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Original Article

Regulatory Mechanisms of Cell Death Induced by *Lactobacillus buchneri, Chlorella vulgaris* Probiotics, and Chitosan-Conjugated Phytochemicals in Pancreatic Cancer Cells

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ABSTRACT

Background and Aim: This study explores the therapeutic potential of natural compounds on pancreatic cancer cell lines (Panc-1). The main objective is to assess the effects of *Lactobacillus buchneri* supernatant, chitosan nanoparticles conjugated with Genistein (GCNPs), chitosan nanoparticles containing Epigallocatechin-3-Gallate (EGCG) and *Chlorella vulgaris*. Pancreatic cancer, especially Pancreatic Ductal Adenocarcinoma (PDAC), is highly aggressive and resistant, with few treatment options and low survival rates due to late diagnosis. The study aims to investigate the combined effects of these compounds on inducing apoptosis and altering gene expression related to cell death in pancreatic cancer cell lines. By evaluating these natural compounds, the research seeks to identify potential therapeutic agents for improving treatment outcomes in pancreatic cancer. **Materials and Methods:** To evaluate the effects of treatments on Panc-1 cells, IC_{50} values were determined using MTT assays at 24 and 48 hr. Cells were treated with *Lactobacillus buchneri* supernatant, chitosan nanoparticles conjugated with Genistein (GCNPs), chitosan nanoparticles containing EGCG and *Chlorella vulgaris*. Post-treatment, RNA was extracted for gene expression analysis via qPCR, focusing on CASP9, CYCLIND1, MMP2, VEGF, Bax, and Bcl-2. The expression levels were quantified to assess apoptosis induction. Additionally, cytotoxicity assays were performed to evaluate cell viability and proliferation, confirming the dose-dependent effects of the treatments on apoptosis and cancer cell inhibition. **Results:** The IC_{50} values for the treatment were 389.7 µg/mL and 297.1 µg/mL at 24 and 48 hr, respectively, significantly increasing the pro-apoptotic gene CASP9 while decreasing CYCLIND1, MMP2, and VEGF expression. GCNPs were synthesized and characterized, showing cytotoxic effects with IC_{50} values of 21 µg/mL at 24 hr and 9.859 µg/mL at 48 hr. These nanoparticles enhanced apoptosis by increasing Bax expression and decreasing Bcl-2, while also suppressing MMP2 and MMP9, which are linked to metastasis. Chitosan nanoparticles containing EGCG demonstrated notable anti-cancer effects, reducing cell proliferation and inducing apoptosis. Additionally, *Lactobacillus* strains from *Chlorella vulgaris* exhibited strong probiotic and anti-cancer properties. Cytotoxicity assays revealed a dose-dependent inhibition of Panc-1 cell viability and induction of apoptosis, highlighting the potential of these natural compounds in cancer therapy. **Conclusion:** In conclusion, the study reveals that *Lactobacillus buchneri* supernatant, chitosan nanoparticles conjugated with genistein, and chitosan nanoparticles containing EGCG significantly induce apoptosis in Panc-1 pancreatic cancer cells. The IC_{50} values indicate strong cytotoxic effects, particularly with GCNPs. The upregulation of CASP9 and downregulation of Bcl-2 highlight the treatments' potential to promote apoptosis. These findings emphasize the promising role of natural compounds and nanotechnology in developing effective pancreatic cancer therapies.

Keywords: Apoptosis, Chitosan nanoparticles, *Chlorella vulgaris*, Epigallocatechin-3-Gallate (EGCG), Gene expression, Genistein, *Lactobacillus buchneri*, Pancreatic cancer.

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INTRODUCTION

Pancreatic cancer is a formidable adversary in the realm of oncology, often referred to as a "silent killer" due to its insidious onset and late-stage diagnosis. With a five-year survival rate that remains dismally low, the need for innovative and effective therapeutic strategies has never been more urgent (Hu and O'Reilly, 2024). Traditional treatment modalities, including surgery, chemotherapy, and radiation, frequently fall short, underscoring the necessity for alternative approaches that can enhance treatment efficacy and improve patient outcomes (Hu and O'Reilly, 2024). In recent years, there has been a growing interest in the potential of natural compounds and nanotechnology to revolutionize cancer therapy. Among these,

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probiotics have emerged as a promising area of research (Zafar *et al*., 2025). *Lactobacillus buchneri*, a strain of lactic acid bacteria, has demonstrated various health benefits, including immunomodulatory and anticancer properties (Kadam *et al*., 2025). Its supernatant, rich in bioactive compounds, may play a crucial role in modulating cellular responses and enhancing the efficacy of conventional therapies (Riaz *et al*., 2023). Furthermore, the use of chitosan nanoparticles as a drug delivery system has garnered significant attention. Chitosan, derived from chitin, is biocompatible and biodegradable, making it an ideal candidate for encapsulating therapeutic agents (Desai *et al*., 2023). When conjugated with genistein, a naturally occurring flavonoid known for its anti-cancer effects, chitosan nanoparticles can enhance the solubility and bioavailability of genistein, allowing for more effective targeting of cancer cells (Cai *et al*., 2017). Additionally, collagen-formulated Epigallocatechin-3-Gallate (EGCG), a potent polyphenol found in green tea, has been recognized for its ability to induce apoptosis and inhibit tumor growth (Mokra *et al*., 2022). EGCG's multifaceted mechanisms of action, including the modulation of signaling pathways involved in cell survival and death, make it a valuable component in the fight against pancreatic cancer (Kciuk *et al*., 2023). This study aims to investigate the synergistic effects of *Lactobacillus buchneri* supernatant, chitosan nanoparticles conjugated with genistein, and collagen-formulated EGCG on inducing apoptosis and regulating gene expression related to cell death in pancreatic cancer cell lines.

MATERIALS AND METHODS

Nanoparticle Synthesis and Characterization

Chitosan from Merck (Cas No: 448877) and TPP from Merck (Cas No: 72061) were sourced from GolpaChemie Company for nanoparticle synthesis, following the methodology outlined by Rajesh Sreedharan Nair *et al*. A 0.2% w/v chitosan solution was prepared in dilute acetic acid (2% v/v), and its pH was adjusted to 5 using 4 M NaOH. The chitosan solution was stirred at 1000 rpm, and a 2 mg/mL epigallocatechin-3-gallate solution was added dropwise. The mass ratio of chitosan to TPP was maintained between 3:1 and 5:1. The resulting mixture was centrifuged for 20 min to obtain nanoparticle precipitate, which was then washed with deionized water. UV-vis spectroscopy was employed to confirm the formation of epigallocatechin-3-gallate-conjugated nanochitosan, analyzing samples in the 300-800 nm wavelength range. Dynamic Light Scattering (DLS) assessed particle size and distribution in liquid, complementing Scanning Electron Microscopy (SEM), which evaluates particle size in a dry state. DLS is crucial for applications in pharmaceuticals and biotechnology, while SEM provides high-resolution imaging and elemental analysis through Energy Dispersive Spectroscopy (EDS), allowing for the detection of light elements like oxygen and carbon.

Chitosan from the Merck brand (Cas No: 448877) and TPP from Merck (Cas No: 72061) were acquired from Golpa Chemie Company. To prepare 5 mL of 1% acetic acid, start by adding 5 mL of distilled water into a sterile falcon tube. Then, remove 50 µL of the distilled water and add 50 µL of 100% acetic acid to the falcon tube. This will yield a solution of 5 mL of 1% acetic acid. For the preparation of a 0.2% (w/v) chitosan solution in a total volume of 5 mL, weigh out 0.01 g of chitosan powder and transfer it into an Erlenmeyer flask. Next, add 5 mL of the prepared 1% acetic acid to the flask and place it on a stirrer to ensure that the chitosan powder dissolves completely through stirring.

Preparation of 0.1% TPP (Sodium triphosphate pentabasic) in 1 mL of distilled water. To prepare a 0.1% (w/v) TPP solution in a volume of 1 mL, weigh 0.001 grams of TPP powder, add 1 mL of distilled water to it, and then add the resulting solution dropwise to the stirring chitosan. Next, we allow the final solution to stir on the stirrer for 1 hr and 30 min.

Nanoparticles was carried out according to pervious study. Briefly, a solution of chitosan (0.2% w/v) in dilute acetic acid (2% v/v) was prepared and the pH was adjusted to 5 using 4 M NaOH. Finally, the chitosan solution was stirred at 1000 rpm on a magnetic stirrer and 2 mg/mL epigallocatechin-3-gallate solution was added dropwise. The solution was added dropwise to this mixture to obtain a mass ratio of chitosan and TPP between 3:1 and 5:1. Finally, the prepared solution was centrifuged at high speed for 20 min to prepare the nanoparticle precipitate. In the next step, the precipitate was washed with deionized water.

Extraction genistein, Synthesis and properties of green genistein-conjugated chitosan nanoparticles

To prepare a soy extract, dissolve 10 g of ground soybeans in 100 mL of distilled water and heat the mixture at 60°C for 2 hr until fully dissolved. After cooling, filter the solution through filter paper and store the extract in clean containers for future analysis or applications. For a 0.1% TPP (Sodium triphosphate pentabasic) solution, weigh 0.001 g of TPP powder and mix it with 1 mL of distilled water. This solution is then added dropwise to stirring chitosan, which is stirred for 1 hr and 30 min. Ultraviolet-visible (UV-vis) spectroscopy is employed to confirm the formation of genistein-conjugated nanochitosan in an aqueous medium, with analysis conducted using a spectrophotometer across a wavelength range of 300-800 nm. A sample of 100 µL is analyzed for this purpose. Additionally, Fourier Transform Infrared (FTIR) spectroscopy is utilized to identify functional groups in the synthesized genistein-conjugated nanochitosan, using a CORDOUAN TECHNOLOGIES, VASCO device to examine a 5 mL sample within the spectral range of 4000 cm^{-1} to 400 cm^{-1}. Dynamic Light Scattering (DLS) is used to assess particle size and distribution in liquids, providing insights into particle behavior, which is crucial for applications in pharmaceuticals and biotechnology. DLS and Scanning Electron Microscopy

(SEM) analyses complement each other, with DLS offering a method for evaluating the dimensional stability and aggregation of nanoparticles. SEM, capable of high magnification imaging, is used to analyze surface properties and morphology, while Energy Dispersive Spectroscopy (EDS) allows for elemental analysis, particularly effective in detecting light elements like oxygen and carbon.

