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ORIGINAL ARTICLE

Cytotoxicity Effect of *Shigella flexneri* Fraction on Breast Cancer Cell as a New Compound for Cancer Therapy

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KEYWORDS

Cell death,
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ABSTRACT: Breast cancer is a major cause of death among women worldwide. Accordingly, conventional medical treatments using high levels of cytotoxic drugs have some side effects for patients. Different secondary metabolites and enzymes from *Shigella* are considered in this case. This research aimed to study the effect of *Shigella flexneri* lysate on the induction of cell death in breast cancer cell line. For this purpose, *Shigella* lysate was prepared at different concentrations. 4T1 breast cancer cells were treated with different concentrations of bacterial lysate and sediment. The cell death was evaluated by PI color and cell viability was also measured by MTT assay. Analysis of bacterial lysate (IC_{50} 250 µg/ml) for 24 hours has shown the cytotoxic effect on 4T1 breast cancer cells. Moreover, the sediment of bacteria (IC_{50} 500 µg /ml) for 24 hours has shown a cytotoxic effect on 4T1 breast cancer cells. The cell death was confirmed using PI staining. Additionally, the bacterial lysate has shown a direct toxic effect on breast cancer cells. This protein may be used as a new therapy for cancer in future.

INTRODUCTION

Cancer is the second leading cause of death following cardiovascular diseases, which annually affects millions of people around worldwide. Among several types of cancer, the incidence of breast cancer in most developing countries has been increasing [1]. Diagnosis and treatment are important factors at early stage of the disease, which improve the healing process in patients with breast cancer [1, 2]. Often, modern treatments of breast cancer are not effective and have undesirable side effects. So, considering the lack of response to treatment and the rapid growth of the disease, finding more effective drugs with less toxicity is essential. In some types of cancer, timely treatment can help in their early detection [3]. Nowadays, many efforts have been made to find suitable methods to treat the disease, including the discovery of new anticancer drugs based on bacterial metabolites. As a result, there is an increasing interest in the application of bacterial products such as proteins and toxins for cancer treatment. *Shigella* is a genus of enteric gram-negative bacteria with numerous virulence factors. This bacterium has shiga toxin that results in shigellosis, which is an intestinal infection and its most common

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symptoms are diarrhea, fever, nausea, vomiting, stomach cramps, and flatulence. It is also known for the treatment of immunocompromised patients in some countries [4, 5].

This bacterium due to having multiple virulence factors is a potential pathogen, but these factors in vitro can be used as a tool for the treatment of untreatable diseases. Using these factors can also be useful to introduce new mechanisms of therapeutic agents. There are few studies on these bacterial products and their effects. Additionally, the functional aspects of these compounds have not been studied yet. Effective responses to these metabolites will open a new window of opportunity for treating cancer. In this study, we evaluated the effect of *Shigella flexneri* lysate on the induction of cell death in breast cancer cell line.

MATERIALS AND METHODS

Bacterial strain and lysate preparation

Shigella flexneri ATCC 2067 was obtained from the Pasteur Institute of Iran (Tehran, Iran). The disruption of bacterial cells was done by sonication, and afterward the cells were collected by centrifugation for 20 min at 10,000 rpm. The supernatant was transferred to a clean microcentrifuge tube. The sample concentration was determined using a spectrometer at 280 nm and the stock solution was stored at -20 °C before performing analysis [22].

Cell line and culture condition

In this study, 4T1 mice, a breast cancer cell line, was purchased from the cell bank of Pasteur Institute of Iran (Tehran, Iran). The cells were cultured in RPMI 1640 Medium (Gibco ™) containing 10% (v/v) fetal bovine serum (FBS; Gibco ™) and then maintained at 37°C in a humidified atmosphere of 5% CO_2 and under 95% air condition. The standard growth curve was established to determine the best time range of 4T1 cells seeded in 96-well plates. MTT assay was performed in the logarithmic phase of the cells.

Tumor cell line cytotoxicity assay

4T1 cells were suspended in RPMI 1640 medium at a density of 1×10^4 /ml [10, 11]. In the next step, serial dilutions of bacterial lysate suspension were added to tumor cells. One group, as a negative control, was cultured and the infected cells were further incubated in 96-well microplates for 24 h. All these experiments were performed in triplicate wells. Thereafter, 20µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (Merck) (5mg/ml in Phosphate-buffered saline PBS) was added to each well and 4T1 cells were incubated for 4 hr at 37°C. Subsequently, the supernatant of each well was removed and 100µl of Dimethyl sulfoxide (DMSO) (Sigma-Aldrich) was added to dissolve formazan crystals. The absorbance was read by a microplate reader at 570 nm and then evaluated based on Stimulation Index: SI, which was calculated as follows: [12]

$$\text{Viability } \% = \text{OD570 of each test} \;/\; \text{OD 570 of negative control} \times 100$$

Cellular viability assay

Cell growth and viability were checked using trypan blue. For this purpose, trypsinization of the treated cell groups was separately done with recombinant protein. Afterward, the cells were exposed to trypan blue and living cells were counted using hemocytometer under an optical microscope [13].

