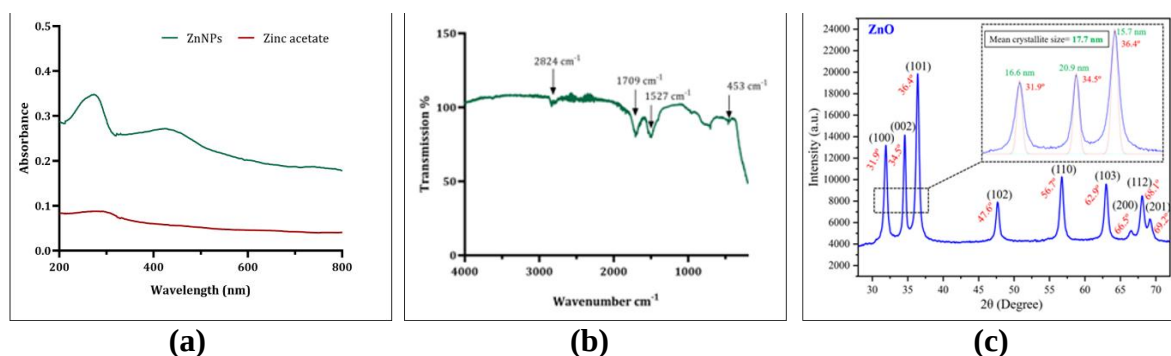


Figure 1. The absorption spectrum of BLZnO-NPs. **(a)** UV-Visible spectroscopy showed a peak at 430 nm, confirming Zinc nanoparticle formation. **(b)** FTIR analysis revealed functional units on the nanoparticles. **(c)** XRD plot for the as-prepared nanoparticles presented the characteristic peaks and crystallite size analysis.

3.4. Microscopic analysis.

The microscopy analyses presented in Figure 2 substantiate the morphology and particle size of the synthesized BLZnO-NPs. SEM micrographs (Figure 2b–c), acquired at low and high magnifications, reveal predominantly near-spherical nanoparticles with a relatively uniform distribution. Particle size analysis based on SEM images indicates a mean diameter of approximately 34 nm. Consistent with SEM observations, TEM micrographs (Figure 2d–e) further confirm near-spherical nanoparticles, with a smaller mean particle size of ~20 nm, suggesting finer structural resolution under TEM.