Antioxidant assay by DPPH

The free radical scavenging activity of genistein was assessed using the DPPH method. In this procedure, an aqueous solution of DPPH (Sigma Chemical Co., USA) at a final concentration of 1000 µg/mL was prepared for the reaction mixture. A total of 50 µL of genistein was combined with 150 µL of the DPPH solution in a 96-well microliter plate, with DMSO serving as a negative control. The reaction mixture was incubated for 30 min, after which the change in absorbance at 517 nm was measured using a microplate reader (Thermo Electron Corporation, Finland). All measurements were conducted in triplicate. The free radical scavenging activity is reported as IC_{50} values, which represent the concentration of the sample needed to scavenge 50% of the free radicals. This value is calculated using the following equation:

%Scavenging=(Ac-At)/Ac x100

where Ac=absorbance of control, At=absorbance of test solution.

Cell culture

In this study, PANC-1 cell lines were sourced from the Iranian Center for Genetic and Biological Resources and cultured using standard protocols in DMEM medium with 10% fetal bovine serum and 1% penicillin-streptomycin at 37ºC in a humidified 5% CO_2 environment. The cells' morphology, health, and count were monitored with an inverted microscope. Upon reaching 70% confluence, the cells were detached using 0.25% trypsin, centrifuged at 1500 RPM for 10 min, and the resulting suspension was prepared. Cell viability was assessed under a light microscope, ensuring no contamination before proceeding with further experiments.

Preparation of Lactobacillus buchneri and probiotics Chlorella vulgaris

Lactobacillus buchneri was cultured in sterile MRS broth at 37ºC for 24-48 hr under anaerobic conditions until reaching the stationary phase. The culture was centrifuged at 4ºC and 4000 rpm for 15 min to pellet the bacterial cells. The supernatant was collected and centrifuged again at 8000 rpm for 10 min to ensure the complete removal of bacterial cells. It was then filtered through a 0.22-micron sterile syringe filter. A sterile MRS broth processed similarly served as a negative control. The supernatant was stored at 4ºC for up to one week or at -20ºC for longer periods, with sterility verified by culturing on MRS agar before use.

Determination of cytotoxicity of Lactobacillus buchneri and Chlorella vulgaris Supernatant, Chitosan Nanoparticles Conjugated with Genistein, chitosan-Formulated Epigallocatechin-3-Gallate (EGCG)

To investigate the effect of lactic acid on the proliferation of PANC-1 cancer cells, the MTT method is used. The cells are first transferred to 96-well plates. Then, using the MTT test, the 50IC value is measured. To each well, 20 µL of MTT solution with a concentration of 0.5 mg/mL is added. The plates are kept in the incubator away from light for 4 hr. After that, the supernatant is discarded, and 150 µL of 100% DMSO are added to turn the insoluble formazan crystals into a coloured solution. In less than 20 min, the absorbance of the produced colour is read using an ELISA device at a wavelength of 570 nm. Cells are treated with *Lactobacillus buchneri* (0, 50, 150, 200, 250, 300, 350, 450, 500 µg/mL) and *Chlorella vulgaris* (0, 1.5625, 3.125, 6.25, 12.5, 25, 50, and 100 µg/mL) Supernatant, Chitosan Nanoparticles Conjugated with Genistein (0, 3, 75, 15, 6, 31, 20, 62.5, 125, 250, and 500 µg/mL), chitosan-Formulated Epigallocatechin-3-Gallate (EGCG) (0, 10, 25, 50, 100, 150, and 200 µg/mL) after 24 and 48 hr of incubation. The cells are cultured at 37ºC, 5% CO2, and 95% O2 for an additional 3 hr. Finally, the cell viability percentage and IC_{50} is calculated.

Examination of the expression of genes using the Real-Time PCR technique after treatment with Lactobacillus buchneri and Chlorella vulgaris Supernatant, Chitosan Nanoparticles Conjugated with Genistein, Collagen-Formulated Epigallocatechin-3-Gallate (EGCG)

To evaluate the changes in the expression of the target genes, the Real Time PCR technique was utilized. For this purpose, PANC-1 cells were cultured in a 6-well plate and exposed to different concentrations of IC_{50} supernatant for 24 hr. Then, RNA extraction was performed from the cells. For this purpose, after counting the PANC-1 cells, a volume of the cell suspension containing 10^6 cells were transferred to the wells of a 6-well plate, and complete DMEM culture medium was added to it. The plate was incubated in a CO_2 incubator at 37ºC for approximately 24 hr. The next day, based on the IC_{50} results obtained from the MTT assay, an appropriate concentration of the supernatant was prepared separately. Then, the contents of the wells where PANC-1 cells had been passaged the previous day were emptied, and appropriate concentrations of them were transferred to each well and incubated in a CO_2 incubator at 37ºC. In this study, GAPDH primer was used as the reference gene. The table below shows the sequences of the Forward and Reverse primers (Table 1).

Ethical Statement

This study did not involve human or animal samples. The research was conducted using a cell line, as detailed in the manuscript.

Statistical Analysis

The data are presented as mean values with Standard Error of the Mean (SEM). Statistical analysis was performed using the 0.9 Prism Graphpad software, and significance was calculated using the t-test and two-way one-way ANOVA methods, with $p<0.0001$ considered statistically significant.

RESULTS

Antioxidant activity of Epigallocatechin-3-Gallate (EGCG)

Based on Figure 1, the antioxidant activity of the aqueous extract of EGCG was examined. The EC_{50} of the green tea aqueous extract and quercetin as a positive control were 0.66 mg/mL and 0.017 mg/mL, respectively.

Characterization of Nanoparticles

Using UV-vis spectroscopy, the synthesis of chitosan nanoparticles with green tea was confirmed. According to the figure below, the solution containing chitosan-green tea nanoparticles shows maximum absorption around the 260 nm region (Figure 2). The presence of a peak in the specified region is evidence of the synthesis of chitosan-green tea nanoparticles

The key results section presents the particle size distribution of the sample, with three distinct peaks observed. The first peak has a diameter of 54.05 nm and accounts for 25.1% of the volume. The second peak has a diameter of 10.26 nm and accounts for 74.3% of the volume. The third peak has a much larger diameter of 2706 nm and accounts for only 0.7% of the volume (Figure 3).

The key findings include a zeta potential of -16.2 mV with a standard deviation of 10.1 mV, indicating a slightly negative surface charge, an electrophoretic mobility of -1.268 µm·cm/V·s, and various plots such as the frequency shift, phase plot, zeta potential voltage and current, and statistics graph, which offer insights into the particle dynamics and behavior of the sample (Figure 4). This comprehensive report can be valuable for applications involving colloidal systems or the characterization of nanoparticles.

This is a high-resolution Field emission Scanning Electron Microscope (Fe-SEM) micrograph showing the detailed microstructure of the sample (Figure 5). The use of a field emission electron source in the SEM allows for a narrower electron beam

Table 1: Primers used in Real Time PCR.

Gene Name		Sequence (5'to3')	Length	GC%	Tm (°C)
Bax	F	CCACCCTGGTCTTGGATCCAGCCC	24	66.67	68.71
	R	CCTGTGCACCAAGGTGCCGGAACT	24	62.50	69.46
BCL2	F	TTGTGGCCTTCTTTGAGTTCGGTG	24	50.00	63.77
	R	GGTGCCGGTTCAGGTACTCAGTCA	24	58.33	66.18
GAPDH	F	TGCCTCCTGCACCACCAAC	19	63.16	62.79
	R	CGGAGGGGCCATCCACAG	18	72.22	62.18
MMP-2	F	AGCGAGTGGATGCCGCCTTTAA	22	54.55	64.84
	R	CATTCCAGGCATCTGCGATGAG	22	54.55	61.70
MMP-9	F	GCCACTACTGTGCCTTTGAGTC	22	54.55	61.44
	R	CCCTCAGAGAATCGCCAGTACT	22	54.55	61.27
p53	F	CCCCTCCTGGCCCCTGTCATCTTC	24	66.67	68.25
	R	GCAGCGCCTCACAACCTCCGTCAT	24	62.50	69.48
CASP-3	F	TTCATTATTCAGGCC TGCCGAGG	24	52.17	62.88
	R	TTCTGACAGGCCATG CATCC TCA	24	52.17	64.12
CASP-7	F	ATGCAGATGGCTGGAGAACC	20	55	60.11
	R	GTTAAAGTACAGTTCTTTTGTCAGCATCG	29	50	61.60
Cyclin D	F	TGGAACTGGGTCGAATTTGA	20	45	57.06
	R	CCTCCTTGCTGACATTGGAA	20	50	57.59
VEGF	F	GAGATGAGCTTCCTACAGCACC	22	54.55	60.48
	R	TCACCGCCTCGGCTTGTCAT	20	60	64.31
Caspase 9	F	GTTTGAGGACCTTCGACCAGCT	22	54.55	62.50
	R	CAACGTACCAGGAGCCACTCTT	22	54.55	62.24

F: Forward; R: Reverse.

and higher resolution imaging compared to a conventional thermionic emission SEM. The image was captured at a high magnification of 50,000x, with a working distance of 11.28 mm and an accelerating voltage of 20 keV. The in-beam secondary electron (In-Beam SE) detector was used to capture the surface topography and morphology of the sample in high detail. The resulting micrograph reveals a highly textured, porous surface structure composed of irregularly shaped grains or particles clustered together in a sponge-like arrangement. This type of microstructure is often seen in advanced materials like porous ceramics, polymers, or natural biomaterials such as chitosan.

