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Apoptotic Induction in Glioblastoma Cells by Staphylococcus aureus Cytoplasmic Extract: An Approach to Targeting *Bax* and *Bcl-2* Pathways

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Authors
Maryam Rasouli, *MSc*[1]
Bahareh Hajikhani, *PhD*[2*]
Hossein Fahimi, *PhD*[1]
Mahdyeh Neghabi Hajiagha, *PhD*[2]

[1] Department of Genetics, Faculty of Advanced Science and Technology, Tehran Medical Science, Islamic Azad University, Tehran, Iran
[2] Department of Microbiology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

**** Correspondence***
Department of Microbiology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran.
E-mail: hajikhani@sbmu.ac.ir

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ABSTRACT

Background: Glioblastoma multiforme (GBM) is one of the most aggressive and prevalent primary brain tumors with limited treatment options. The median survival rate remains low, highlighting the need for innovative therapeutic strategies. *Staphylococcus aureus* (*S. aureus*) extract has shown the potential to induce apoptosis in cancer cells, offering a promising avenue for glioblastoma treatment. This study aimed to investigate the effects of *S. aureus* cytoplasmic extract on the U87 glioblastoma cell line, focusing on its ability to induce apoptosis and modulate key apoptotic genes, *Bax* and *Bcl-2*.
Materials & Methods: U87 cells were cultured under standard conditions, and *S. aureus* cytoplasmic extract was prepared using sonication. The extract protein concentration was determined using the Lowry assay. Cell viability was assessed by MTT assay, and the expression levels of *Bax* and *Bcl-2* were measured via quantitative real-time PCR (qRT-PCR) following treatment with the extract at concentrations ranging from 10 to 30 µg/mL.
Findings: The cytoplasmic extract significantly reduced U87 cell viability in a concentration-dependent manner with the highest cytotoxicity (30 µg/mL) ($p< .05$). The extract increased *Bax* expression and decreased Bcl-2 expression, indicating apoptosis induction. Statistical analysis confirmed significant differences in gene expression between the treated and control groups ($p< .05$).
Conclusion: The findings demonstrate that *S. aureus* cytoplasmic extract effectively inhibits U87 glioblastoma cell proliferation and promotes apoptosis through the modulation of apoptotic genes. These results suggest that bacterial extracts could serve as a potential therapeutic agent for glioblastoma, warranting further research into their mechanisms and clinical applications.

Keywords: Glioblastoma, *Staphylococcus aureus*, Apoptosis, Cell proliferation, Cell line

CITATION LINKS

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Introduction

Glioblastoma multiforme (GBM) is among the most aggressive and prevalent primary malignant brain tumors, accounting for approximately 15% of all brain tumors and over 60% of malignant gliomas globally [1, 2]. Despite significant advancements in treatment modalities, including surgical resection, radiation therapy, and chemotherapy, the prognosis for GBM patients remains grim, with a median survival time of 12 to 15 months post-diagnosis and a five-year survival rate of less than 5% [3]. The failure to achieve better outcomes is attributed to the infiltrative nature of the disease, resistance to existing therapies, and the presence of the blood-brain barrier (BBB) restricting effective drug delivery to the tumor site [4]. These issues highlight the urgent necessity for pioneering therapeutic approaches to enhance survival rates and quality of life for patients with GBM.

Given the urgent need for innovative therapeutic approaches, the current research focused on the potential of bacterial products for cancer treatment. The use of bacterial extracts and components in cancer therapy offers a promising approach by selectively targeting cancer cells and inducing apoptosis [5]. These extracts modulate apoptotic genes by upregulating *Bax* and downregulating *Bcl-2*, promoting tumor cell death [6]. Bacteria-based therapies also address challenges like drug resistance and poor tumor penetration [7].

