

Anticancer Effect of Fractions From *Staphylococcus aureus* and *Bacillus atrophaeus* on the Proliferation and Death of Human Breast Cancer Cell Line (MCF-7)



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Abstract

Background: Nowadays, breast cancer is known to be one of the most common cancers among women. Due to the side effects of chemotherapy and the high probability of recurrences in surgery, it is essential to identify and introduce new anticancer drugs of natural origin with fewer complications. In this regard, secondary bacterial metabolites and other microbial products have been considered. In the meantime, pathogenic and environmental bacteria have been investigated.

Objective: The aim of this study is to examine the effects of the interaction between cytoplasmic extract and the cell wall of *Staphylococcus aureus* and *Bacillus atrophaeus* on the proliferation rate of human breast cancer cells.

Materials and Methods: In this experimental study, cytoplasmic and cell wall extracts of bacteria were prepared. Then, SDS-PAGE was used to examine their protein contents. MCF-7 cells, as human breast cancer cells, with bacterial cytoplasmic extract and bacterial cell wall, were treated at different concentrations. Mesenchymal stem cells derived from adipose tissue were treated with different concentrations of bacterial cell wall extract. The effects of cytotoxicity were assessed by MTT assay at 24 and 48-hour intervals. The results were analyzed by SPSS.

Results: The results showed that bacterial cytoplasmic extract had a concentration-dependent cytotoxic effect on cancer cells, suggesting that the increase of concentration significantly ($P < 0.05$) increased cell death. Additionally, the bacterial cell wall extract showed a proliferative effect on cell growth ($P < 0.05$).

Conclusion: The bacterial cytoplasmic extract has a lethal effect and can, therefore, be considered as an anticancer compound in the future. This feature of the bacterium is attributed to the presence of a novel bioactive compound that can be used as an adjunct to other chemotherapy compounds. The bacterial cell wall extract, on the other hand, has cell growth-promoting components and can, therefore, be adopted as a compound for the proliferation of mesenchymal stem cells or wound healing in future studies.

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Background

Nowadays, various cancers incur a large proportion of demises around the globe. In Iran, cancer is the third leading cause of death after cardiovascular diseases and road accidents.¹ Human cancers occur at the molecular level due to biological and biochemical defects linkage that eventually lead to disruption in cell division, repair, and apoptosis. Breast cancer is the second most common cancer worldwide after lung cancer and the fifth most common cause of cancer deaths. It is also the leading cause of cancer deaths in women; 30% of all cancers, 15% of cancer-related deaths in women, and less than 1% of cancers in men are associated with it. The global prevalence of breast cancer is higher than other cancers,

and the incidence of breast cancer is increasing.² The rate of breast cancer in Iran is estimated to be 33.4 per 110 000 women. The reported data indicate that the disease in Iran has also been increasing, with one in eight women being reported to have succumbed to it at some point in their lives.³

There are different approaches to cancer treatment including surgery, chemotherapy, radiotherapy, hormone therapy, immunotherapy and so on. For breast cancer, there are effective drugs, such as tamoxifen, raloxifene, and anastrozole that, along with radiation therapy and adjuvants, have saved the lives of many women over the past 30 years.⁴ Notwithstanding, several disadvantages, including recurrence of the disease due to metastasis

within some months after treatment, side effects of drug consumption on glucocorticoid metabolism, and cancer risks after breast cancer chemotherapy, have posed some restrictions on the use of these therapeutic systems. In fact, the disease still remains as one of the major problems in women's health.⁵

In addition to the high cost of chemotherapy and myriad side effects, as well as the low efficiency of the drugs used in chemotherapy, another major problem in tumor treatment is the reduction of tumor cell susceptibility to drugs or, in other words, drug resistance.⁶ Therefore, investigating the creation and development of strategies to address these issues is one of the main priorities of public and private organizations worldwide such as the National Cancer Institute of America (NCI) and considerable funds and sums of money are being dedicated to and invested in research on new therapeutic methods. The use of anticancer agents derived from natural sources that have fewer side effects is deemed to be a vital strategy in this regard.⁷

