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Antiproliferative Activity of Crude Marcescin on Human Laryngx Epidermoid Carcinoma (Hep-2) Cell Line *in Vitro* Study

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Abstract

Marcescin is a bacteriocin that is extracted from isolates of *Serratia marcescens*, which is a gram-negative bacterium that belongs to the family Enterobacteraeaceae and is considered a nosocomial pathogenic bacteria. In this research, the toxic effect of crude marcescin extracted from the *S. marcescens* bacteria on the human laryngx epidermoid carcinoma (Hep-2) cell line was investigated. *S. marcescens was* isolated from blood, urine, and stool samples of patients from Nursing House Hospital, Baghdad Teaching Hospital, and Teaching Laboratories of Medical City by using a series of dilutions to concentrations of (0.00, 1.95, 3.9, 7.8, 15.62, 31.25, 62.5, 125, 250, 500, and 1000) µg/ml and an exposure time of (24, 48, and 72) hours. The results showed significant differences ($P \leq 0.05$) in the IR values (100–2%) at the concentrations (1000–1.95) µg/ml when compared with the control (100%); there were also significant differences after 48 and 72 hours. The results showed that high concentrations caused a high rate of inhibition at concentrations (1000, 500, and 250) µg/m where the rate of inhibition was (100, 67, and 58) after 24 hours, then the rate of inhibition began to decrease gradually, reaching 2% at the lowest concentration of 1.95. It is clear that Hep-2 cells are sensitive to marcescin, and their sensitivity increases exponentially with increasing concentration and time of exposure.

Keywords: *Serratia marcescens*, Marcescin, Cytotoxicity, Hep-2 cell line, Inhibition rate.

1. Introduction

Despite major improvements in diagnosis and treatment, cancer remains the top cause of death in the world (1). In 2020, 10 million people worldwide died from cancer, one of the major causes of death. The most prevalent cancers include melanoma, lymphoma, leukemia, sarcoma, and carcinoma, which can originate in a variety of body organs (2). In men, the prostate, lung and bronchus, colon and rectum, and urinary bladder, respectively, have the largest percentages of cancer types. Breast, lung, and bronchus; colon and rectum; uterine corpus; and thyroid cancer prevalence are higher in women, correspondingly (3). Today's anticancer agents fall into several groups (4). Modern treatments, including hormone-, immuno-, and antibody-based therapies, are gaining popularity. Toxins, antibiotics, bacteriocins, non-ribosomal peptides, polyketides, phenylpropanoids, phenylflavonoids,

purine nucleosides, short-chain fatty acids (SCFAs), and enzymes that fight cancer have been the focus of studies. The majority of these compounds selectively target cancer cells, either directly or indirectly via particular pathways (5). According to reports, the use of microorganisms or their products to treat cancer dates back to the eighteenth century. For example, people with tumors that couldn't be removed were given Coley's toxins, which are made from the culture fluids of bacteria such as Streptococcus pyogenes and S. marcescens. Evidently, those who received this toxin treatment had their malignant tumors shrink, and they recovered their health (6, 7). We desperately need new compounds with improved selectivity and specificity against cancer cells. Numerous proteins and peptides having anticancer activities are present in microorganisms, particularly bacteria. For example, bacteriocins have biological effects because of their distinctive structural characteristics. Initially believed to be limited to preventing bacterial growth, their roles have since been expanded to include suppressing a number of cancer cell lines (8). Several bacteriocins have demonstrated specific cytotoxicity against cancer cells compared to healthy cells. Due to this, they are viable candidates for additional studies and clinical trials (9). Many bacterial species synthesize bioactive peptides known as bacteriocins (10). Bacteriocins exist in all microbial communities, indicating their significance for bacterial survival in complex microbial environments (11). Bacteriocin can help with some of the most difficult issues with multi-drug-resistant organisms, which have become a severe challenge in disease control (12). A class of protein-based antibiotics called marcescin destroys sensitive microorganisms. Hamon and Peron reported that 76 of 85 isolates of *S. marcescens* produced the bacteriocins known as marcescins over ten years ago (13). The chromosome carries the marcescin genes (14). Research has revealed the existence of both broad-spectrum and targeted antibacterial action against unrelated pathogens. Researchers have also demonstrated that bacteriocins can stop the growth of certain cancer cell lines (15). Due to their capacity to distinguish between cancerous and non-cancerous cells, bacteriocins have potent anticancer capabilities (16).