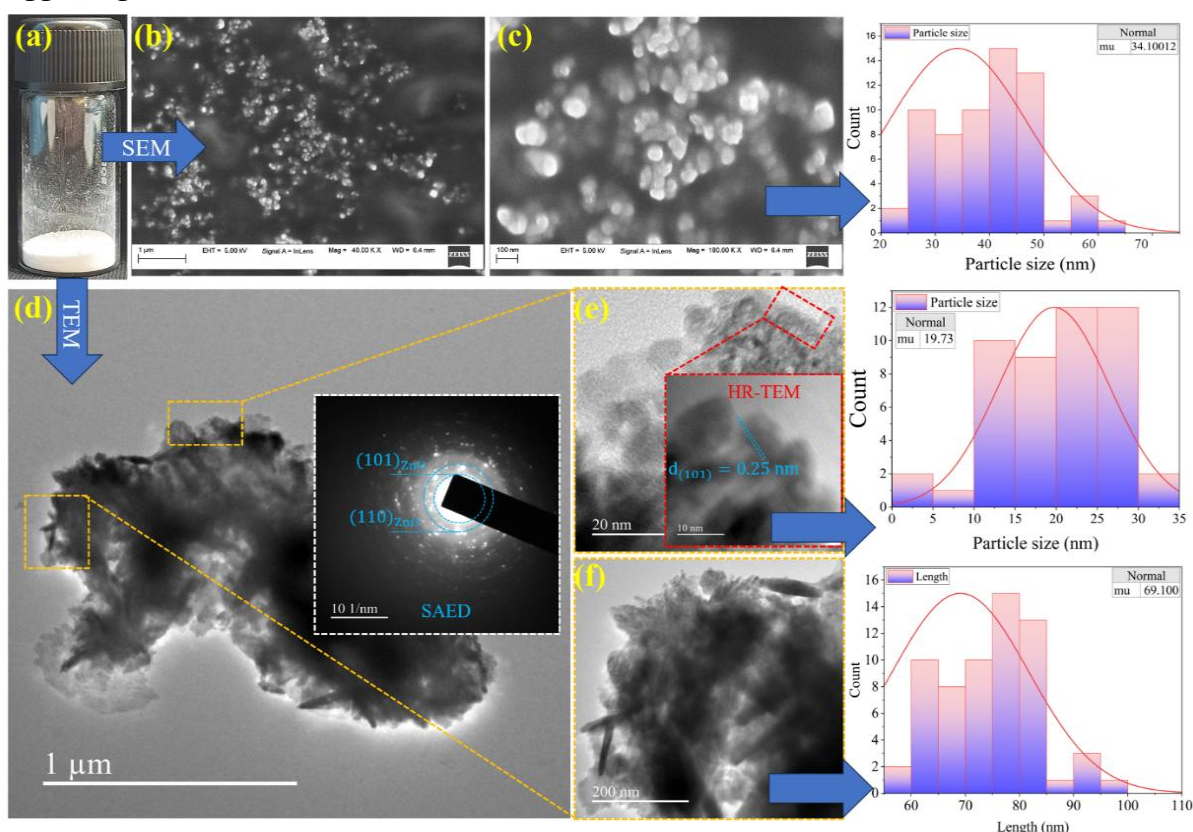


Figure 2. Morphological and Structural Characterization of BLZnO-NPs by SEM and TEM Analyses. **(a)** Visual appearance of synthesized nanoparticles; **(b)** and **(c)** SEM micrographs at low and high magnifications with corresponding particle size distribution histogram (mean $\approx$ 34 nm); **(d)** TEM image showing overall morphology with inset SAED pattern confirming crystalline ZnO planes ((101) and (110)); **(e)** HR-TEM image displaying lattice fringes with d-spacing of 0.25 nm corresponding to the (101) plane; **(f)** TEM image highlighting nanorod morphology with corresponding length distribution histogram (mean $\approx$ 69 nm).

The selected area electron diffraction (SAED) pattern demonstrates distinct diffraction rings corresponding to the (101) and (110) crystallographic planes of ZnO, confirming the crystalline nature of the nanoparticles. HR-TEM analysis (Figure 2e) further validates this finding, with clearly resolved lattice fringes exhibiting a d-spacing of 0.25 nm, characteristic of the (101) plane of ZnO. TEM images also reveal nanorod-shaped structures with an average length of approximately 69 nm (Figures 2d and 2f). Similar mixed morphologies have been reported in previous studies [26,27]. The coexistence of nanospheres and nanorods may enhance biomedical performance, as anisotropic nanostructures have been shown to influence cellular interactions and therapeutic efficacy [28].

3.5. MTT assay.

The cytotoxic effects of BLZnO-NPs and cisplatin were evaluated in KB oral carcinoma cells over a concentration range of 12.5–1000 µg/mL (Figure 3a). Both agents exhibited a clear dose-dependent increase in cytotoxicity. BLZnO-NPs induced $15.17 \pm 1.66\%$ cytotoxicity at 12.5 µg/mL, which progressively increased with concentration, reaching a maximum of $82.84 \pm 1.47\%$ at 1000 µg/mL. Cisplatin also demonstrated concentration-dependent cytotoxicity, producing $63.24 \pm 0.19\%$ inhibition at the lowest tested dose (12.5 µg/mL). The cytotoxic effect increased with concentration, peaking at 500 µg/mL ($85.10 \pm 0.03\%$) and remaining relatively stable at 1000 µg/mL ($85.48 \pm 0.11\%$). IC₅₀ values of BLZnO-NPs were found to be 50.64 µg/mL, and that for cisplatin was 34.81 µg/mL.