Characterization of Nanoparticles

The UV-vis graph illustrates the absorption spectra associated with the production of chitosan using genistein. The graph exhibits a significant peak at a low wavelength, signifying the principal absorption feature of the chitosan-genistein complex (Figure 6). The absorbance diminishes progressively with increasing wavelength, which is characteristic of biopolymer and antioxidant interactions. The initial peak may be linked to the interaction of polyphenols from genistein with the chitosan matrix (340 nm). The progressive decline in absorbance indicates a slow transition

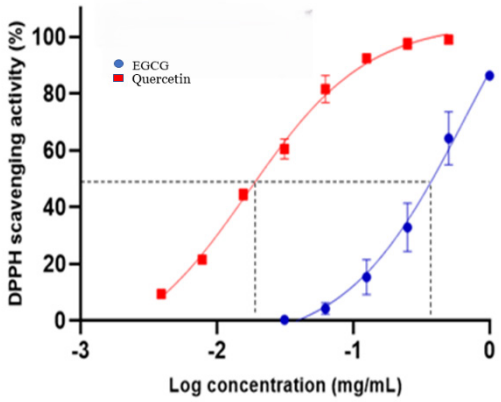

Figure 1: Result of aqueous extract of EGCG along with quercetin as a positive control.

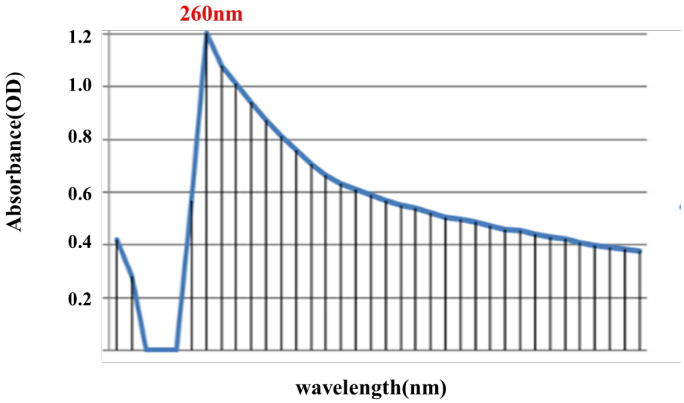

Figure 2: UV-visible of chitosan NPS.

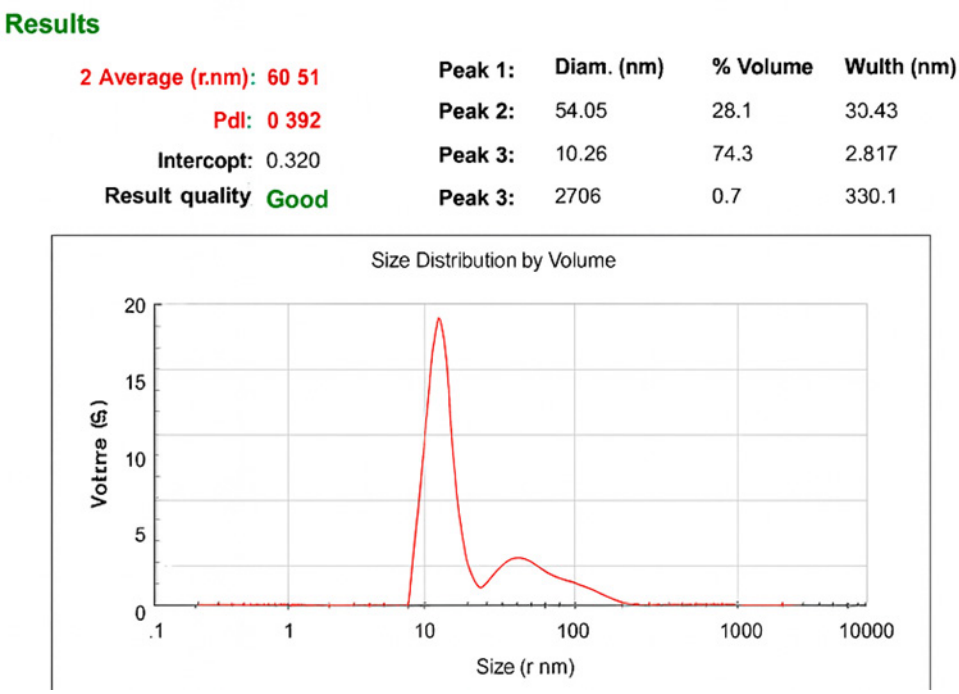

Figure 3: DLS result of chitosan NPS.

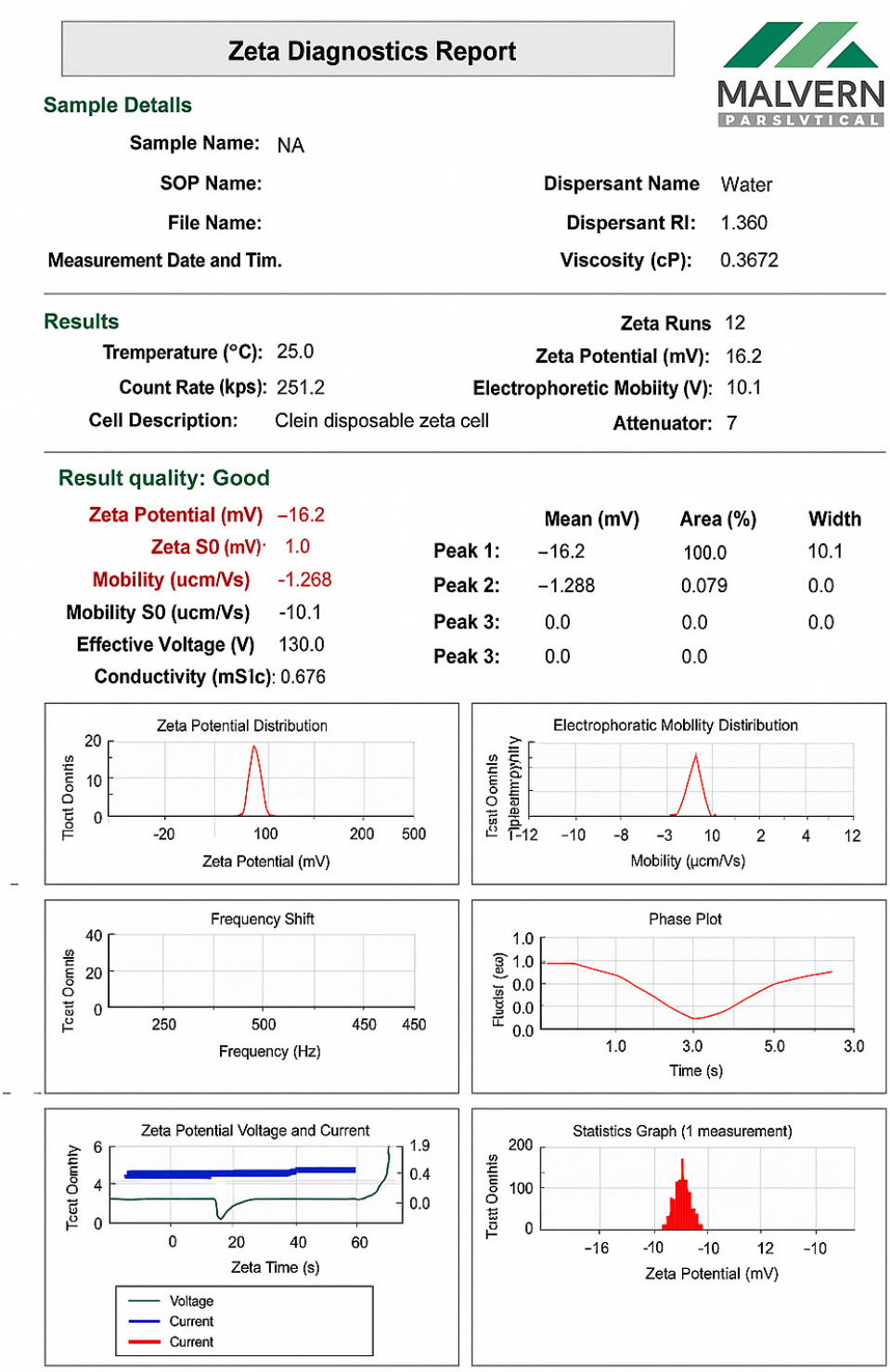

Figure 4: Zeta Potential of NPS.

and dispersion of the particles inside the solution. This pattern signifies effective synthesis and appropriate interaction between chitosan and genistein components.