This study aims to provide a comprehensive understanding of the anticancer activities of marcescin and their potential as therapeutic agents for the disease.

2. Materials and Methods

2.1. Isolation and Identification of *S. marcescens*.

Two hundred samples were collected from various clinical sources, including blood, urine, and stool from patients in hospitals of Baghdad Teaching Hospital, Private Nursing Home Hospital, and Teaching Laboratories of Medical City in Baghdad city, and then they were cultured on culture media. The bacteria were diagnosed based on cultural characteristics, microscopic examination, and biochemical tests, and then the Vitek-2 system was used to confirm their identity.

2.2. Extraction of marcescin

Marcescin was extracted from isolates of *S. marcescens* from blood, urine, and stool samples of hospitalized patients (17). As follows: The isolates of *S. marcescens were* cultured in test tubes containing 6.5 ml of sterile BHI broth medium at a temperature of 37°C for 18 hours. We prepared the B.H.I. Broth Medium + 5% Glycerol in suitable flasks, each with a capacity of 250 ml, autoclaved it, and then let it cool. The next day, we inoculate each prepared medium with a tube from the bacterial isolate. The flasks are then incubated in a shaking incubator for 14 hours at a rate of 150–200 cycles per minute to reach a number of cells of about 3×10^4.The flasks receive a dose of 2 μg/ml of mitomycin C before being placed back in the shaking incubator for an additional 3 hours. We use a cooled centrifuge for 30 minutes at

a speed of 7000 cycles per minute to separate the bacteria in each flask after incubation. Finally, we separated the supernatant from the precipitate for each flask containing marcescin. Next, we used the Bradford method to measure the concentration of Marcescin (18). We measured the effectiveness of Marcescin using the well method (19).

2.3. Study the toxic effects of marcescin on the Hep-2 cancer cell line *in vitro*.

The study of the toxic effect of marcescin in different concentrations (0.0, 1.95, 3.9, 7.8, 15.62, 31.25, 62.5, 125, 250, 500, 1000 µg/ml) on the human laryngx cancer cell line (Hep-2). It is grown on RPMI-1640 medium supplemented with 5% fetal calf serum (FCS).

2.4. Marcescins' cytotoxic effects on cancer cells

The study was carried out using 96 flat-bottomed, multiple-tissue culture microtiter plates, which involved three steps:

2.4.1. Seeding of cells.

After the cells had grown and multiplied, the monolayer-containing containers were taken out, and the cells were then harvested using a trypsin-versin (T.V.) solution. Each container received 20 ml of the culture medium, including serum, which was added and thoroughly mixed. Following that, the cells were counted using a (Haemocytometer) and 1% trypan blue dye, as suggested by Freshney (20). (0.1) ml of the cell suspension was pipetted into each well of the plate using a micropipette. (1×10^{4}) cells/wells were contained in each well. In order to facilitate cell attachment, the surface of the plate was then covered with a specific kind of sterile, transparent adhesive paper. The plate was then gently rotated before being incubated at 37 °C overnight.

2.4.2. Exposure of cancerous cells to the marcescin

Serial concentrations of marcescin were prepared using tissue culture medium devoid of (FCS), and added to the pits containing adherent cancer cells, taking into account the preparation of material solutions immediately upon use, and using three replicates for each treatment. After removing the adhesive paper, the culture medium was emptied from the wells. Due to the addition of 0.2 ml of serum-free culture media, column No. 1 was regarded as a negative control. While the dilutions of the marcescin, which were made as (0.2 ml/ well/ concentration) were applied to the columns from 2 to 12, a new piece of adhesive paper was then placed on the plate's surface. The plates were exposed for 24, 48, and 72 hours each at a temperature of 37°C.

2.4.3. Cytotoxicity assay

The assay MTT test was used to detect the cytotoxic effect of marcescin on cancer cells, according to the following steps:

In order to verify that all traces of the test material and non-adherent cells were eliminated, the culture media and suspended cells in the plates were removed after the specified incubation period and then washed three times with phosphate-buffered saline. Then, a volume of 10 µl of MTT dye solution (with a final concentration of 0.5 mg/ml) was added to each well and left for 4 hours at a temperature of 37 °C in a carbon dioxide incubator. The excess dye was removed from the cells by repeatedly washing them in PBS. To ensure the violet granules were totally dissolved, 100 µl of DMSO were added when the dishes were completely dry. At a wavelength of 500 nanometers, results were read using an ELISA microplate spectrophotometer, keeping in mind that the work should be done with MTT dye away from light (21, 22).