The cytocompatibility of BLZnO-NPs was further assessed in L929 murine fibroblast cells across a concentration range of 12.5–200 µg/mL (Figure 3b). BLZnO-NPs maintained cell viability at $\geq 95\%$ across all tested concentrations, indicating favorable cytocompatibility and minimal toxicity toward normal fibroblasts.

In contrast, cisplatin significantly reduced L929 cell viability in a dose-dependent manner. Cell viability was $87.31 \pm 1.22\%$ at 12.5 µg/mL and decreased to $78.68 \pm 0.78\%$ at 25 µg/mL. A pronounced reduction in viability was observed at higher concentrations, declining to $12.72 \pm 0.70\%$ at 100 µg/mL and $10.34 \pm 0.53\%$ at 200 µg/mL, demonstrating substantial cytotoxic effects on normal fibroblasts.

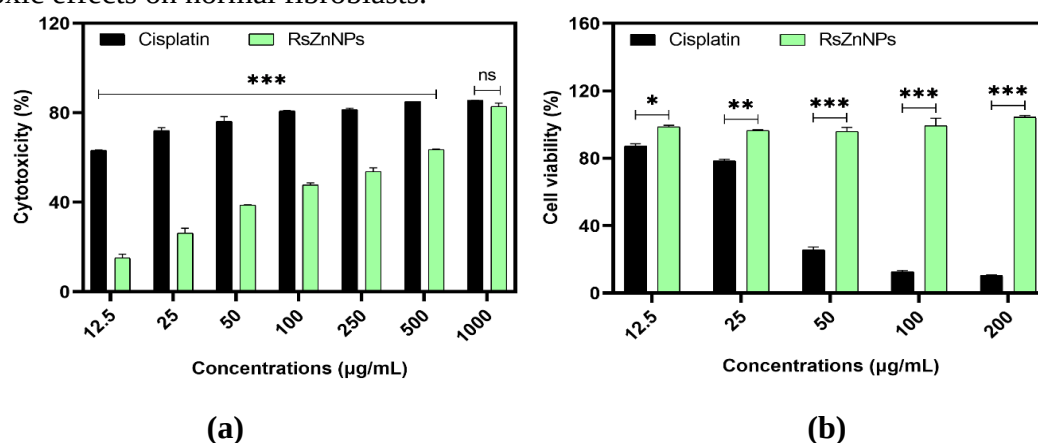


Figure 3. Comparative evaluation of cytotoxicity and cytocompatibility of *BaLac*-derived zinc nanoparticles (BLZnO-NPs) and cisplatin, evaluated using MTT assay. **(a)** Dose-dependent cytotoxic effects of BLZnO-NPs and cisplatin on KB oral carcinoma cells (12.5–1000 µg/mL). **(b)** Cell viability of L929 fibroblast cells following treatment with BLZnO-NPs and cisplatin (12.5–200 µg/mL), demonstrating selective cytocompatibility of BLZnO-NPs toward normal cells. Values are presented as mean cell viability $\pm$ SD ($n = 3$). Statistical significance was determined using two-way ANOVA followed by sidak's multiple comparison test (***) $p < 0.001$, (**) $p < 0.01$ and (*) $p < 0.05$ when compared to cisplatin)

3.6. Detection of apoptosis using AO/EB staining.

The acridine orange/ethidium bromide (AO/EB) dual fluorescence staining assay was performed to elucidate the mechanism of cell death induced by BLZnO-NPs at their IC₅₀ concentration in KB oral carcinoma cells. Fluorescence microscopy analysis revealed distinct morphological features consistent with apoptosis.