The wavelength range of the spectrum was 500-4000 cm^{-1}. FTIR analyses were performed with a resolution of 4 cm^{-1} to identify the potential functional groups in genistein responsible for capping the formed chitosan nanoparticles (Figure 7). The peak observed at 3419.9 cm^{-1} is related to the OH, NH stretching vibration. The broadening or shift in this peak indicates the interaction between the hydroxyl and amine groups after the formation of nanoparticles. The CH stretching vibration, the overlapped C=O and C=C stretching vibrations show peaks at 2922.9 cm^{-1} and 1631.1 cm^{-1}. The changes in these peaks indicate changes in the chemical environment of the C-H groups during the synthesis

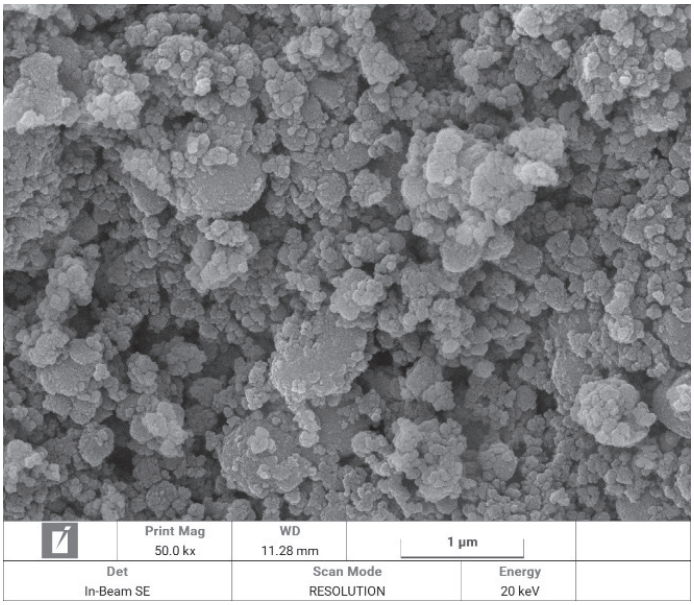

Figure 5: FE-SEM of NPS.

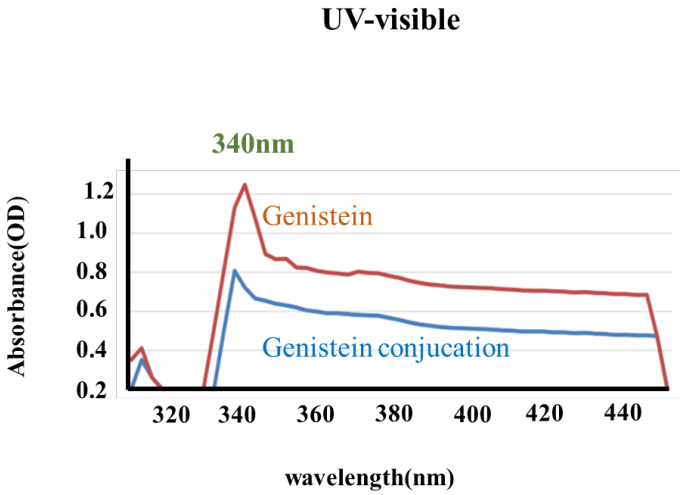

Figure 6: UV-visible spectrum of genistein and chitosan nanoparticle.

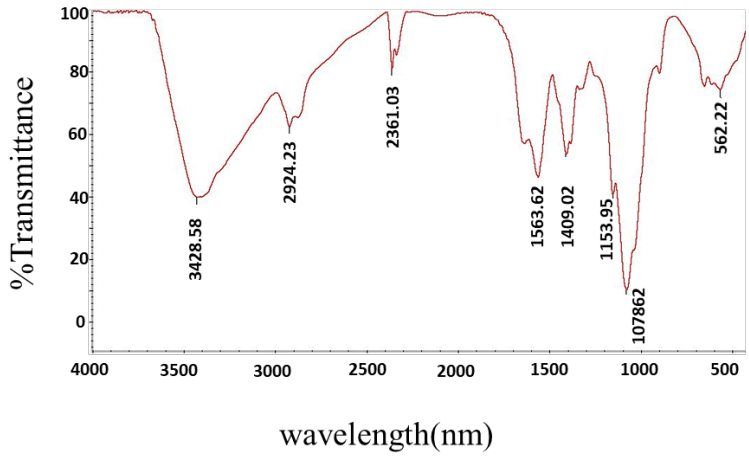

Figure 7: FTIR spectrum of chitosan nanoparticles conjugated with genistein.

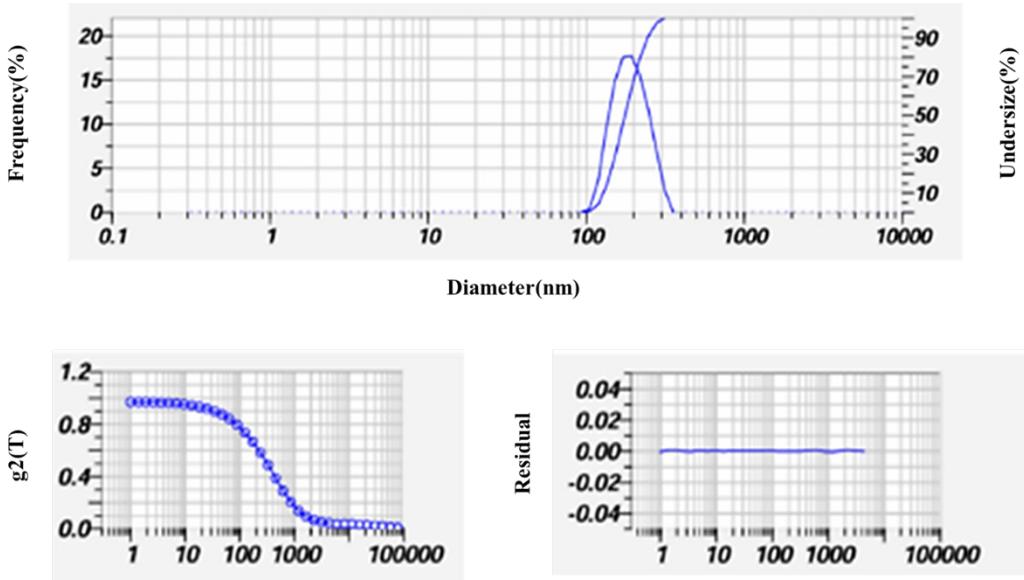

Figure 8: Dynamic Light Scattering (DLS) of the synthesized chitosan nanoparticles.

of nanoparticles. The C-N stretching vibration and N-H bending vibration show peaks at 1566.7 cm^{-1} and 1407.3 cm^{-1}. The peaks recorded at 1073.3 cm^{-1} and 1500-600 cm^{-1} are attributed to the O-C-C vibration and the CH bending vibration. The shift in these peaks indicates the interaction between the amide groups and other ligands. The total peaks of the functional groups in the NPs formulation were shown from the FTIR data analysis and it was confirmed that the NPs were synthesized due to the interaction between the amino groups of chitosan and the phosphate groups of STPP, which was also synthesized.

DLS results indicate an average particle size (Z-Average) of 209.1 nanometers and a Polydispersity Index (PDI) of 0.466, signifying a rather broad particle size dispersion. A single principal peak was detected with a size of 182.2 nanometers, accompanied by a standard deviation of 44.1 nanometers. The sample was assessed at a temperature of 25°C and a viscosity of 0.886 mPa·s, and it was classified as monodisperse. Figures 8 demonstrate a very consistent distribution and reliable data.

Figure 9 shows SEM images of chitosan nanoparticles containing genistein. It is clear from the images that the particles are well dispersed in the chitosan matrix.

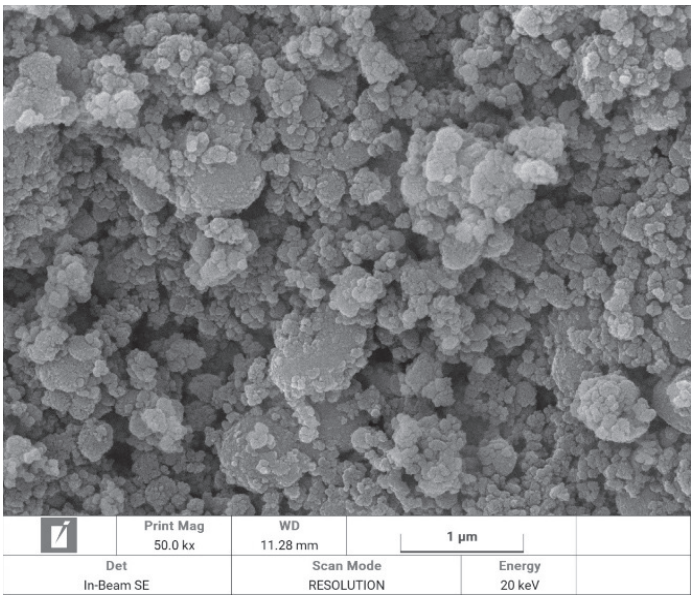

Figure 9: SEM images of chitosan nanoparticles.

Evaluation of antioxidant capacity by DPPH radical method

According to Figure 10, the antioxidant levels of genistein and nanosynthesis of genistein were obtained. According to the table below, the IC_{50} of nanochitosan genistein extract (104.3 mg/mL) was obtained in comparison with quercetin (86.23 mg/mL).

IC_{50} of Lactobacillus buchneri Supernatant

After treating Panc1 cancer cells with different concentrations of *Lactobacillus buchneri* Supernatant at various times (24 and 48 hr), its effect on the viability of Panc1 cells was examined using the MTT method at a wavelength of 630/570 nm. The positive control in this test includes 20% DMSO, which has a very high cytotoxic effect on the treated cells. The negative control included live Panc1 cells that were not treated with the extract in a 2% culture medium (Figure 11).