Statistical analysis

This study was an interventional trial and the results were reported as mean ± standard deviation (SD). The results of different concentrations of recombinant protein were analyzed based on one –way variance analysis (ANOVA). Moreover, the differences between means were determined by the Tukey test. All the analytical features were determined in SPSS 16 and Prism6 software with $P\leq 0.001$. The standard growth curve was established to optimize the best time range of 4T1 cells seeded in 96-well plates. A density of 8,000 cells with the absorbance of 0.6 was selected for further studies

RESULTS AND DISCUSSION

Cytotoxicity assays on the tumor cell line

The differences between different groups were considered as statistically significant by ANOVA (P≤ 0.001). Post hoc analysis indicated that the viability of tumor cells in a concentration of 31µg/ml has reduced by 50% (IC_{50}value) and inhibited breast cancer cell line. Higher inhibition was seen in the concentration of 250 µg/ml (P≤ 0.001) (Figures 1 and 2). Cytotoxicity of this protein was shown to be dependent on time and concentration. By comparing the data obtained after measuring the viability of cancer cells treated with *Shigella* lysate, it was shown that there was a significant statistical difference between the survival rates of the cells at different doses for 24 hours in the treated groups. The results of the MTT test on 4T1 cells treated with *Shigella* lysate for 24 hours are shown in Figure 3. Investigation of viable cells was performed using Trypan blue exclusion assays. The cells treated with *Shigella* lysate at a concentration above 15 µg/µl showed the highest mortality rate. The concentration of 125 µg/µl caused more than 50% mortality compared to the control groups (viability of 97%). The viability of the treated cells was evaluated by both MTT and Trypan blue assays. Correspondingly, these assays were well-provided to confirm the results of other studies. The MTT assay was used to monitor the cell viability after 24 h. It was shown that at the concentration above 250 µg/µl, a significant decrease occurred in the number of viable cells. The concentration of 500 µg/µl caused the highest mortality rate (more than 50%) in the treated groups compared to the control groups. Our results show that *Shigella* lysate had a higher (Figures 4).

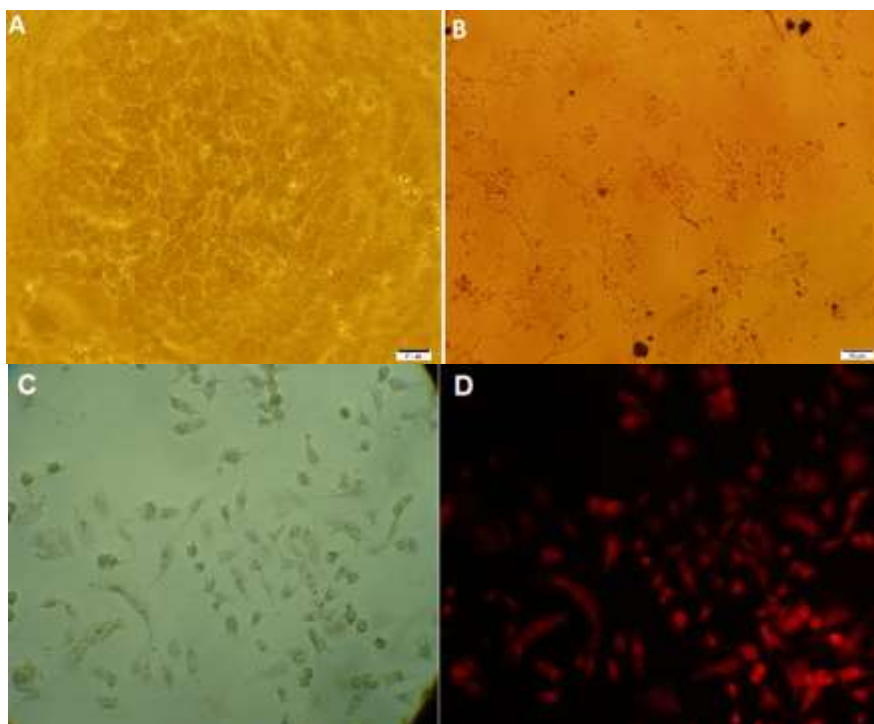

Figure 1. A: untreated 4T1 breast cancer cell line.B: Treated 4T1 breast cancer cell line with *Shigella* lysate by light microscopy. C: treated by light microscopy. D: Treated 4T1 breast cancer cell line with *Shigella* lysate. Red fluorescence is due to Propedium Iodide staining and observed under fluorescent microscope. Observations done at 600× magnification.