Untreated control cells showed uniformly green fluorescent nuclei, indicating viable cells with intact membrane integrity. In contrast, cells treated with BLZnO-NPs exhibited greenish-yellow fluorescent nuclei, reflecting early apoptotic changes characterized by chromatin condensation and nuclear fragmentation. A marked increase in the proportion of early apoptotic cells was observed in the treated group compared with the control.

These observations indicate that BLZnO-NPs induce cytotoxicity predominantly through the activation of early apoptotic pathways in KB cells. Representative photomicrographs of control and BLZnO-NPs-treated KB cells are presented in Figure 4.

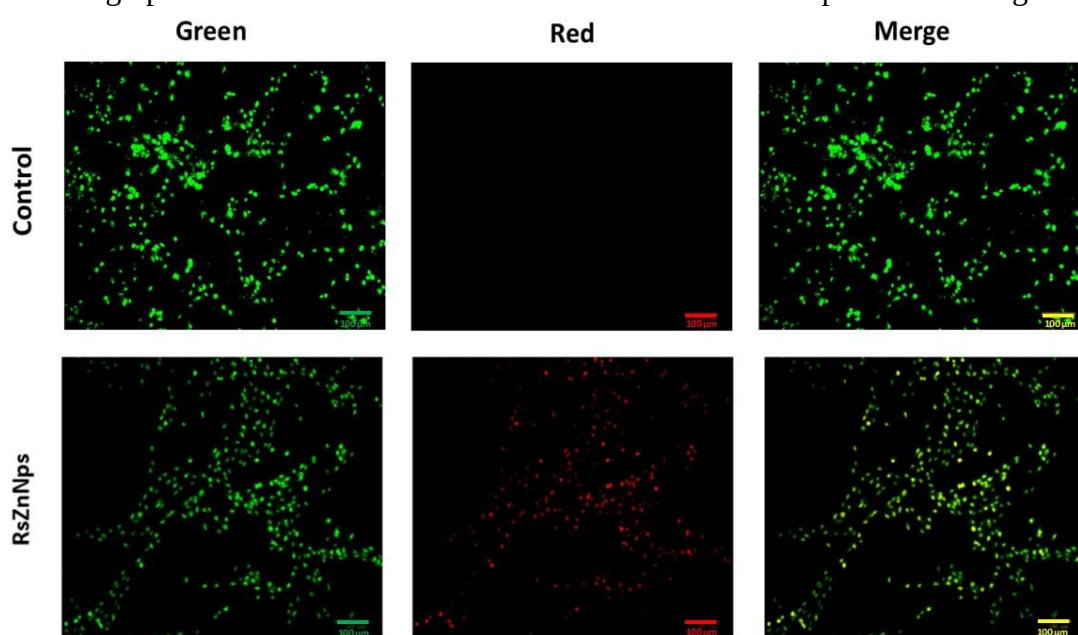


Figure 4. AO/EB Dual Fluorescence Staining of KB Oral Carcinoma Cells Following BLZnO-NPs Treatment. Representative fluorescence micrographs showing green (acridine orange), red (ethidium bromide), and merged channels for control and BLZnO-NPs-treated KB cells at the IC₅₀ concentration. Control cells exhibit uniformly green nuclei, indicating viable cells, whereas BLZnO-NPs-treated cells display increased yellow/red fluorescence, consistent with early apoptotic changes. (Magnification 20X).

3.7. Discussion.

The present study demonstrates the successful Biosynthesis of ZnO-NPs using *BaLac*, a classical Ayurvedic formulation of *Berberis aristata* processed in goat's milk, and evaluates their anticancer potential against OSCC cells. The integration of traditional medicinal knowledge with nanotechnology provides a scientifically rationalized approach for developing safer and potentially more selective anticancer nanomaterials that warrant further investigation. In our synthesis, zinc acetate was reacted with sodium hydroxide under an alkaline environment. Initially, zinc ions formed zinc hydroxide intermediates (Zn (OH)₂) upon continuous stirring in the ethanol–water system, and in the presence of *BaLac* extract, the hydroxide exhibited in situ dehydration to form ZnO nuclei [29]. The UV- Visible absorption peak observed at ~430 nm confirms the formation of ZnO-NPs, consistent with previously reported characteristic absorption profiles of Zinc nanostructures. The absence of any peak in