IC_{50} of genistein-conjugated chitosan

The genistein-conjugated chitosan and genistein were administered to Panc-1 cells at concentrations of 0, 3, 75, 15, 6, 31, 20, 62.5, 125, 250, and 500 µg/mL for a 24-hr period (Figure 12). The activity against Panc-1 cells was most potent at a concentration of 500µg/mL of genistein-conjugated chitosan and genistein, which was statistically significant in comparison to the control cells (p<0.001). Nevertheless, the concentration

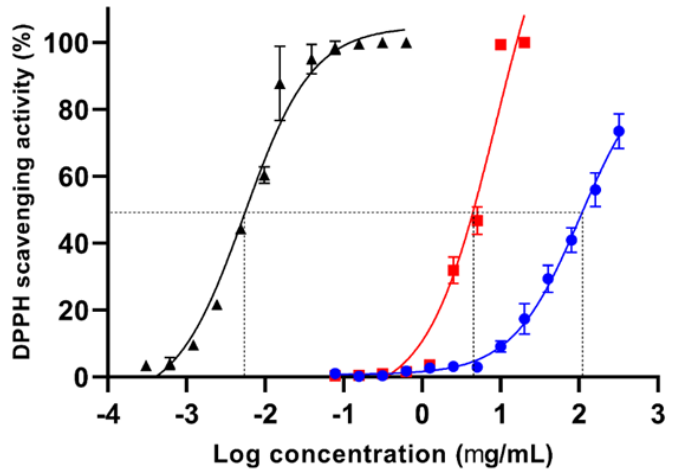

Figure 10: Results of antioxidant genistein and chitosan nanoparticles. Black graph: quercetin, red graph: chitosan nanoparticles, and blue graph: genistein.

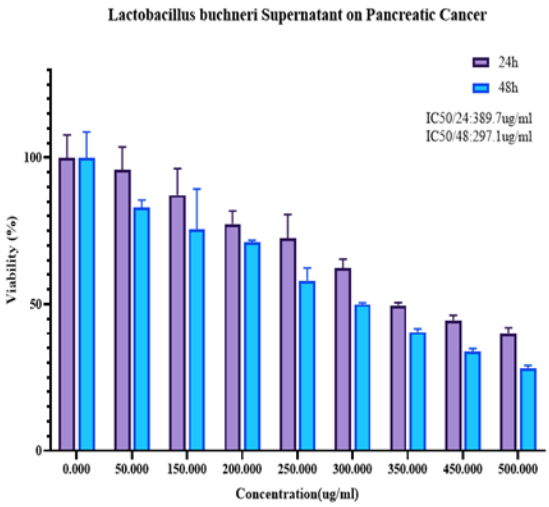

Figure 11: Effect of *Lactobacillus buchneri* Supernatant on PANC-1 cell viability. Cell viability curves for *Lactobacillus buchneri* Supernatant at 24 and 48 hr, showing a dose-dependent decrease in viability with IC_{50} values of 389.7 µg/mL at 24 hr and 297.1 µg/mL at 48 hr.

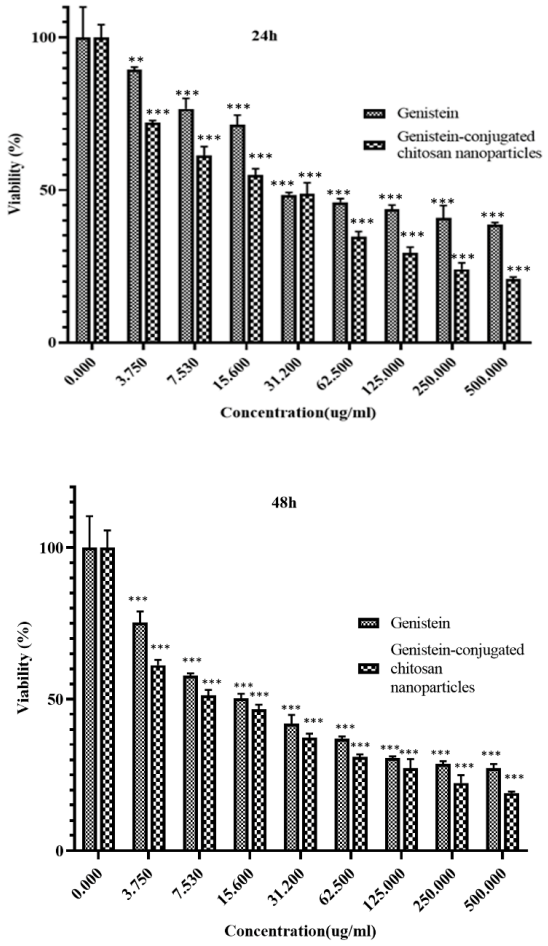

Figure 12: The results of the MTT assay in Panc-1 cell treated with the genistein-conjugated chitosan nanoparticles and genistein after 24 hr and 48 hr (results are reported as viability in comparison with control samples ***$p \leq 0.001$, **$p \leq 0.01$) (n=3).

of 3.75 µg/mL exhibited the least anti-proliferation effect, which was identical to the control group ($p<0.01$). The IC_{50} values of genistein-conjugated chitosan and genistein were 21 and 82.10 µg/mL, respectively. Genistein-conjugated chitosan and genistein had IC_{50} values of 9.859 and 22.7 µg/mL, respectively (Figure 12).

IC_{50} of Chitosan/Epigallocatecin-3-Gallate (EGCG)

Figure 13 shows the concentration- and time-dependent effects of Epigallocatechin Gallate (EGCG), a polyphenol compound found in green tea, on cell viability. The x-axis represents the log concentration of EGCG in micrograms per milliliter, while the y-axis represents the percentage of cell viability. The graph shows two lines, one for the 24-hr treatment and the other for the 48-hr treatment, indicating that as the concentration of EGCG increases, cell viability decreases for both treatment periods, with the 24-hr treatment showing higher cell viability than the 48-hr treatment at the same concentration of EGCG. At lower concentrations of EGCG (about 1.0-1.5 µg/mL), cell viability remains relatively high (about 80-100%), but with increasing concentration above 2.0 µg/mL, cell viability decreases significantly, reaching about 20-40% at the highest concentration (3.0 µg/mL), time-dependent concentration.

IC_{50} of Chlorella vulgaris

Based on Figure 14, the graph shows a gradual decrease in cell viability as the concentration of the supernatant increases. At lower concentrations, the effect on cell viability is minimal, but as the concentration increases, a more pronounced decrease in cell viability is observed, indicating a dose-dependent cytotoxic effect. The IC_{50} (Half-Maximal Inhibitory Concentration) is reached at a higher concentration, showing that the 24-hr treatment has a less potent effect compared to 48 hr. On the other hand, the 48-hr

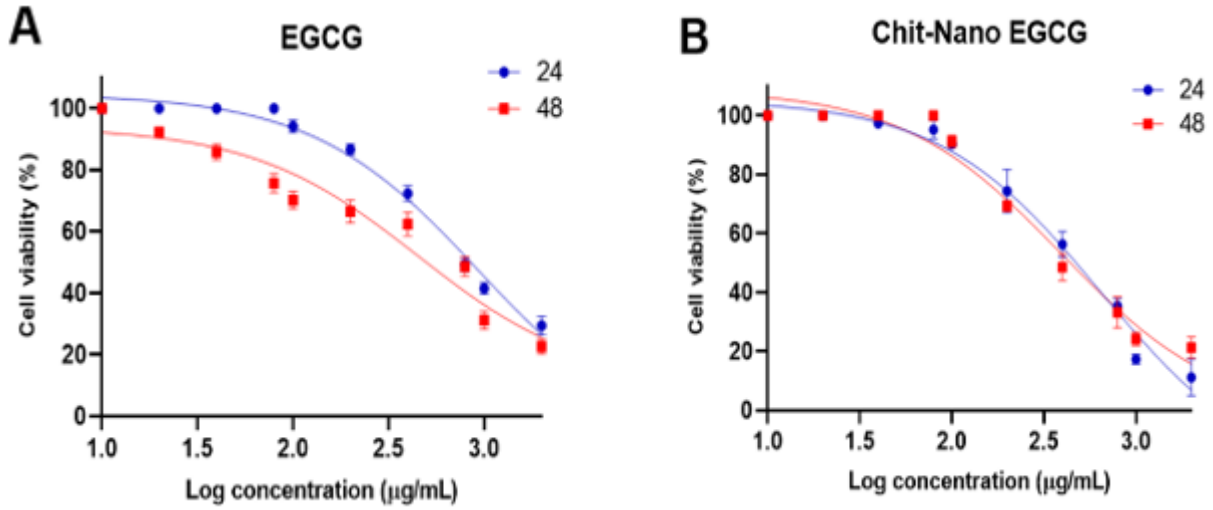

Figure 13: Result of cell viability using EGCG and Chito-Nano/EGCG against PANC-1 in various times (24 and 48 hr).

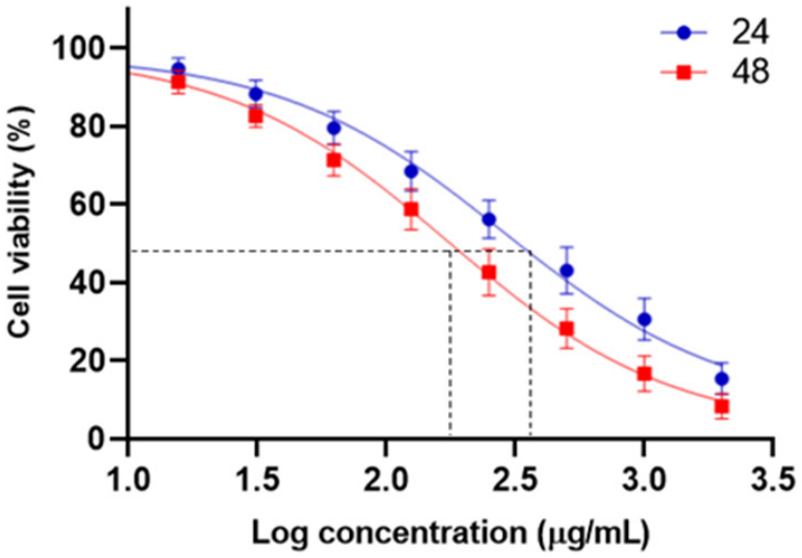

Figure 14: Dose-dependent cytotoxicity of supernatant from probiotic bacteria in *Chlorella vulgaris* against PANC-1 cancer cells after 24 and 48 hr of treatment. IC_{50} 24 hr: 285.2 and 48 hr: 174.3.