Bacterial products have emerged as promising anticancer agents, leveraging their ability to induce apoptosis, modulate immune responses, and potentially penetrate the BBB [7]. *Clostridium butyricum* extracts inhibit tumor growth via Wnt signaling in colon cancer models [8], and *Escherichia coli* supernatant triggers caspase-mediated apoptosis in bladder cancer cells [5]. However, studies on the effects of bacterial extracts on GBM are limited. *S. aureus* enterotoxins (e.g., SEB) suppress GBM growth by targeting TGF-β/smad pathways, and live *S. aureus* infections enhance the survival of GBM patients via microglial activation [9]. However, the use of *S. aureus* cytoplasmic extracts, which contain a complex mixture of toxins (e.g., alpha-hemolysin, PSMs) and metabolites, in GBM treatment remains unexplored.

S. aureus, a Gram-positive bacterium, is of particular interest as it produces a variety of toxins and enzymes, which could influence apoptosis [10], a form of programmed cell death critical for maintaining cellular homeostasis [11]. *S. aureus* NCTC 8325 was chosen in this study due to its standardized use in over 1,000 studies, enabling reproducible cytoplasmic extract preparation rich in apoptosis-inducing components. Its genetic stability is suitable for mechanistic studies of *Bax/Bcl-2* modulation in GBM.

Disruption of apoptosis is a hallmark of cancer, characterized by uncontrolled cell proliferation and resistance to cell death [12]. Apoptosis is regulated by the *Bcl-2* protein family, with pro-apoptotic proteins such as *Bax* promoting cell death and anti-apoptotic proteins like *Bcl-2* inhibiting it [13]. Targeting these proteins to restore apoptotic pathways offers a promising approach to combating cancer.

Bacterial cytoplasmic extracts have shown the potential to induce apoptosis and inhibit tumor growth in various cancer models [14-16]. However, the specific effects of *S. aureus* cytoplasmic extract on GBM cells, particularly in the widely used U87 glioblastoma cell line, remain largely unexplored. The U87 cell line, derived from human glioblastoma, is a critical model for studying glioblastoma biology and testing therapeutic interventions [17]. Understanding how *S. aureus* cytoplasmic extract affects apoptosis, mainly through regulating *Bax* and *Bcl-2* expression, could

open new avenues for GBM treatment.

Objectives: This study aimed to investigate the potential effects of *S. aureus* cytoplasmic extract on U87 glioblastoma cells to elucidate the therapeutic potential of bacterial components in GBM treatment and assess their ability to induce apoptosis and affect key apoptotic markers.

Materials and Methods

Preparation of *S. aureus* cytoplasmic extract: *S. aureus* NCTC 8325 was purchased from the Microorganism Bank at the Iranian National Center for Genetic and Biological Resources (Iranian Biological Resources Center: IBRC) and cultured in tryptic soy broth (TSB; BD Biosciences, USA) at 37 °C with agitation until reaching mid-logarithmic phase. Bacterial cells were harvested by centrifugation at 5,000 × g for 10 min at 4 °C, washed twice with phosphate-buffered saline (PBS), and resuspended in lysis buffer (20 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1 mM EDTA; 1 mM PMSF). Cell lysis was achieved through sonication on ice (six 30-second cycles with 30-second intervals). The lysate was centrifuged at 12,000 × g for 20 min at 4 °C to remove cell debris, and the supernatant containing the cytoplasmic extract was filtered through a 0.22 µm membrane to ensure sterility. Protein concentration was determined using the Lowry assay (Bio-Rad Laboratories, USA) based on the manufacturer›s instructions. Briefly, 5 µL of the sample was mixed with 25 µL of alkaline copper reagent and 200 µL of Folin-Ciocalteu reagent in a 96-well plate and incubated for 15 min at room temperature, and the absorbance was measured at 750 nm. A bovine serum albumin standard curve (0–2 mg/mL) was used to determine protein concentration.