One of the functional mechanisms of anticancer drugs is the induction of apoptosis that causes the death of cancer cells.⁸ Therefore, researchers' efforts to develop anticancer drugs have increased in achieving anticancer drugs from biotoxins that have pharmacological and toxic effects.⁹ In the meantime, bacterial products, including bacterial proteins and toxins, which have been shown to have different impacts on human cells as available and cost-effective sources, have received due attention as novel therapeutic approaches to cancer treatment. The benefits of this treatment are the reduction of the side effects due to the direct and specific effects of the bacteria and their products on the cancer cells and precisely on the tumors. Live bacteria as antitumor agents and bacterial vectors as enzyme producers are considered as a robust strategy for treatment; notably, this is more so for the recombinant bacterial toxins, which are currently being treated as toxin-based anticancer agents.¹⁰ In this study, in order to introduce a new biological treatment for breast cancer, the effects of different fractions of Gram-positive pathogenic bacteria, namely *Staphylococcus aureus* and *Bacillus atrophaeus* as an environmental bacterium, have been investigated on human breast cancer cell line (MCF-7).

Materials and Methods

Preparation of Supernatants and Bacteria Pellet

The purified strains of *S. aureus* were obtained from the bacteriology laboratory of Shahid Beheshti University, Iran, with the approval of an expert in this laboratory. The strain of *B. atrophaeus* was isolated and purified at the Environmental and Industrial Microbiology Research Laboratory of Shahid Beheshti University. To perform the bacterial treatments, each bacterium was first cultured in a neutral broth for 24 hours. Then, the optical absorption of the medium containing the bacterium was read at

600 nm. Under these conditions, the bacterial count reached 10^8 colony forming unit (CFU) according to the McFarland standard.¹¹ Bacteria were then separated by centrifugation at 6000 rpm for 20 minutes. Next, they were weighed and suspended in sterile PBS. The sonication method (Ibra Cell, USA, serial No. 50456) was used to prepare the bacterial extracts. In this method, the bacterial suspension was lysed by a sonicator for 30 minutes and then centrifuged again at 10000 rpm for 10 minutes¹².

The prepared lysates were filtered through a 0.22 μm sterile filter; before and after filtering, the pellets, as well as the supernatants, were applied on a plate containing nutrient agar to ensure the completion of lysis of the bacteria and the sterilization of the samples. After 24 hours of incubation, bacterial growth was evaluated. If bacteria did not grow on the plates, the samples for treatment on the cells would be approved and selected. Then, supernatants as lysate or cytoplasmic extract and pellets as cell wall extract were separated and stored at -20°C .

SDS-PAGE Gel

To investigate the protein content of cytoplasmic and cell wall extracts obtained from bacteria, SDS-PAGE (Bio-Rad) gel was used.

According to the Bio-Rad protocol, the top and bottom gels and the specified place for injection were prepared. Then, each sample was injected into the wells at 10 μL concentration and then ran at 80 mA.

Preparation of the Cells

Human breast cancer cells (MCF-7) used in this study was obtained from Tarbiat Modarres University Cell Bank. Cells were cultured in DMEM/F12 high glucose medium (Gibco, USA) supplemented with 10% FBS (Gibco, USA), 100 units of penicillin-streptomycin antibiotics (Gibco, USA) and incubated at 37°C and 5% CO_2 .¹³

The Analysis of the Growth Rate

To measure cell growth and death, MTT assay was performed according to the protocol. Cells were trypsinized and collected from flasks. Neobar lam was used to count the total number of the cells. A total of 5000 cells were transferred to each 96-well plate containing 200 μL of culture medium. The cells were then treated with different doses of the bacterial cytoplasmic and cell wall extracts, referred to as pellets, were considered as treated and the remaining wells were considered as controls. The exact specifications of the plates and groups of treatments are as follows:

24-hour plates containing 30, 20, 15, 10, and 5 $\mu\text{g}/\text{mL}$ concentrations of bacterial cytoplasmic extract of *S. aureus* and 10, 7, 5, 3, and 1 $\mu\text{g}/\text{mL}$ concentrations of cell wall extracts (pellet) of *S. aureus* were specified. Additionally, 40, 30, 20, 10, and 5 $\mu\text{g}/\text{mL}$ concentrations of bacterial

cytoplasmic extract of *B. atrophaeus* and 20, 15, 10, 5, and 2.5 µg/mL of cell wall extracts (pellet) of *B. atrophaeus* were applied. In addition, 6 wells without treatment were considered as controls. Additionally, 3 wells were considered as 3 replicates for each concentration. A 48-hour plate was also prepared with these concentrations.

All plates were incubated in a CO₂ incubator (5%) at a temperature of 37°C for 24 and 48 hours. After the incubation time for each plate, 10 µL of MTT solution was added to each well and was incubated at 37°C for 1 hour during which the formazan crystals formed. Then, 100 µL of DMSO was added to each well and after a few minutes of incubation at room temperature, its optical absorption was read at 570 nm with an absorbance reader (ELx800™, BioTek, USA).¹⁴

Statistical Analysis

Statistical analysis was performed using the SPSS software. Results were analyzed by one-way ANOVA and all data were reported as mean ± SD and $P < 0.05$ was considered statistically significant. All tests were repeated three times.

Results

MTT Test

Figure 1 shows the viability of cells treated with different concentrations of cytoplasmic extract of *S. aureus*. As depicted, by decreasing the concentration of cytoplasmic extract, the survival rate increased and less death was observed. The concentration of 15 µg/mL was set as IC₅₀. The analysis of the two time intervals of 24 and 48 hours revealed that bacterial lysate has concentration and time-dependent toxicity which increases over time.

Figure 2 shows the stimulation index of the cell wall extract of *S. aureus* at different concentrations. While the concentration of 10 µg/mL had the highest stimulation index at the 24-hour interval, as well as the 48-hour interval compared to the control group, it did not significantly increase the cell proliferation. Therefore, it can be concluded that increasing the concentration of cell wall extract results in the elevation of the growth stimulation and more cell proliferation. This pattern was observed at both 24 and 48-hour intervals.

Figure 3 shows the viability of cells treated with different concentrations of cytoplasmic extract of *B. atrophaeus*. As can be seen, there is no discernible pattern in the concentration of bacterial cytoplasmic extract and cell viability, and the concentrations of 40 µg/mL at 24-hour period and 10 µg/mL at 48-hour period were determined as IC₅₀. Comparison of the two different periods (i.e., 24 and 48 hours) shows that bacterial lysates have time-dependent toxicity which increases with time.

Figure 4 represents the stimulatory index of cell wall extract of *B. atrophaeus* at different concentrations. It can be concluded that with increasing the concentration of cell wall extract, growth stimulation increased and more cell proliferation was observed. This trend was observed

at both the 24- and 48-hour intervals.

Figure 5 shows the effect of cell wall extract of *B. atrophaeus* and *S. aureus* on human mesenchymal stem cell growth. According to this figure, the pellets of both bacteria have a time and concentration dependent effect and the bacterial pellets of *B. atrophaeus* have a higher stimulation index on this cell line than *S. aureus*.

SDS-PAGE Gel

As displayed in Figure 6, the bacterial cell wall extract has more bands that show richer protein contents *vis-à-vis* bacterial cytoplasmic extract.

Discussion

Nowadays, machine-oriented lifestyle and malnutrition have accelerated cancer tsunami in most countries, especially developing countries. According to statistics from the cancer institutes of Iran, breast cancer is one of the most common cancers, particularly among women.¹⁵ Although we have seen many advances in modern cancer therapies including cell therapy and gene