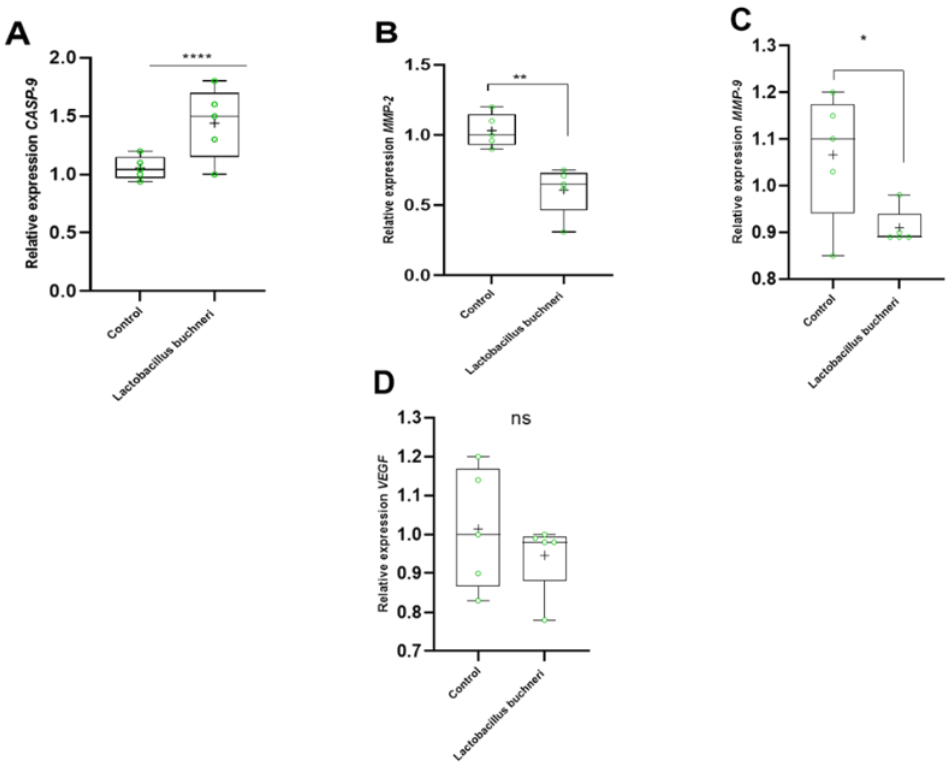

Figure 15: Effect of *Lactobacillus buchneri* Supernatant on gene expression in panc-1 cancer cells. Relative expression levels of caspase9 (A), MMP2 (B), CYCLIND (C) And VEGF were measured by qRT-PCR. Data are presented as box plots, with each box representing the median, interquartile range, and whiskers representing the minimum and maximum values. ***p*<0.01, *** *p*<0.001, and *****p*<0.0001 compared to untreated cells.

treatment curve shows a more rapid and pronounced decrease in cell viability with increasing concentration of the supernatant compared to the 24-hr treatment. The IC_{50} is reached at a lower concentration, suggesting that the longer exposure time increases the cytotoxic effect of the supernatant on PANC-1 cells.

Gene expression after Lactobacillus buchneri Supernatant

In this study, we examined the effects of *Lactobacillus buchneri* Supernatant on the expression of critical genes involved in apoptosis and inflammation in PANC-1. Figure 15A-D showed that the expression of caspase 9 was significantly increased,

and MMP2 genes were significantly increased, and VEGF was not significantly decreased. The observed downregulation of these genes suggests that *Lactobacillus buchneri* Supernatant may be exerting anti-inflammatory effects. *CASP9, CYCLIND1, MMP2*, and *VEGF* are all involved in inflammation and tumor progression. Therefore, these findings suggest that the treatments could potentially inhibit cancer cell invasion and metastasis. *Lactobacillus buchneri* Supernatant the expression of CASP9, CYCLIND1, MMP2, and VEGF on pancreatic cancer. These findings suggest a potential anti-inflammatory and anti-metastatic role for these agents. Further investigation into the underlying mechanisms and *in vivo* validation are warranted to elucidate their therapeutic potential.

Gene expression after genistein-conjugated chitosan

The dose of IC_{50} (9.859 µg/mL) was used to evaluate the expression of Bax, Bcl-2, MMP2 and MMP9 genes after 48 hr, the results are presented in Figure 16 illustrates the comparative expression levels of four genes (Bax, Bcl-2, MMP-2, and MMP-9) in Panc-1 cells subjected to treatment with genistein-conjugated chitosan nanoparticles. The x-axis denotes the treatment group (control, genistein-conjugated chitosan nanoparticles), while the y-axis indicates the relative gene expression. The genistein-conjugated chitosan nanoparticles markedly increased Bax expression relative to the control group. This indicates that treatments may trigger apoptosis in Panc-1 cells. Effects of genistein-conjugated chitosan nanoparticles on the expression of Bcl-2, MMP-2, and MMP-9. Genistein-conjugated chitosan nanoparticles markedly reduced the levels of MMP2 and MMP9. Genistein-conjugated

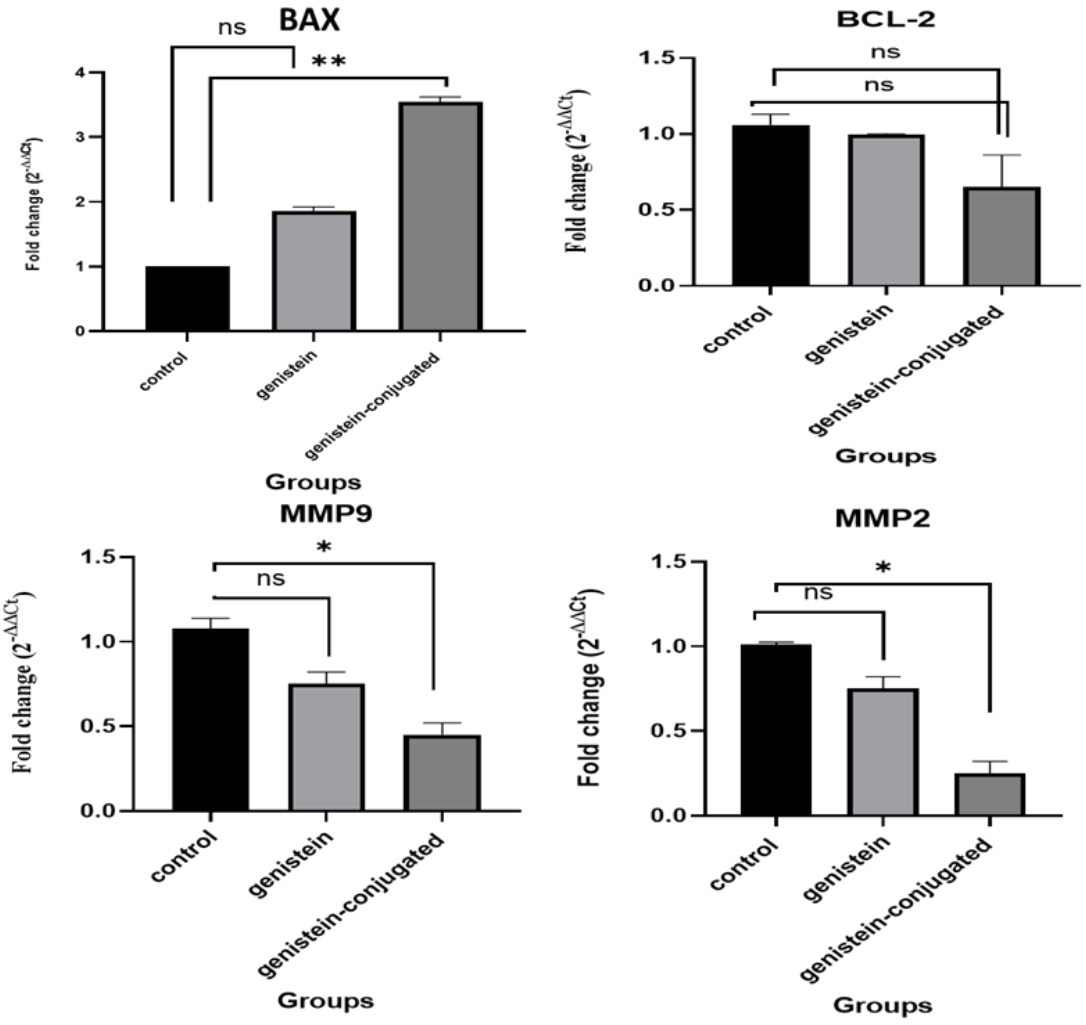

Figure 16: The effect of genistein-conjugated chitosan nanoparticles on the relative expression of apoptotic and metastatic markers in cancer cells. (A) Expression of pro-apoptotic gene BAX was significantly increased ($p<0.0001$) following treatment. (B) The anti-apoptotic gene BCL-2 was significantly downregulated ($p<0.001$). (C) MMP-2 expression was significantly reduced ($p<0.001$), and (D) MMP-9 expression was also significantly downregulated ($p<0.0001$), suggesting reduced metastatic potential. Data are presented as mean±standard deviation ($n=3$).