U87 cell culture: The U87 cell line, originally derived from human malignant glioma, was obtained from the National Center for Genetic and Biological Resources of Iran. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; BIOSERA, USA) supplemented with 10% fetal bovine serum (FBS; GIBCO, USA) and 1% penicillin-streptomycin (Thermo Fisher Scientific). Cultures were maintained at 37 °C in a humidified atmosphere containing %5 CO_2, with the culture medium replaced every 48 hrs.

MTT viability assay and treatment of U87 cells: Following cell counting, a 48-well culture plate was prepared. Initially, 10 µL of U87 cells were dispensed into each well. Then 200 µL of specific culture medium was added to each well to facilitate optimal cell growth, and the plate was placed in a 37 °C incubator for 24 hrs.

After incubation, the bacterial extract was introduced into the wells at concentrations of 10, 15, 20, 25, and 30 µg/mL in triplicate alongside a control group. The plate was incubated for an additional day to allow interaction between the bacterial extract and glioblastoma cells.

Cell viability was then assessed using the MTT assay. After the treatment period, the culture medium was carefully removed, and 100 µL of MTT stock solution prepared at a concentration of 5 mg/mL in PBS was added to each well. The plate was incubated at 37 °C for 4-3 hrs to allow MTT to be metabolized by viable cells.

Following incubation, the supernatant was removed, and 100 µL of DMSO was added to each well to dissolve the formazan crystals formed. The mixture was incubated for an additional 15 min. Finally, the optical density (OD) at a wavelength of 570 nm was quantified using a spectrophotometer to measure the cell viability post-treatment.

RNA extraction and quantitative real-time PCR (qRT-PCR): Total RNA was extracted using the RNeasy mini kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. RNA concentration

and purity were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Germany). cDNA synthesis was performed with one µg of total RNA using the iScript cDNA synthesis kit (Bio-Rad Laboratories). qRT-PCR was conducted using SYBR Green Master Mix (Addbio, Korea) on a StepOnePlus real-time PCR system (Applied Biosystems). Specific primers for *Bax*, *Bcl-2*, and GAPDH (housekeeping gene) were selected based on published sequences (Table 1). Amplification conditions for *Bax* and *Bcl-2* genes were set as follows: an initial cycle at 95 °C for 5 min, followed by 40 cycles consisting of 15 s at 95 °C, 25 s at 60 °C, and 20 s at 72 °C. Each reaction was conducted in duplicate to guarantee precision and consistency. Relative expression levels of *Bax* and *Bcl-2* were calculated using the 2^−ΔΔCt method and normalized to GAPDH expression.

Statistical analysis: Experiments were performed in triplicate, and data were presented as mean ± standard deviation (SD). Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test, with $p < .05$ considered statistically significant. Analyses were conducted using GraphPad Prism software (GraphPad Software, San Diego, CA, USA).

Ethical considerations: This study received ethical approval from the Ethics and Research Committee of Islamic Azad University, adhering to established protocols (Approval No. IAU.PS.REC.1400.456).

Findings

Preparation and evaluation of *S. aureus* cytoplasmic extract: *S. aureus* cytoplasmic extract was successfully prepared through sonication, which resulted in cell lysis. The supernatant obtained after centrifugation was confirmed to contain cytoplasmic proteins, as evidenced by the presence of protein bands on SDS-PAGE. The protein concentration was determined to be 16.48 µg/µL using the Lowry assay, which was suitable for further experiments. The SDS-PAGE results demonstrated effective lysis of bacterial cells. Bands corresponding to cytoplasmic proteins were observed, indicating successful extraction. The positions of these bands aligned with molecular weight markers, confirming the presence of proteins in the cytoplasmic extract. In fact, *S. aureus* cytoplasmic extract primarily contained soluble proteins and metabolites with minimal DNA/RNA contamination due to filtration (0.22 µm).