the precursor zinc acetate solution further validates that nanoparticle formation occurred only after interaction with the *BaLac* extract, indicating a phytochemical-mediated reduction process. FTIR analysis revealed functional groups corresponding to C–H, C=O, and aromatic C=C vibrations, along with a distinct Zn–O stretching band in the 400–600 cm⁻¹ region. These findings suggest that phytoconstituents present in *B. aristata* and bioactive peptides from goat's milk likely acted as reducing, capping, and stabilizing agents during nanoparticle synthesis. Such biomolecular capping may enhance colloidal stability and modulate biological interactions.

XRD analysis confirmed the crystalline nature of the synthesized BLZnO-NPs, with diffraction peaks indexed to the hexagonal wurtzite structure of ZnO (ICDD 00-036-1451) and an average crystallite size of 17.7 nm. The absence of impurity peaks indicates phase purity and successful synthesis without secondary phases. SEM and TEM analyses further corroborated the nanoscale dimensions and morphology. While SEM revealed near-spherical particles with a mean size of ~34 nm, TEM analysis showed smaller particle dimensions (~20 nm), reflecting higher-resolution imaging and reduced aggregation effects.

The difference between the crystallite size obtained from XRD and the particle size observed in SEM and TEM arises due to the fundamental distinction between the concept of crystallite size and particle size. The XRD-derived crystallite size, calculated using the Scherrer equation, represents the size of coherently diffracting domains. These domains may be smaller than the actual particle size if a particle consists of multiple crystallites. In contrast, SEM and TEM provide direct morphological visualization, where the measured size corresponds to the physical particle size. In many nanostructured materials, a single particle may contain multiple crystallites, leading to larger sizes observed in SEM or TEM compared to XRD. Additionally, peak broadening in XRD can be influenced by microstrain and instrumental factors, which may further reduce the calculated crystallite size. In the current work, as per the SAED pattern presented in Figure 2d, the synthesized particle is polycrystalline in nature. So, it is obvious that the crystallite size will be smaller than that of the particle size, as the particle itself contains multiple crystallites [30]. The presence of distinct SAED rings and HR-TEM lattice fringes with a d-spacing of 0.25 nm corresponding to the (101) plane confirms crystallinity at the atomic level. Interestingly, the coexistence of nanospheres and nanorods suggests anisotropic growth, which may influence cellular uptake dynamics and therapeutic performance, as particle morphology is known to affect internalization efficiency and intracellular interactions. Biological evaluation revealed that BLZnO-NPs exert significant dose- dependent cytotoxic effects on KB oral carcinoma cells, comparable to cisplatin at higher concentrations. However, unlike cisplatin, which demonstrated pronounced toxicity toward L929 fibroblasts, BLZnO-NPs maintained high viability (≥95%) in normal cells within the tested concentration range. The selectivity index was calculated to be 13.82, which further confirmed the selectivity of BLZnO-NPs toward KB cancer cells compared to L929 normal cells, inferring its strong anticancer potential against OSCC cells. This selective cytotoxicity observed *in vitro* is a critical finding, as a major limitation of conventional chemotherapeutic agents is their lack of tumor specificity and associated systemic toxicity. The improved cytocompatibility of BLZnO-NPs may be attributed to biomolecule-mediated surface functionalization, which can modulate nanoparticle cell interactions and reduce nonspecific cytotoxic effects.