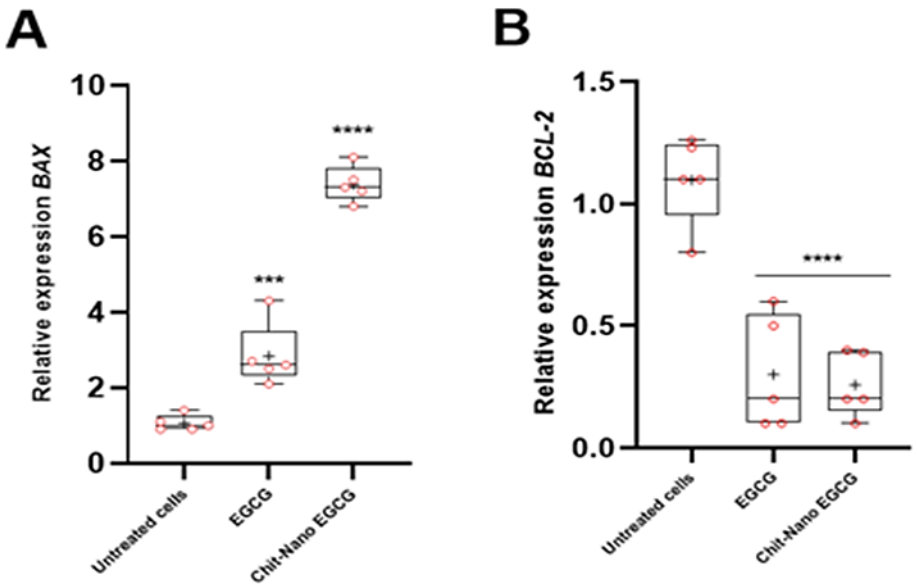

Figure 17: Relative expression of pro-apoptotic (BAX) and anti-apoptotic (BCL-2) genes in PANC-1 cells following treatment with EGCG and Chit-Nano EGCG.

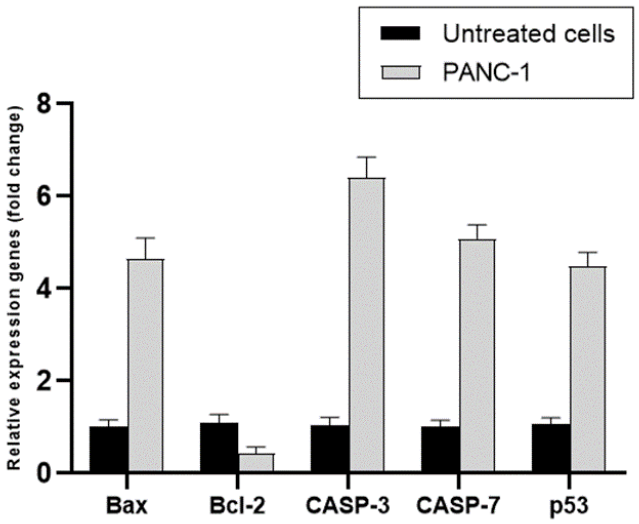

Figure 18: Relative expression of apoptotic-related genes in PANC-1 cells compared to untreated cells. The expression of pro-apoptotic genes (Bax, CASP-3, CASP-7, and p53) is significantly upregulated in PANC-1 cells, while the expression of the anti-apoptotic gene Bcl-2 is downregulated. Data indicate activation of apoptosis in PANC-1 cells, contributing to cell death mechanisms. Values are presented as fold change ± standard error of the mean (SEM).

chitosan nanoparticles demonstrated anticancer properties in Panc-1 cells by causing apoptosis and perhaps preventing tumor invasion. Treatments markedly increased Bax expression, while decreasing Bcl-2, MMP2, and MMP9 expression. The results indicate that genistein-conjugated chitosan nanoparticles may possess therapeutic potential for pancreatic cancer.

Gene expression after chitosan/Epigallocatecin-3-Gallate (EGCG)

In Figure 17, the relative expression levels of the pro-apoptotic gene *Bax* in PANC-1 cells are compared across three groups: untreated cells, cells treated with EGCG, and cells treated with Chit-Nano EGCG. The expression levels of *Bax* increase significantly when treated with EGCG and even more so with Chit-Nano EGCG. The higher expression of *Bax*, especially in the Chit-Nano EGCG group, suggests that this treatment induces a stronger pro-apoptotic response in the cells compared to EGCG alone. The data show statistical significance, with $p<0.001$ for EGCG treatment and $p<0.0001$ for Chit-Nano EGCG treatment.

Gene expression after Chlorella vulgaris

Figure 18 illustrates the relative expression levels of apoptosis-related genes in PANC-1 cells in comparison to untreated counterparts. The analyzed genes include Bax, Bcl-2, CASP-3, CASP-7, and p53, which are frequently implicated in the regulation of apoptotic and cellular death signaling pathways. Bax is recognized as a pro-apoptotic protein that triggers cell death by enhancing the permeability of the mitochondrial membrane, culminating in the release of cytochrome c and the subsequent activation of caspases. The pronounced upregulation of Bax in PANC-1 cells signifies the promotion of apoptosis within these cells. Bcl-2 serves as an anti-apoptotic protein that obstructs cell death by inhibiting the function of pro-apoptotic proteins, such as Bax. In the presented figure, the comparatively low expression of Bcl-2 in PANC-1 cells relative to untreated cells indicates a reduction in the anti-apoptotic defensive mechanism, thereby facilitating cellular demise. The upregulation of caspase-3 in PANC-1 cells, a pivotal executioner caspase within the apoptotic cascade, denotes the activation of apoptosis, leading ultimately to the degradation of cellular constituents. Analogous to caspase-3, caspase-7 represents another executioner caspase, and its elevated expression in PANC-1 cells as opposed to untreated cells corroborates the notion that apoptotic pathways are highly

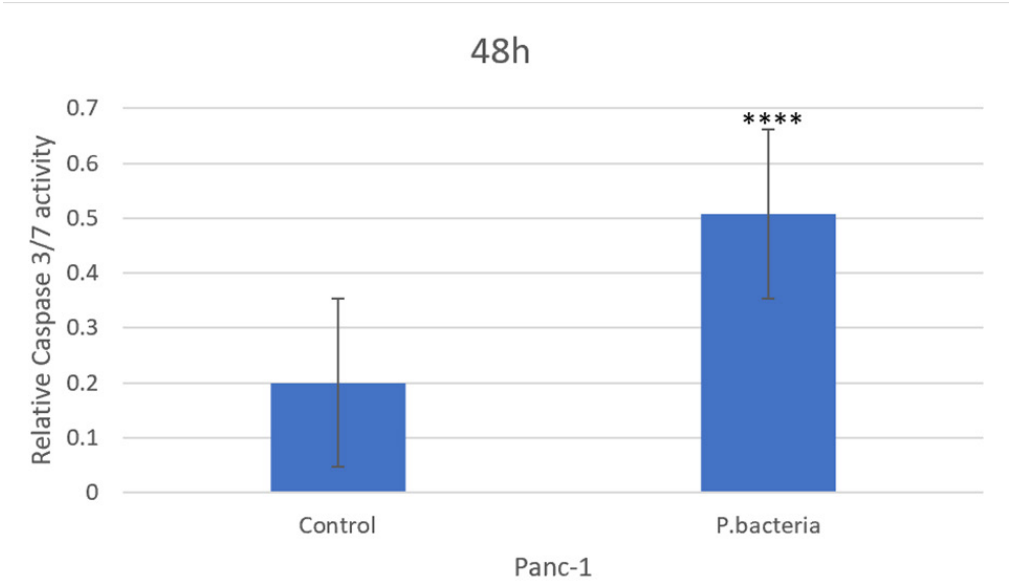

Figure 19: Caspase 3/7 activity Panc-1 cells treated with different samples. Data represented means ± standard deviations (n=3). For all charts, ****p<0.0001.

engaged. p53, a crucial tumor suppressor gene, initiates apoptosis in response to cellular stress and DNA damage. The upregulation of p53 in PANC-1 cells indicates that the cellular stress response is active, instigating the onset of apoptosis. Overall, the empirical evidence indicates that PANC-1 cells demonstrate an elevation in apoptotic activity, as reflected by the upregulation of pro-apoptotic genes (Bax, CASP-3, CASP-7, and p53) and the downregulation of anti-apoptotic genes (Bcl-2). This gene expression profile aligns with the initiation of apoptosis in neoplastic cells, which represents a vital mechanism for regulating the proliferation and survival of cancer cells. The results shown in Figure 19 also indicate that the activity of caspase-3/7 was significantly increased in the presence of the probiotic bacteria from *Chlorella vulgaris* extract on Panc-1 cell line.

DISCUSSION

The study aims to investigate the combined effects of these compounds on inducing apoptosis and altering gene expression related to cell death in pancreatic cancer cell lines. By evaluating these natural compounds, the research seeks to identify potential therapeutic agents for improving treatment outcomes in pancreatic cancer. The IC_{50} values for the treatment were 389.7 µg/mL and 297.1 µg/mL at 24 and 48 hr, respectively, significantly increasing the pro-apoptotic gene CASP9 while decreasing CYCLIND1, MMP2, and VEGF expression. GCNPs were synthesized and characterized, showing cytotoxic effects with IC_{50} values of 21 µg/mL at 24 hr and 9.859 µg/mL at 48 hr. These nanoparticles enhanced apoptosis by increasing Bax expression and decreasing Bcl-2, while also suppressing MMP2 and MMP9, which are linked to metastasis. Chitosan nanoparticles containing EGCG demonstrated notable anti-cancer effects, reducing cell proliferation and inducing apoptosis. Additionally, *Lactobacillus* strains from *Chlorella vulgaris* exhibited strong probiotic and anti-cancer properties. Cytotoxicity assays revealed a dose-dependent inhibition of Panc-1 cell viability and induction of apoptosis, highlighting the potential of these natural compounds in cancer therapy.