MTT viability assay and treatment of U87 cells: The cytotoxic effects of *S. aureus* cytoplasmic extract on U87 glioblastoma cells were evaluated using the MTT assay. The results indicated a concentration-dependent decrease in cell viability. At concentrations of 10, 15, 20, 25, and 30 µg/mL, the cell survival rates were 67.42% ± 11.77, 63.69% ± 11.14, 65.21% ± 2.92, 55.87% ± 3.86, and 42.71%

Table 1) Primer specifications for gene expression analysis

Genes	Primer Sequences	Product Length	TM	Accession Number
Bax forward	CGCCCTTTTCTACTTTGACA	152	55.3 C°	001291430/1
Bax reverse	GTGACGAGGCTTGAGGAG		°58.2 C	
Bcl2 forward	TGGTCTTCTTTGAGTTCGG	120	54.5 C°	000633/2
Bcl2 reverse	GGCTGTACAGTTCCACAA		53.7 C°	
Gapdh forward	CTTTGGTATCGTGGAAGGAC	197	57.3 C°	001289745/2
Gapdh reverse	GCAGGGATGATGTTCTGG		56 C°	

± 5.33, respectively, compared to the control group (100% ± 11.84) (Figure 1). Statistical analysis using one-way ANOVA revealed a significant effect of treatment on cell viability (F = 14.32, p< .0001, R^2 = 0.8565). Post hoc analysis using Tukey's test showed significant differences in cell viability between the control and all treated groups (p< .05). However, no significant difference was observed between different extract concentrations (p> .05). The analysis highlighted a consistent reduction in cell viability with increasing *S. aureus* concentration, demonstrating a clear dose-response relationship (Figure 1).

Microscopic evaluation: Microscopic examination of U87 cells post-treatment revealed morphological changes indicative of cell death, with a notable reduction in cell density correlating with increasing bacterial extract concentrations (Figure 2). These observations support the quantitative findings from the MTT assay.

RNA extraction and quantitative real-time PCR (qRT-PCR): The expression levels of the pro-apoptotic gene *Bax* and the anti-apoptotic gene *Bcl-2* were analyzed using qRT-PCR. The results showed a significant upregulation of *Bax* expression in the treated group compared to the control group (p= .034), with a mean relative expression of 0.4893 ± 0.2508 in the treated group versus 0.0303 ± 0.0141 in the control group (Figure 3). Conversely, *Bcl-2* expression was significantly downregulated in the treated group (p= .0459), with a mean relative expression of 0.4726 ± 0.2133 in the treated group compared to 1.324 ± 0.4689 in the control group (Figure 3).

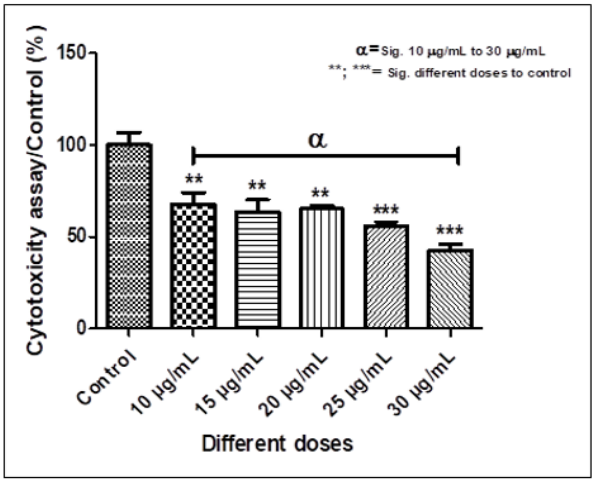

Figure 1) Cytotoxicity of *S. aureus* extract on U87 cells. Cell viability (%) of U87 cells after treatment with *S. aureus* cytoplasmic extract (10–30 µg/mL), showing a general concentration-dependent decrease compared to the control group (100%), with significant reductions at higher concentrations (p< .05, ANOVA with Tukey's post hoc test).