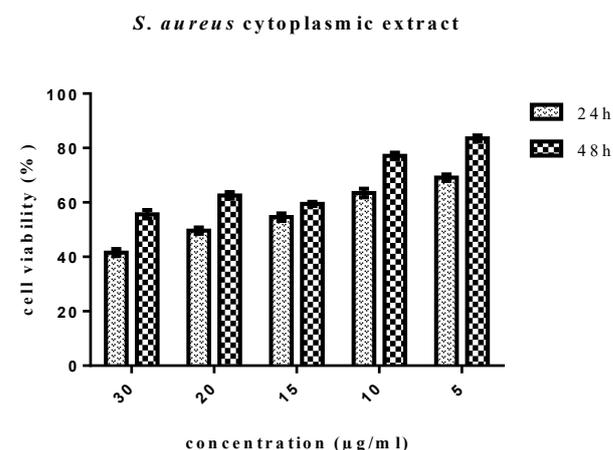


Figure 1. Cytotoxic Effect of Cytoplasmic Extract of *S. aureus* on Breast Cancer Cell at 24 and 48-Hour Intervals by MTT Assay.

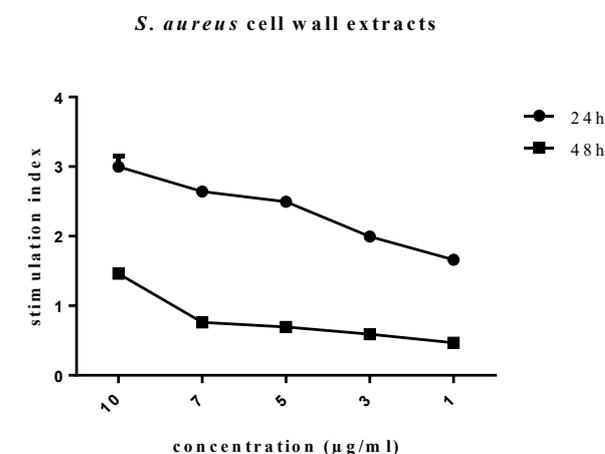


Figure 2. Stimulatory Effect of Cell Wall Extracts of *S. aureus* on Breast Cancer Cell at 24 and 48-Hour Intervals by MTT Assay.

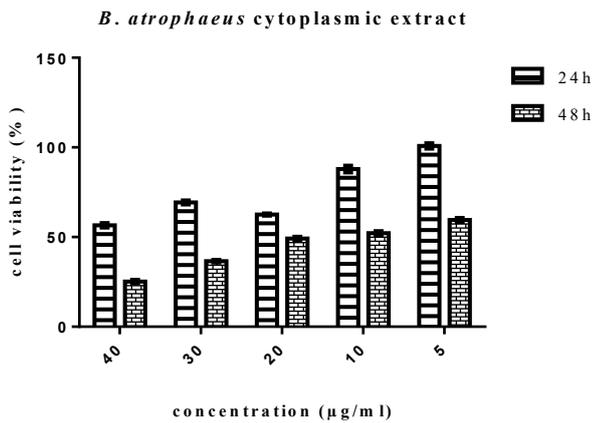


Figure 3. Cytotoxic Effect of Cytoplasmic Extract of *B. atrophaeus* on Breast Cancer Cell at 24 and 48-Hour Intervals by MTT Assay

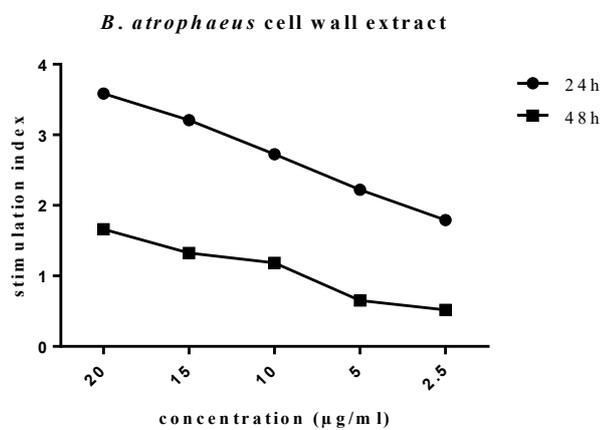


Figure 4. Stimulatory Effect of Cell Wall Extract of *B. atrophaeus* on Breast Cancer Cell at 24 and 48-Hour Intervals by MTT Assay.