The rate of cancer cell growth inhibition (Inhibitory Rate) (I.R) was calculated according to the equation indicated by (23).

$$IR = \frac{A-B}{A} \times 100\% \quad (1)$$

Where:
IR= Inhibitory Rate
A= Absorbancy for Negative Control
B= Absorbancy for Test

3. Results

Out of 200 samples, 33 isolates of *S. marcescens* were identified based on phenotypic characteristics, microscopic examination, and biochemical tests and confirmed using the Vitek-2 system. Then the efficient isolates produced were selected using the cap assay technique (24), and isolate S23 was chosen as the best isolate using the Well Method (19), as it gave the highest zone of inhibition, the highest activity, and the highest concentration, which was used in this research.

3.1. Toxic effect of marcescin after 24 hours.

The toxic impact was studied in the Hep-2 cell line. The percentage of cell growth inhibition was mostly determined by the concentration utilized and the exposure time.The maximum percentage of growth inhibition was reported at a concentration of 1000 µg/ml, where the effect was recognized when exposed for the first 24 hours and reached 100% before decreasing. When the concentration was gradually reduced, the percentage inhibition of cancer cell growth reached 2% at the concentration of 1.95 µg/ml. It was observed that there are significant differences in the first 24 hours ($P \leq 0.05$) for concentrations (1000–1.95) µg/ml, where the inhibition rate starts from 100% until it reaches 2% when compared to the control's 100% viability, while there are no significant differences when compared between the values of IR **(Figure 1)**.

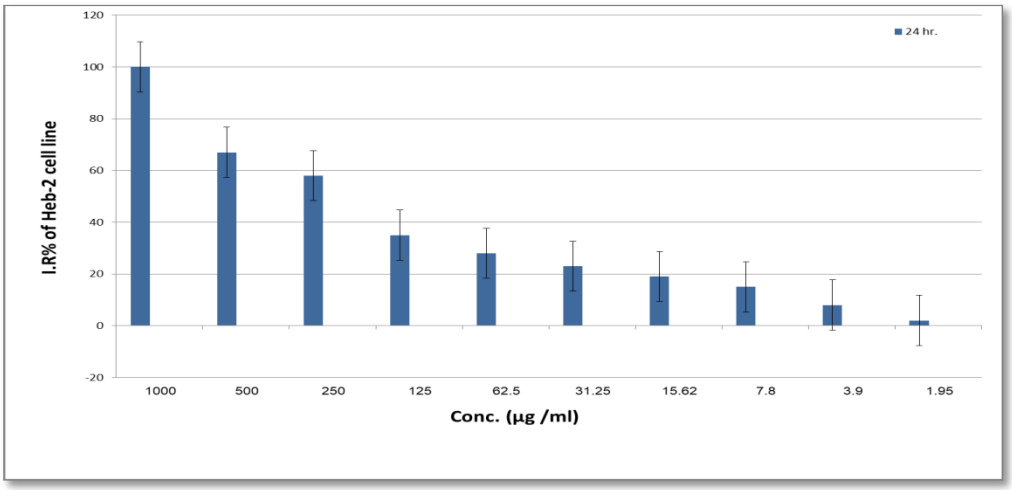

Figure 1. Effect of marcescin on Hep-2 cell line after 24 hours.

3.2. Toxic effect of marcescin after 48 hours

With regard to the effect of the marcescin during 48 hours, it increased more than in the first 24 hours, where the effect (IR) was observed when the concentration was 1000 µg/ml is 100%, then the decrease in the effect began gradually with a decrease in the concentration, that it was more than the effect in the first 24 hours, where the inhibition was during the first 24 hours when the concentration was 500 µg/ml is 67%, while the inhibition is 73% at the same concentration after 48 hours. Even in low concentrations, it was observed that inhibition in 48 hours was greater than inhibition in the first 24 hours. It was observed that there are

significant differences in the 48 hours (P≤0.05) for concentrations (1000–1.95) µg/ml, where the inhibition rate starts from 100% until it reaches 6% when compared to the control 100% viability. Also, there are significant differences between the concentrations (500–250 and 7.8–3.8) µg/ml, while there are no significant differences between the rest of the concentrations (**Figure 2)**.