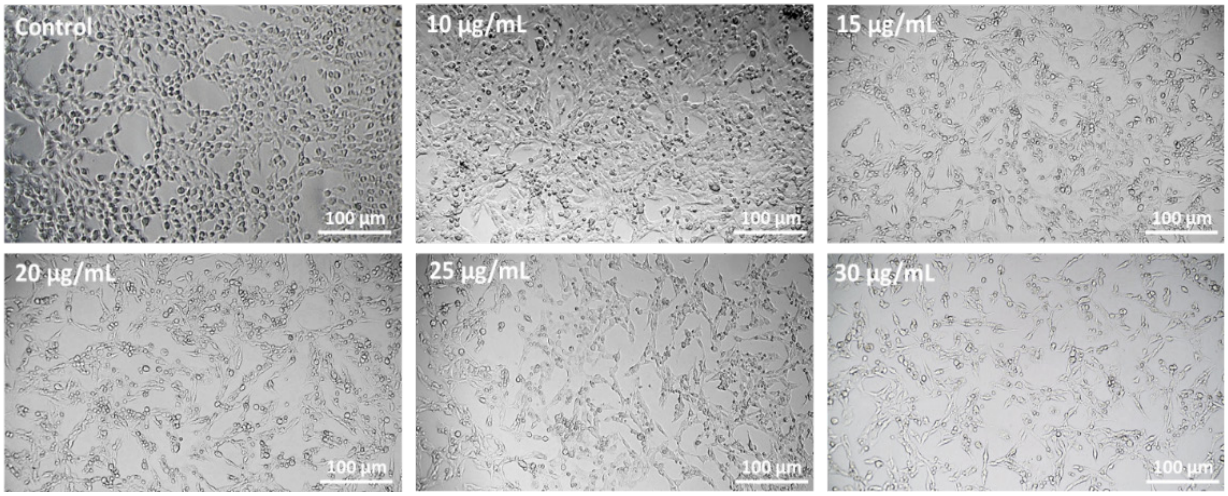

Figure 2) Cytotoxic effects of *S. aureus* extract on U87 cells. The figure shows the concentration-dependent reduction in U87 cell viability after treatment with *S. aureus* cytoplasmic extract (30–10 µg/mL) compared to the control group. Significant cytotoxicity was observed at higher concentrations, consistent with MTT assay results (p< .05).