therapy, chemotherapy is still one of the most common ways to treat cancer. One of the major problems faced in chemotherapy is the side effects of chemotherapy drugs, cancer cell resistance, high dose of drugs, and high cost of treatment.¹⁶ Therefore, the search for effective compounds with lower cost and lower side effects is a quintessential step in the development of cancer treatment. On the other hand, significant advances in the production of analogues from naturally occurring products make bacterial metabolites increasingly one of the most valuable sources of modern pharmaceutical raw materials and of particular interest to sciences. In the meantime, bacterial toxins, whose cytotoxicity and apoptosis induction mechanisms have been identified, as immunotoxins have been capable of outperforming other biomaterials during advanced clinical stages of cancer treatment.¹⁷

In this study, the effects of different fractions of *S. aureus* as Gram-positive pathogenic bacteria and *B. atrophaeus* as environmental bacteria on MCF-7 human breast cancer cells were studied. The effect of cytoplasmic extracts of *S. aureus* and *B. atrophaeus* on MCF-7 cells revealed that increasing the concentration of cytoplasmic extract

decreases the survival rate of the cells and increases their mortality rate. Furthermore, the evaluation of the decrease in the survival rate *vis-à-vis* the increase in cytoplasmic extract concentration demonstrated a concentration-dependent relationship as a response to treatment in the studied sample. The cause of cell death can be attributed to the presence of toxic compounds in the cytoplasmic extract of the bacteria. Alpha hemolysin is one of these compounds in the cytoplasmic extract of *S. aureus*. According to a study by Bantel et al, by activating the caspase pathway in the cell, this toxin induces apoptosis and cell death.¹⁸

Moreover, in 2014, Swofford et al documented the toxicity and anticancer effects of *S. aureus* toxin on MCF-7 cells.¹⁹ The environmental bacterium *B. atrophaeus* produced a compound called surfactin, which is one of the most potent biosurfactants. In 2007, Kim et al reported that surfactin induces apoptosis in human cells.²⁰ Cao et al also found that surfactin produced by *B. subtilis* inhibited the proliferation of human breast cancer cells (MCF-7) in a dose and time-dependent manner.²¹ Another study on *Bacillus* spp. was conducted by Vidhyalakshmi and Vallinachiyar. They reported that

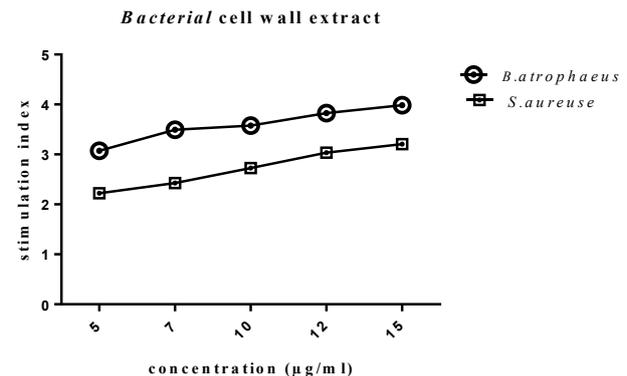


Figure 5. Stimulatory Effect of *B. atrophaeus* and Cell Wall Extracts of *S. aureus* (Pellets) on Mesenchymal Stem Cells in 24 Hours by MTT Assay.

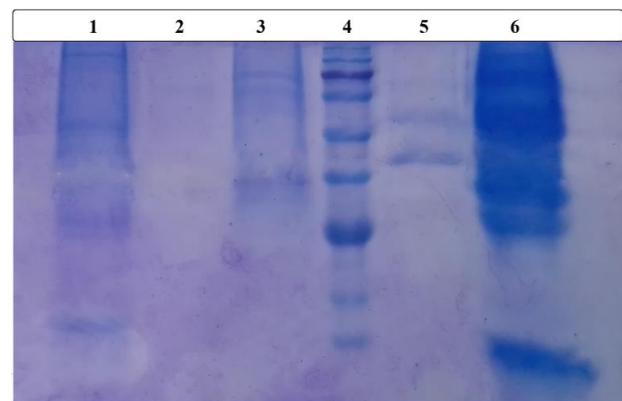


Figure 6. SDS-PAGE Gel. Bands: (1) *S. aureus* Pellet (2) Cytoplasmic Extract of *S. aureus* (3) *B. atrophaeus* Pellet (4) Ladder (5) Cytoplasmic Extract of *B. atrophaeus*.