For many years, bacteria were viewed predominantly as harmful to human health. However, this perspective is evolving, as certain bacteria, particularly probiotics, are recognized for their health benefits. Probiotics are live microorganisms that, when consumed, positively influence the host's health by enhancing the body's microbial flora (Das *et al.*, 2022). They are primarily found in the human intestine, where they coexist with other bacteria and play a protective role against various diseases. The intestinal microflora is a complex ecosystem containing over one hundred trillion bacteria, categorized as beneficial, harmful, or neutral (Rinninella *et al.*, 2019). A healthy balance between these bacteria is crucial; disruptions caused by factors such as antibiotics, chemotherapy, and environmental toxins can lead to an overgrowth of harmful bacteria, increasing susceptibility to diseases like diarrhea and weakened immunity. Probiotics help restore this balance by promoting beneficial bacteria, allowing the gut to repair itself (Patangia *et al.*, 2022). Research indicates that probiotics may also play a role in cancer prevention, particularly breast cancer, by modulating gut bacteria and influencing the immune system. Probiotics support the body's natural microbial balance, contributing to disease prevention and immune support. They are known for enhancing digestion, boosting immune function, and promoting overall health, with notable strains including *Lactobacillus* and *Bifidobacterium* (Wang *et al.*, 2022). Probiotics are commonly found in fermented foods like yogurt and kefir, as well as dietary supplements. Their health benefits depend on the strain, dosage, and individual health conditions.

Emerging studies suggest probiotics may also impact mental health and have therapeutic benefits beyond gastrointestinal health, such as lowering cholesterol and exhibiting anti-cancer properties (Kaur *et al*., 2022). A promising strategy for improving health involves dietary fortification with bioactive substances, shifting focus from drug dependency to regular consumption of functional foods. Probiotics, particularly lactic acid bacteria, have gained attention as potential nutraceuticals, enhancing immune function without adverse effects. Regular intake of probiotics is linked to improved immune resistance against diseases (Damián *et al*., 2022). Pancreatic Cancer (PC) is one of the deadliest cancers, with a low survival rate. Current treatments include surgery, chemotherapy, and emerging immunotherapy, but they often fail to significantly improve survival due to issues like drug resistance and side effects (Patel *et al*., 2019). Novel therapeutic strategies are crucial for improving patient outcomes. Numerous studies have explored the effects of various *Lactobacillus* strains on cancer, demonstrating their potential to enhance immune responses and inhibit tumor growth. For instance, *Lactobacillus acidophilus* and *Lactobacillus casei* have shown promise in improving survival rates in animal models of breast cancer by stimulating immune responses (Cho *et al*., 2024). Other studies have highlighted the immunomodulatory effects of probiotics in breast cancer-bearing mice and the potential of *Lactobacillus plantarum* as a preventive agent. Research into the cytotoxic effects of *Lactobacillus buchneri* on colorectal cancer cells has also shown promising results, indicating its ability to induce apoptosis and inhibit cell proliferation (Torabi *et al*., 2025). Overall, the growing body of evidence supports the beneficial role of probiotics in cancer prevention and treatment, highlighting their potential as therapeutic agents in modern medicine. Continued research is essential to fully understand their mechanisms and optimize their use in clinical settings (Sankarapandian *et al*., 2022). Pancreatic cancer is a leading cause of cancer-related deaths globally, often diagnosed at advanced stages when treatment options are limited. The prognosis is poor, with a 5-year survival rate of only about 5%. This dire situation has spurred research into alternative therapies, including the application of nanotechnology in medicine. Nanocarriers such as nanoparticles and liposomes can deliver drugs effectively, but challenges remain in finding biodegradable and biocompatible options (Stoffel *et al*., 2023). Chitosan, a natural polysaccharide derived from crustacean shells and fungi, has gained attention for its biocompatibility and potential health benefits. It can influence the immune system and has antimicrobial properties, although its high viscosity and insolubility in water limit its applications (Ke *et al*., 2021). Genistein, an isoflavone found in soy, has shown promise in cancer prevention, particularly in pancreatic cancer, by modulating apoptosis-related genes. This study investigates the effects of Genistein-Conjugated Chitosan Nanoparticles (GCNPs) on pancreatic cancer cell lines, focusing on the expression of key genes involved in apoptosis and metastasis (Javed *et al*., 2021). Chitosan, a natural polysaccharide, has shown promise in enhancing pancreatic cancer therapy through nanoparticle formulations. Studies have explored its use in delivering chemotherapeutic agents like gemcitabine and metformin. Additionally, Epigallocatechin-3-Gallate (EGCG), a polyphenolic compound from green tea, exhibits anti-cancer properties by inducing apoptosis and modulating the cell cycle (Aydemir *et al*., 2024). The anticipated global population increase to 9.5 billion by 2050 necessitates the exploration of sustainable food sources, with microalgae emerging as a promising solution. Microalgae, particularly *Chlorella vulgaris*, are rich in essential nutrients and bioactive compounds, offering health benefits such as antioxidant, anticancer, and cholesterol-lowering effects (Kiran and Venkata Mohan, 2021). *Chlorella* is recognized as a Generally Recognized as Safe (GRAS) food source and has been incorporated into various products (Albulaihed Y. *et al*., 2026), including cheese, where it enhances lactic acid bacteria counts. Probiotics, live microorganisms that provide health benefits, are known for their roles in immune modulation and disease prevention. They can also induce apoptosis in cancer cells, particularly through mechanisms involving the BCL2 gene family, which regulates cell death pathways (Patel *et al*., 2021). Pancreatic cancer, with a low survival rate and limited treatment options, underscores the need for safer therapies. Gemcitabine, the standard chemotherapy, offers minimal survival benefit and is associated with significant toxicity (Ying *et al*., 2012) (Albulaihed *et al*., 2026). The research involves assessing apoptosis-related gene expression and Caspase-3/7 activity. Initial findings indicate that the *Chlorella* extract induces apoptosis in Panc-1 cells by modulating key apoptotic markers. Similar studies have shown that probiotics can effectively inhibit cancer cell proliferation and migration, highlighting their potential as complementary cancer therapies

CONCLUSION

In conclusion, this study reveals that *Lactobacillus buchneri* supernatant, chitosan nanoparticles conjugated with genistein, and chitosan nanoparticles containing EGCG significantly induce apoptosis in Panc-1 pancreatic cancer cells. The IC_{50} values indicate strong cytotoxic effects, particularly with GCNPs. The upregulation of CASP9 and downregulation of Bcl-2 highlight the treatments' potential to promote apoptosis. These findings emphasize the promising role of natural compounds and nanotechnology in developing effective pancreatic cancer therapies. Furthermore, this study underscores the importance of utilizing nanomaterials to enhance drug delivery and therapeutic effects. Chitosan nanoparticles serve as effective drug carriers, efficiently delivering genistein and EGCG to target cells, thereby amplifying their therapeutic effects. This innovative approach could be considered a new strategy in pancreatic cancer

treatment, potentially improving clinical outcomes for patients. Additionally, the research suggests that combining *Lactobacillus buchneri* supernatant with chitosan nanoparticles may serve as a complementary method in pancreatic cancer therapy. Given that pancreatic cancer is one of the most challenging types of cancer, these findings could pave the way for further investigations into natural compounds and nanotechnology, contributing to the development of new and effective treatments.

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ABBREVIATIONS

PDAC: Pancreatic Ductal Adenocarcinoma; **Panc-1:** Pancreatic Cancer Cell Line 1; **GCNPs:** Genistein-Conjugated Chitosan Nanoparticles; **EGCG:** Epigallocatechin-3-gallate; **MTT**: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **IC_{50}:** Half Maximal Inhibitory Concentration; **qPCR:** Quantitative Polymerase Chain Reaction; **CASP9:** Caspase-9; **Bax:** Bcl-2-associated X protein; **Bcl-2:** B-cell lymphoma 2; **CYCLIND1:** Cyclin D1; **MMP2:** Matrix Metalloproteinase-2; **MMP9:** Matrix Metalloproteinase-9; **VEGF:** Vascular Endothelial Growth Factor. **TPP:** Sodium triphosphate pentabasic**; UV-vis:** Ultraviolet-Visible spectroscopy; **DLS:** Dynamic Light Scattering; **SEM:** Scanning Electron Microscopy; **EDS:** Energy Dispersive Spectroscopy; **FTIR:** Fourier Transform Infrared spectroscopy; **DPPH:** 2,2-diphenyl-1-picrylhydrazyl; **DMSO:** Dimethyl Sulfoxide; **MRS:** Man, Rogosa, and Sharpe**; FE-SEM:** Field Emission Scanning Electron Microscope; **PDI:** Polydispersity Index; **NPS:** Nanoparticles; Chit-Nano **EGCG:** Chitosan-Nanoparticle Epigallocatechin-3-gallate; **SEM:** Standard Error of the Mean.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

FUNDING

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AUTHOR CONTRIBUTIONS

R.M. conceived and designed the study, supervised the overall research process, and contributed substantially to data interpretation and manuscript preparation. Na.J. conducted the experimental procedures, including the assessment of regulatory mechanisms of cell death induced by *Lactobacillus buchneri, Chlorella vulgaris* probiotics, and chitosan-conjugated phytochemicals in pancreatic cancer cells, and assisted in data collection and statistical analysis. Ne.J. contributed to the literature review, methodological development, and participated in drafting and revising the manuscript. All authors read and approved the final version of the manuscript and agreed to be accountable for all aspects of the work.

SUMMARY

The study reveals that *Lactobacillus buchneri* supernatant, chitosan nanoparticles conjugated with genistein, and chitosan nanoparticles containing EGCG significantly induce apoptosis in Panc-1 pancreatic cancer cells. The IC_{50} values indicate strong cytotoxic effects, particularly with GCNPs. The upregulation of CASP9 and downregulation of Bcl-2 highlight the treatments' potential to promote apoptosis. These findings emphasize the promising role of natural compounds and nanotechnology in developing effective pancreatic cancer therapies.

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