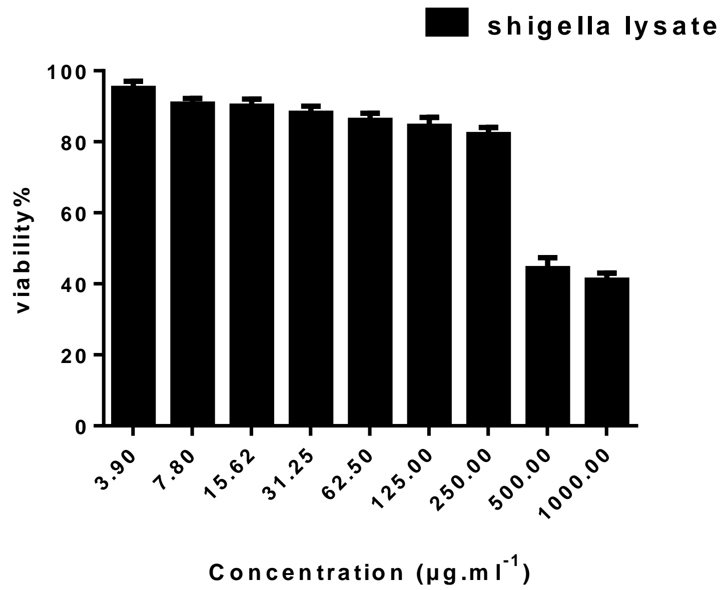

Figure 2. Evaluation of cellular viability that was treated with *Shigella* lysate, MTT assay based on stimulation index (SI) and Percentage of Viability (P<0.001).

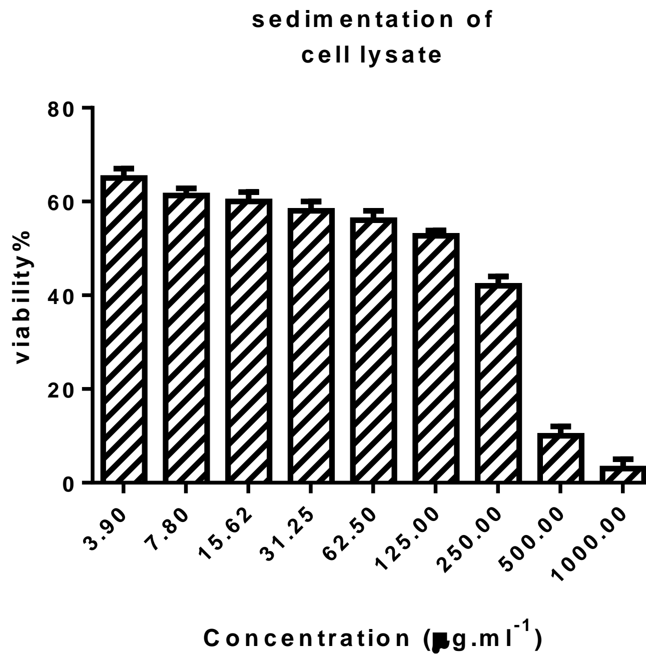

Figure 3. Evaluation of cellular viability that was treated with *Shigella* sedimentation, MTT assay based on stimulation index (SI) and Percentage of Viability (P<0.001).