Mechanistically, AO/EB dual staining demonstrated that BLZnO-NPs predominantly induce early apoptosis in KB cells, as evidenced by chromatin condensation and greenish-yellow nuclear fluorescence. The induction of apoptosis rather than necrosis is therapeutically

advantageous, as apoptotic pathways minimise inflammatory responses and collateral tissue damage. ZnO nanoparticles have been reported in previous studies to generate intracellular reactive oxygen species (ROS), disrupt mitochondrial membrane potential, and activate caspase-dependent pathways [31-33]. ROS generation is a possible pathway rather than a confirmed mechanism in this study, as direct experimental evidence is lacking. However, the limitations of this study include that the interpretation was based on the observed cytotoxic and apoptotic morphological changes and well-established literature reports demonstrating that ZnO-based nanoparticles induce cancer cell death predominantly through ROS-mediated mitochondrial dysfunction and subsequent caspase cascade activation. The ROS induction and caspase activity could not be studied experimentally in our investigation. Further, the KB cell line is reported to be HeLa cross-contaminated, yet they retain the epithelial carcinoma characteristics and is used as a model to evaluate the anticancer activity of the BLZnO-NPs. Apoptotic conclusions are based on qualitative evidence and are not quantified by selective assays such as flow cytometric analysis. The phytochemicals from *B. aristata*, including alkaloids (e.g., berberine), flavonoids, and phenolic compounds, might synergistically enhance ROS-mediated apoptotic signaling or modulate redox-sensitive pathways in cancer cells. Additionally, bioactive peptides from goat's milk have been reported to possess anticarcinogenic properties, which may further contribute to the observed anticancer activity through complementary mechanisms [34]. Further, studies suggest that the proteins present in goat's milk are thought to reduce the silver ions during the synthesis of silver nanoparticles [35]. The rationale for selecting *BaLac* as a reducing and stabilizing matrix is further supported by both classical Ayurvedic literature emphasizing its role in oral tumor management and prior experimental evidence demonstrating anticancer effects of *B. aristata*. On the other hand, Goat's milk-derived bioactive peptides have been found to have an anti-apoptotic effect on colon cancer cell lines [34]. Thus, the present study not only validates traditional knowledge through a nanotechnological framework but also suggests a possible role of phytochemical-rich formulations in serving dual functions as bio-reductants in nanoparticle synthesis and as bioactive contributors to therapeutic efficacy.

In conclusion, *BaLac*-mediated biosynthesis produced phase-pure, crystalline ZnO-NPs with controlled nanoscale dimensions and mixed morphology. These nanoparticles showed significant dose-dependent cytotoxicity against KB cells while maintaining high cytocompatibility in normal fibroblasts, primarily by inducing early apoptosis. The integration of traditional Ayurvedic formulations with nanotechnology represents a promising preliminary approach for developing selective and biocompatible anticancer nanomaterials, which require further mechanistic investigation and *in vivo* validation for OSCC therapy.

Study Limitations: The present study has certain limitations. The anticancer activity was evaluated *in vitro* using a single OSCC cell line, and the precise molecular pathways underlying apoptosis induction were not elucidated. Further investigations involving ROS quantification, mitochondrial membrane potential assays, caspase activation studies, and gene expression profiling are warranted. *In vivo* validation and pharmacokinetic studies will also be essential to establish translational relevance.

4. Conclusions

The present study successfully demonstrates the Biosynthesis of ZnO-NPs using *BaLac*, a traditional Ayurvedic formulation of *B. aristata* processed in goat's milk. The synthesized BLZnO-NPs were confirmed to be crystalline, phase-pure, and predominantly

nanoscale in dimension, as evidenced by UV-Vis spectroscopy, FTIR, XRD, SEM, and TEM analyses. The presence of characteristic ZnO diffraction planes and lattice fringes further validated the structural integrity of the nanoparticles. Phytochemicals from *B. aristata* and bioactive components from goat's milk likely played a critical role in the reduction, capping, and stabilization processes during synthesis.