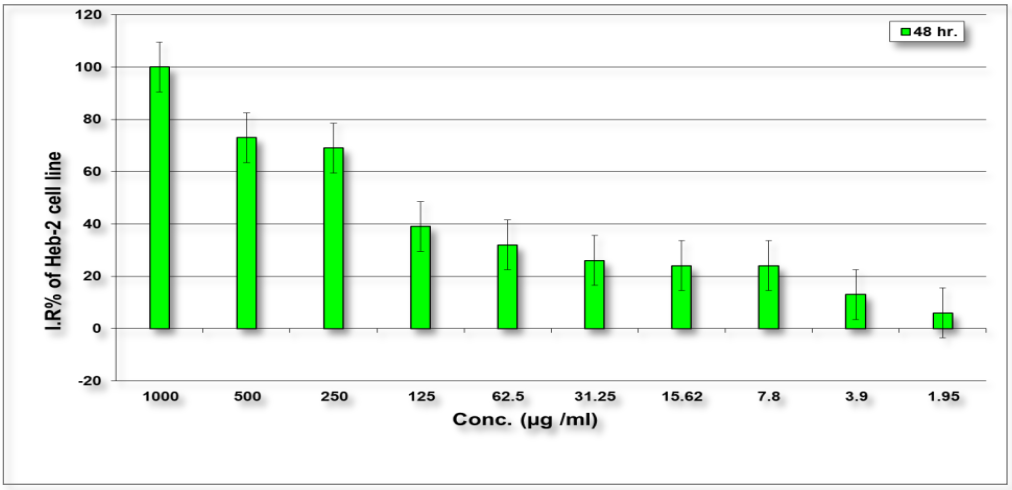

Figure 2. Effect of marcescin on Hep-2 cell line after 48 hours.

3.3. Toxic effect of marcescin after 72 hours

The inhibitory effect of marcescin in the 72 hours was greater than the inhibition in the 48 hours and 24 hours. The inhibition was noticed at the concentration of 1000, which was 100%, and at the concentration of 500 in the 72 hours, it was 78%, which is greater than the inhibition in the 48 hours, which was 73 at the same concentration, where it was 67% at the 24 hours. The inhibition rate decreased by gradually decreasing the concentration to reach an inhibition rate of 14% at a concentration of 1.95 µg/ml, which is greater than the inhibition rate at the same concentration at 24 and 48 hours. It was observed that there are significant differences in the 72 hours (P≤0.05) for concentrations (1000–1.95) µg/ml, where the inhibition rate starts from 100% until it reaches 14% when compared to the control's 100% viability (**Figure 3)**.

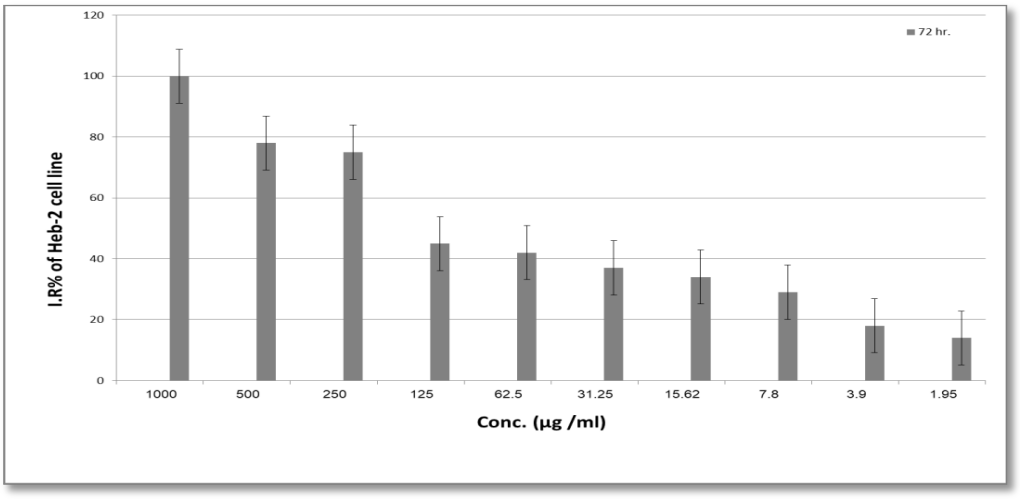

Figure 3. Effect of marcescin on Hep-2 cell line after 72 hours.