some bacterial polysaccharides produced by *Bacillus* spp. and *Pseudomonas* strains have anticancer properties and induce apoptosis in MCF-7 human cancer cells.²² Moreover, in 2014, Kumar et al in a study on the cytotoxic and anti-cancer effects of *Bacillus cereus* and *Bacillus pumilus* extracts isolated from soil on HEP2 and HEP G2 cancer cell lines found that the extract of these bacteria destroys the DNA constructs in the cancer cells. However, the effects seemed to be smaller on the healthy cells; hence, given the close proximity of this family of *Bacillus* to *Bacillus atrophaeus* and the confirmation of the cytotoxic impact of the cytoplasmic extract of *B. atrophaeus* through MTT assay, the results could be generalized.²³ Aldeewan et al demonstrated the anticancer effect of these proteins in a study on parasporins and the Bt toxins produced by *Bacillus thuringiensis* on cancer cells.²⁴ The cytoplasmic extract of *S. aureus* and *B. atrophaeus* examined in this study unveiled concentration- and time-dependent toxic effects on the cells. It was also contended that the toxicity of the treatment would increase over time. The results of this study on MCF-7 cells also showed that this combination can cause cell death.

Treatment of the cells with the cell wall extract of *S. aureus* and *B. atrophaeus* showed that as the pellet concentration increased, the number of cells increased. Comparison of cell numbers after 24 and 48 hours unveiled that with time, the stimulation of cell growth increased. In 2015, Senthilraja and Kathiresan probed the effect of three yeast extracts (i.e., *Candida albicans*, *Kuraishia capsulate*, and *Sacchromyces cerevisiae*) on normal Vero cells and two HEPG2 and MCF-7 cancer cell lines. They observed that low doses of extracts would stimulate cell growth and division and high doses of yeast extract cause cancer cell death and have time-dependent toxicity.²⁵ Saberian et al reported a significant increase in stem cell growth using *Lactobacillus acidophilus* and MRS culture medium.²⁶ Additionally, Takeuchi et al referred to the presence of both TLR2 and TLR4 and observed that with the increase in the concentrations of the bacterial cell wall extract, the stimulation of growth will increase, and greater cell proliferation will be observed in Gram-positive and Gram-negative bacterial cell wall extracts. They showed that both compounds induce FNFA production and consequently affect the proliferation and differentiation of human cells.²⁷ Therefore, the pellets of these bacteria contain cell growth-promoting agents and can therefore be used as compounds for stem cell proliferation, such as mesenchymal stem cells, which is a low-cost and viable way of cell proliferation in the future. This trend was observed at both the 24 and 48-hour intervals.

Conclusion

Due to their toxic compounds such as hemolysin and surfactin, cytoplasmic extracts of *S. aureus* and *B. atrophaeus* have cytotoxic effects on cancer cells and may be a viable alternative for anticancer therapy in the future.

These compounds could potentially proffer new therapies against cancer cells and treating tumors. On the other hand, the pellets of these two bacteria are considered as a stimulus for cell growth and can therefore be used for mesenchymal stem cells proliferation, which is a low-cost and practical way to proliferate mesenchymal stem cells.

Authors' Contributions

SA: performing laboratory operations and writing original draft; NS: Conceptualization, methodology, Supervision, validation

Ethical Approval

This research was conducted in accordance with the protocol approved by the Shahid Beheshti University, Faculty of Biological sciences and technology, Iran.

Conflict of Interest Disclosures

The authors declare that they have no conflict of interests.

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