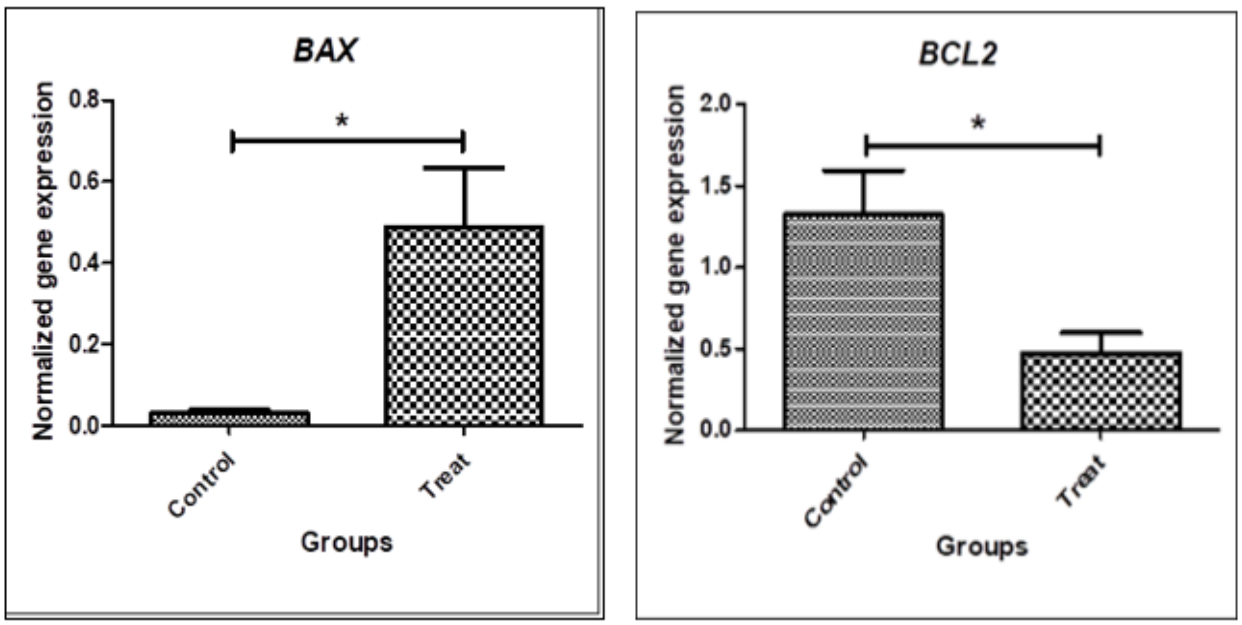

Figure 3) Relative expression of *Bax* and *Bcl2* in treated and control groups. The figure displays the normalized expression levels of the pro-apoptotic gene *Bax* and the anti-apoptotic gene *Bcl-2* in U87 cells treated with *S. aureus* cytoplasmic extract compared to the control group. Although *Bax* expression was significantly upregulated in the treated group, a significant decrease in *Bcl-2* expression was observed ($p < .05$).

The relative expression of *Bax* and *Bcl-2* showed moderate variability, likely due to biological heterogeneity in U87 cells; however, significant differences were observed ($p < .05$). These findings suggest that *S. aureus* cytoplasmic extract induces apoptosis in U87 cells by modulating the expression of key apoptotic genes.

Discussion

The current study explored the use of *S. aureus* cytoplasmic extract as a potential therapeutic agent against U87 glioblastoma cells. This exploration was built upon the landscape of novel cancer therapies, which focus on harnessing the biological activity of microbial derivatives to initiate apoptosis, a critical pathway in the suppression of malignant tumors. Glioblastoma, known for its aggressive nature and poor prognosis, necessitates innovative therapeutic approaches, making this study highly relevant and necessary.

The *Bax/Bcl-2* pathway is a critical regulator of apoptosis, serving as a balance between pro-apoptotic and anti-apoptotic signals within the cell. *Bcl-2* is an anti-apoptotic protein that inhibits cell death by preventing the release of cytochrome c from mitochondria, thereby blocking the activation of caspases that are essential for the apoptotic process. In contrast, *Bax* is a pro-apoptotic protein that promotes apoptosis by facilitating mitochondrial outer membrane permeabilization, leading to the release of cytochrome c and subsequent activation of the apoptotic cascade [18]. The interplay between *Bax* and *Bcl-2* is crucial for cell fate determination, and dysregulation of this pathway is often implicated in various cancers, making it a potential target for therapeutic intervention.

This study demonstrated that *S. aureus* NCTC 8325 cytoplasmic extract induced significant concentration-dependent cytotoxicity in U87 glioblastoma cells, reducing cell viability to 42.71% ± 5.33 at a concentration of 30 µg/mL ($p < .05$) and promoting apoptosis through *Bax* upregulation (0.4893 ± 0.2508, $p = .034$) and *Bcl-2* downregulation (0.4726 ± 0.2133, $p = .0459$). These findings align with prior reports on the anticancer ef-

fects of bacterial extracts, such as a 50% reduction in bladder cancer cell viability by *E. coli* supernatant at 20 µg/mL via caspase-3 activation [5], although our study uniquely targeted GBM, a malignancy with distinct therapeutic challenges like the blood-brain barrier [4]. Compared to *S. aureus* enterotoxins, which inhibit GBM growth via TGF-β/smad2/3 suppression (e.g., 60% viability reduction with 10 µg/mL of SEB), our crude extract likely has broader effects due to multiple components (e.g., alpha-hemolysin, PSMs), potentially reducing resistance risks [15]. Unlike Ibrahim et al. (2020) [21], who reported that *Bacillus amyloliquefaciens* extract increased the *Bax/Bcl-2* ratio by 2-fold in breast cancer cells, the *Bax* upregulation in the present study was less pronounced (1.6-fold), possibly due to GBM intrinsic resistance to apoptosis [17].