3.4. Comparative between effect of marcescin on Hep-2 cell line in different exposure time.

The effect of marcescin on the Hep-2 cell line at 48 hours is higher than at 24 hours, and at 72 hours; it is higher than at 48 and 24 hours. Significant differences were found when comparing the concentrations at different exposure times. Therefore, the effect of marcicin on the Hep-2 cell line after 72 hours of exposure was the highest because the inhibition rate was highest at the concentration of 500 µg/mL, where it was 78%, while at the same concentration after 24 and 48 hours it was 67% and 73%, respectively (**Figure 4)**.

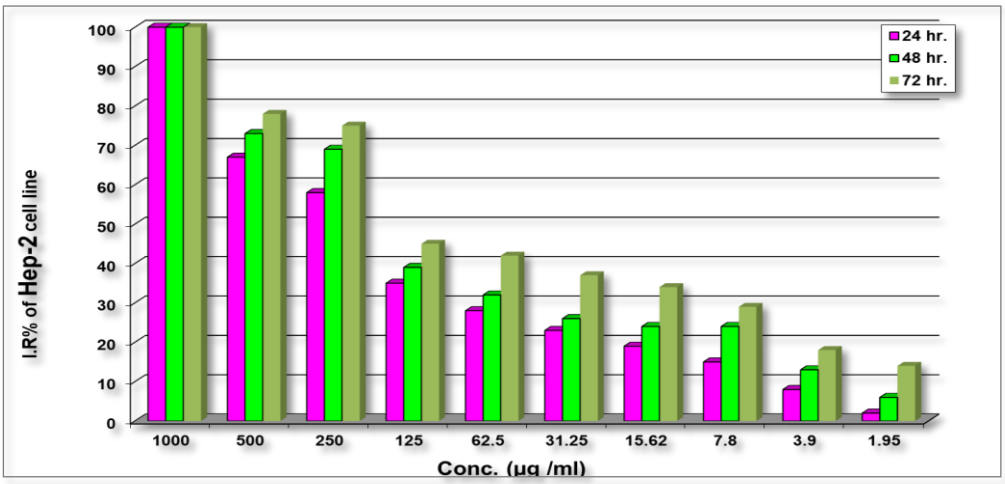

Figure 4. Comparative between effect of marcescin on Hep-2 cell line after different exposure time.

4. Discussion

Studies (25, 26 and 27) showed that bacteriocins can inhibit the division of different types of cancer cells, but it depends on the dose and time and increases exponentially. Another study by (28) The results of this study were consistent with the results of the research, as this study confirmed that the bacteriocins cause inhibition of the growth of cancer cells depending on the concentration of the bacteriocin and the periods of exposure to the bacteriocin. The results of the research were consistent with the study (29), the period of exposure, and the type of extract. The highest rate of inhibition was recorded at a concentration of 400 µg/ml for the four types of extracts, and it decreases as the concentration decreases from the first day of exposure (24 hours) and increases to give the highest percentage of inhibition after 72 hours. According to (30) and (31), the effect of bacteriocins (colicin, pyocin, and vibriocin) on cancer cells is not restricted to concentration alone, and the cell growth phase also has a significant impact on survival. Marcescin destroys the cancer cells by forming pores in the cell wall that lead to cell death. This agrees with the study by (32). The formation of pores in the cytoplasmic membrane of cancer cells affects the efflux of intracellular substances, including potassium and amino acids. Cancer cell death results from this depolarization of the membrane. Another investigation revealed that the *S. marcessens* bacteriocin 28b has the ability to generate pores, suggesting that this may be the bacteriocin's mode of action for killing (33). It was found that partially purified bacteriocin causes the death of cancer cells through programmed cell death by affecting the DNA and breaking it into pieces. This effect begins after the first hour of exposure and reaches its highest peak after six hours (34). Some colicins, including HSC10, affect the cell cycle of cancer cells (T-cell leukemia), causing their division to be inhibited, making them unable to reach phases (S, G2, M), and congregating in the G1 phase (35).

5. Conclusions

The results showed that Hep-2 cell lines are sensitive to marcescin, and this sensitivity increases with increasing concentration and the longer the exposure period to marcescin. Marcescin does not promote the proliferation of cancer cells. So this bacteriocin may be used to treat cancer.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

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Ethical approval

The study was conducted in accordance with the ethical principles. It was carried out with patients verbal and analytical approval before the sample was taken. The University of Baghdad College of Science, a local ethics committee, reviewed and approved the study protocol, subject information, and consent form on 26 September 2022, using the document number CSEC/0922/0089.

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