Biological evaluation revealed that BLZnO-NPs exhibit significant dose-dependent cytotoxicity against KB cells while maintaining high cytocompatibility toward normal L929 fibroblasts. Compared to cisplatin, BLZnO-NPs demonstrated a more favorable selectivity profile, indicating reduced toxicity toward normal cells. Mechanistic assessment using AO/EB staining confirmed that the anticancer effect is primarily mediated through the induction of early apoptosis.

Overall, this study provides preliminary evidence that *BaLac*-derived ZnO-NPs exhibit selective cytotoxicity toward oral squamous carcinoma cells while maintaining cytocompatibility toward normal fibroblasts. These findings support the potential of integrating traditional Ayurvedic formulations with nanotechnology as a strategy for developing biocompatible anticancer nanomaterials. However, further mechanistic investigations, *in vivo* validation, and pharmacokinetic evaluations are necessary to validate their therapeutic potential and establish translational relevance.

Author Contributions

Conceptualization, A.K.B.; methodology, A.K.B., A.P., R.S., B.S., and R.K.S.; validation, R.S. and B.S.; formal analysis, A.P.; investigation, K.K., R.S., B.S., and R.K.S.; data curation, K.K.; writing—original draft preparation, A.K.B. and A.G.N.; writing—review and editing, A.K.B. and A.G.N.; visualization, A.P., R.S., and B.S.; supervision, R.K.S.; funding acquisition, A.K.B., R.S., and R.K.S. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement

The raw data linked to this study can be obtained from the corresponding author upon reasonable request.

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Conflicts of interest

The authors declare no conflicts of interest.

Artificial Intelligence disclosure

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Abbreviations

Abbreviation	Definition
OSCC	Oral squamous cell carcinoma
TEM	Transmission electron microscopy
HR-TEM	High-resolution TEM
SEM	Scanning Electron Microscopy
FTIR	Fourier Transform Infrared Spectroscopy
UV-Vis	UV- Visible spectroscopy
<i>BaLac</i>	<i>Berberis aristata</i> processed in goat's milk
BLZnO-NPs	<i>BaLac</i> -derived zinc nanoparticles
ZnO-NPs	Zinc Oxide nanoparticles
MTT	Methyl thiazolyl tetrazolium
FBS	Fetal bovine serum
DMEM	Dulbecco's Modified Eagle's Medium
ROS	Reactive oxygen species
AO/EB	Acridine orange–ethidium bromide
PBS	Phosphate-buffered saline

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Supplementary materials

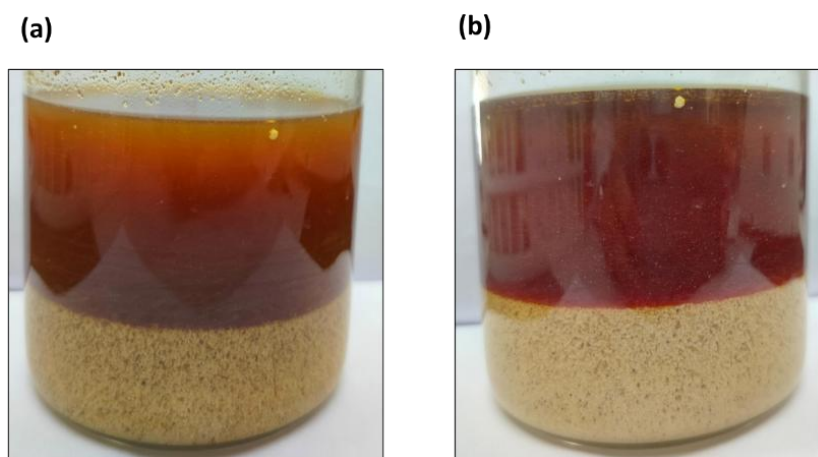


Figure S1. Visual Observation of BLZnO-NPs Formation During Biosynthesis. Representative images showing (left) the reaction mixture during nanoparticle synthesis and (right) the formation of precipitated ZnO nanoparticles after centrifugation, indicating successful bioreduction and nanoparticle formation using BaLac extract.