However, the present study results suggest a therapeutic window (15–30 µg/mL) comparable to that of *C. butyricum* extracts inhibiting colon cancer growth [8]. The moderate variability in gene expression data (SD ~ 51%–45) contrasts with tighter SDs in purified toxin studies, reflecting the complexity of crude extracts but warranting further proteomic analysis to identify active components, as suggested by Zhao et al. (2022) [26]. These comparisons highlight the present study contribution to microbial oncology, offering a novel, multi-component approach to GBM treatment, which merits in vivo validation to address blood-brain barrier challenges [7].

The results demonstrated that various concentrations of the extract significantly inhibited the proliferation of U87 cells, with a notable survival rate of less than 50% observed at concentrations above 15 µg/mL. This finding is consistent with those of other studies showing how bacterial metabolites could induce cytotoxicity in cancer cells [19, 20]. The significant upregulation of the pro-apoptotic gene *Bax* and the downregulation of the anti-apoptotic gene *Bcl-2* provide compelling evidence for the extract pro-apoptotic mechanism of action. This molecular signature is consistent with the results of recent studies demonstrating the capacity of bacterial products to induce programmed cell death in cancer cells. For instance, Ibrahim et al. (2020) reported similar alterations in apoptotic marker expression when treating breast cancer cells with bacterial derivatives, supporting the validity and broader applicability of our findings [21]. While mRNA expression changes strongly suggest modulation of the *Bax/Bcl-2* axis, they do not directly confirm protein-level alterations. Future studies, including Western blot analysis, are warranted to validate corresponding changes in *Bax* and *Bcl-2* protein expression, as mRNA and protein levels may diverge due to post-transcriptional regulation [22].

The present study results contribute to the base of evidence supporting the therapeutic potential of bacterial products in cancer treatment. This aligns with recent studies by Chen et al. (2020), demonstrating tumor-inhibitory effects of bacterial products in various cancer models [8, 23]. The selective cytotoxicity observed in the current study addresses one of the primary challenges in glioblastoma therapy – the need for treatments that selectively target cancer cells while minimizing damage to healthy tissue.

The concentration-dependent response observed in cytotoxicity assays suggests a controllable therapeutic window, similar to findings reported by Ibrahim et al. (2020) using other bacterial derivatives [21]. This characteristic is crucial for potential clinical applications. However, several aspects warrant attention as this approach moves toward therapeutic development, particularly regarding selective host cell death mechanisms, as discussed by

Missiakas and Winstel (2020) [24].

In recent years, exploration of bacteria-based therapies has gained momentum, particularly in treating glioblastoma. A clinical trial reported by Bagley et al. (2024) demonstrated rapid regression of glioblastoma tumors following next-generation CAR-T (chimeric antigen receptor T-cell) therapy, showcasing the potential of harnessing the immune system to combat cancer (25). This is consistent with our findings; bacterial extracts may serve as complementary agents to enhance immune responses in glioblastoma treatment.

Next-generation CAR-T therapy refers to advanced iterations of T-cell-based immunotherapy, designed to enhance efficacy, safety, and applicability over first-generation CAR-T therapies. Unlike earlier versions targeting single antigens (e.g., CD19 for leukemia), next-generation CAR-T therapies incorporate multi-specificity (e.g., dual-targeting CARs), improved T-cell persistence, and reduced toxicities like cytokine release syndrome. For glioblastoma, these therapies target tumor-specific antigens such as EGFRvIII or IL13Rα2, overcoming tumor heterogeneity and immunosuppressive microenvironments. For instance, bivalent CAR-T cells targeting EGFR and IL13Rα2 have shown rapid tumor regression in phase 1 trials for recurrent glioblastoma, with improved infiltration across the blood-brain barrier [25]. Therefore, according to the findings, *S. aureus* cytoplasmic extract immunomodulatory and apoptotic effects could complement such therapies by enhancing tumor cell death and immune activation, potentially improving GBM treatment outcomes.