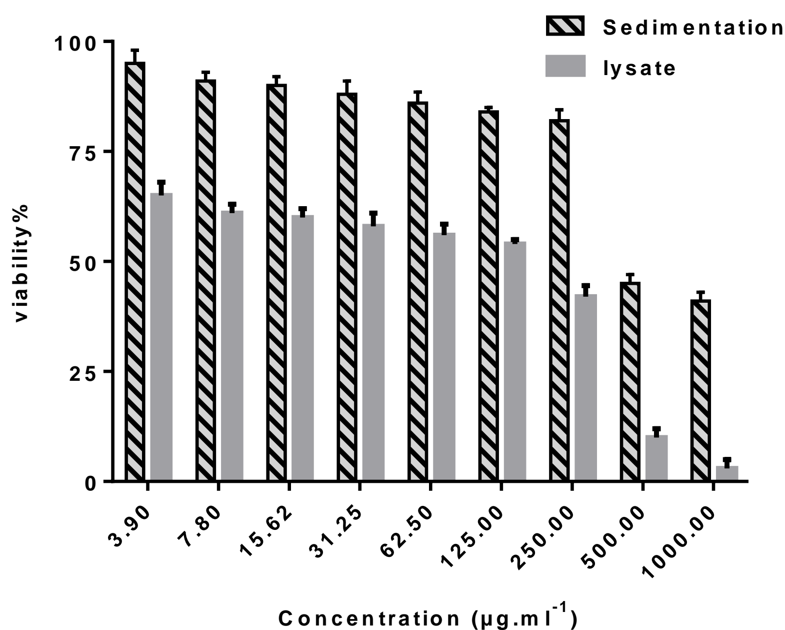

Figure 4. compared of evaluation of cellular viability that was treated with *Shigella* sedimentation and lysate, MTT assay based on stimulation index (SI) and Percentage of Viability (P<0.001).

Recently, by performing a more thorough study of the metabolites and their reactions to the host in vitro, new compounds were introduced for the treatment of cancer [14]. Studies on BCG, *Pseudomonas* toxins, bacterial spores to treat and kill tumor cells also are in progress. In addition, the effects of factors such as CagA and VacA of *Helicobacter pylori* on cell signaling as a candidate have been studied [15]. In 2007, Handa et al. reported that CagA can induce pathological signal transduction pathways and increase cell proliferation in a gastric epithelial cell [16]. Soleimani et al. (2014) evaluated the preparation of chitosan nanoparticles carrying recombinant HP-NAP *Helicobacter pylori* as a candidate to treat the model of breast cancer tumor [17]. In this study, among the listed bacterial metabolites, *Shigella* have been discussed. Several virulence factors have been proposed for *Shigella flexneri* and each one of them was shown to have different effects. Therefore, initial cellular and molecular studies of virulence factors and evaluating them would lead us to understand the exact performance for future functional studies. In 2010, Galmbacher et al. in their study used *Shigella* to prevent tumor development in mice. They reported that *Shigella* could induce apoptosis in macrophages in a mice breast cancer model, which could consequently decrease the rate of transplanted tumor in mice in the laboratory [18]. In another study, Engedal et al. (2011) used two-part *Shigella* toxin for cancer therapy in the laboratory [19]. To eliminate tumor cells, Hakomori et al. (1997) used glycosphingolipid as a receptor [20]. In 1995, Sizemore et al. used *Shigella* as a vector for gene transferring to cancer cells [21]. So far, the application of the lysate of this bacterium has not been still evaluated as a candidate for cancer treatment in the world. In this project, the effects of lysate and sedimentation of *shigella* on the growth of 4T1 cell line were evaluated. The viability of cancer cells using the MTT test showed that lysate of bacteria depends on concentration and significantly affects 4T1 cells. Therefore, with increasing the concentration of lysate, the growth and survival of 4T1 cells dramatically reduced. So, it can be said that *Shigella flexneri* lysate significantly reduces the viability of cancer cells. The results of bacterial sedimentation on 4T1 cancer cells showed that sedimentation containing bacterial cell wall as well as its lysate had a cytotoxicity effect; however, this impact was less than the effect of lysate. It seems that effective compounds on bacterial lysate are impressive in killing cancer cells. In the current study, the results show that the bacterial lysate was toxic and the concentration of 125 μg/ml induces apoptosis in 50% of breast cancer cell lines under in vitro. The cytotoxicity on tumor cells was evaluated

based on Stimulation Index (SI) and ANOVA test showed that the differences between the groups were statistically significant (P<0.001). Moreover, the results show that the sedimentation of *Shigella* was toxic, but with the power of bacterial lysate at a concentration of 500 µg/µl induced death in 50% of breast cancer cell lines under in vitro in mouse models for tumor metastasis.

CONCLUSIONS

This metabolite can be used as an effective tool for therapeutic applications and cancer treatment in future. On the other hand, increasing the efficiency of these metabolites in vitro and animal models have been discussed. This research can be considered as the first way to propose a new approach for cancer therapy.

Availability of data and materials

We will make avail the data based on the request.

Funding Author's

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Ethics approval and consent to participate

Ethical approval for this study was obtained from the ethics committee of Shahid Beheshti University of Medical Sciences in Tehran, Iran (IR.SBMU.RETECH.REC.1397.838). Ethical clearance was received from Institutional Review Board of the College and full written informed consent was obtained from participants. Privacy and strict confidentiality were maintained during the data collection process. No personal details were recorded or produced on any documentation related to the study. We declare that all necessary data's and materials are available in the manuscript and support information section of Infectious Diseases and Tropical Medicine Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran (SBMU/14940).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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