Recent research on apoptosis in cancer cells has shown that bacterial proteins could induce apoptosis through various molecular pathways [26, 27], supporting our findings regarding the complex apoptotic modulation. Furthermore, the concept of PANoptosis, as described by Shi et al. (2023), suggests that bacterial products might trigger multiple cell death pathways simultaneously, potentially enhancing their therapeutic efficacy [28].

In addition to the promising therapeutic effects of *S. aureus* cytoplasmic extract, it is crucial to consider the potential toxicity of natural bacteria, including *S. aureus*, to brain cells and other body tissues. Although our findings indicate that this extract could induce apoptosis in glioblastoma cells, understanding its impact on healthy brain cells and the immune system is essential for evaluating its safety and therapeutic viability. Previous studies have shown that bacterial components could elicit immune responses and may influence the tumor microenvironment, potentially enhancing anti-tumor immunity [5]. However, the risk of unintended cytotoxic effects on normal cells must be carefully assessed. For instance, *S. aureus* toxins have been reported to induce apoptosis in various cell types, raising concerns about their effects on non-cancerous tissues [10]. Future studies should investigate the selective toxicity of *S. aureus* extracts, focusing on their effects on non-cancerous brain cells and other critical tissues. This exploration not only enhances the immunological significance of *S. aureus* as a therapeutic agent but also provides a more comprehensive understanding of its potential risks and benefits in glioblastoma treatment.

Future research directions should focus on elucidating the specific molecular components responsible for the observed effects, investigating potential synergistic interactions with conventional therapies, conducting in vivo studies to validate efficacy and safety, developing strategies to optimize delivery across the blood-brain barrier, and examining potential immunomodulatory effects in the tumor microenvironment.

The remarkable modulation of *Bax*/*Bcl*-

2 expression ratios observed in this study suggests that *S. aureus* cytoplasmic extract could potentially overcome the characteristic therapy resistance of glioblastoma cells. This finding is particularly significant given the limited treatment options currently available for this aggressive malignancy, as highlighted by Silva et al. (2021) in their review of natural anticancer compounds [29]. Although the findings are promising, it is crucial to address potential limitations, including the risk of bacterial lethality and genetic instability. Although this study demonstrated the significant cytotoxic effects of *S. aureus* cytoplasmic extract on the U87 glioblastoma cell line in vitro, it is essential to extend these findings to in vivo models to further validate the therapeutic potential of this treatment. Animal studies, particularly using mouse models with established glioblastoma tumors, will provide critical insights into the pharmacokinetics, biodistribution, and overall efficacy of the bacterial extract in a complex biological environment. Additionally, long-term safety studies and investigation of potential resistance mechanisms will be crucial for clinical translation.

Conclusion

In conclusion, this study elucidated the potent apoptotic effects of *S. aureus* bacterial extract on U87 glioblastoma cells, mediated through the modulation of key apoptotic genes. The significant increase in *Bax* expression and the decrease in *Bcl-2* expression underscore the potential of this extract as a novel therapeutic agent for glioblastoma. The findings contribute to the growing body of evidence supporting the use of microbial derivatives in cancer therapy, opening up new therapeutic options for this challenging disease.

Future research should focus on elucidating the precise mechanisms through which *S. aureus* extract influences apoptotic pathways and assessing its efficacy and safety in clinical trials. Additionally, exploring the therapeutic potential of extracts from various bacterial species may provide valuable insights into optimizing cancer treatments. Integrating bacterial extracts with existing therapies, such as chemotherapy and immunotherapy, could enhance overall treatment efficacy and provide a multifaceted approach to combating glioblastoma.

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Authors' contributions: Conceptualization: MR; data curation and formal analysis: HF; validation: MN and BH; investigation and writing of the original draft: BH; supervision: ADB. All authors reviewed and approved the final manuscript.

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