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Effect of Pseudomonas sp. UW4 Native Strain Metabolites on HT29 Cell Line Proliferation and Apoptosis

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Abstract: *Considering rising antibiotic resistance issues of bacterias, systemic toxicity and drug resistance in chemotherapy, discovering new bio-active compounds is essential. Therefore, introducing new bio-active compounds for treating patients of infectious diseases and cancer can be considered.* In this study, the stability of antimicrobial metabolite produced by Pseudomonas sp.UW4 against lipase enzyme and the anti-cancer effect of it was evaluated through the rate of HT29 cell line proliferation and apoptosis. In order to achieve this goal, Pseudomonas sp. UW4 was isolated from Isfahan soil samples and it was evaluated for production of active bio-compounds and stability of antimicrobial metabolite Pseudomonas sp.UW4 against the lipase enzyme. The evaluation of the stability of the produced metabolite against the lipase enzyme showed that this metabolite is persistent against the enzyme.

Also, treatment with the metabolite showed that by increasing the concentration of the metabolite depended on dose and time, the biotic potential of the cells decreased. The highest effect was observed at the concentration of 20 mg/ml and 72 hours after treatment of the cells. Induction of apoptosis was also dose-dependent and the concentration of 20 mg / ml supernatant induced the highest percentage of apoptosis in HT29 cells. Therefore, bioactive compounds produced by Pseudomonas sp. UW4 may be used to eliminate infections and treat HT29 breast cancer.

Keywords: Pseudomonas sp. UW4, apoptosis, HT29 cell line.

INTRODUCTION

Four types of key genes involved in directing cancer cells include oncogenes, tumor suppressor genes, DNA repairing genes, and apoptosis genes.(Molecular Genetics) If a genetic mutation is generated in the cell, normal cells are deviated from their natural path and are affected by new commanders that progress to cancerous cells (Hanahan D et al, 2000). Antimicrobial metabolites are important factors in reducing many diseases. Many chemical drugs are currently at risk of drug resistance and their effects in treatment are decreasing. More than 60% of anticancer drugs have a natural origin, such as plants and microorganisms. Among several sources of anticancer drugs, microorganisms are more effective in providing the desired drugs (Griffin MR et al, 1987; Liang S et al, 2009; Phonnok S et al, 2010). The probiotic effect of Bifidobacterium bifidium on cacoII cancer cells (colon cancer cell line) was investigated. The results showed that the suppressant percentage of Bifidobacterium supernatant on cancer cells was 55% to 82%.

Which is related to a concentration of 100 µl in 24 hours and 300 µl in 72 hours (Piddock LJ, 2006). Pseudomonas are gram-negative, motile, aerobic, and gram-negative bacteria and some of them produce water-soluble pigments. Pseudomonas are widely found in soil, water, plants, and animals (Ionov Y et al, 2014). So far, some antibiotics have been made by Pseudomonas fluorescent bacteria. The therapeutic application of microbial

metabolites has led to an opportunity for discovering antibiotics e.g. anticancer agents (Daunorubicin, Doxorubicin, Bleomycin, Pentostatin). Systematic poisoning and drug resistance issues in cancer chemotherapy require the continuation of discovering new anticancer agents. The specificity microbial metabolites in the manner of antimicrobial activity are the reason it has been suggested for the process. Pseudomonas is as producer of glycolipid surfactant contains rhamnose and 3-hydroxy fatty acid (Zhao J et al, 2013). Cupredoxin Azurin is a redox protein containing copper that is released by the Pseudomonas aeruginosa pathogen bacteria and is known to be cytotoxic to mcf-7 cells of human breast carcinoma. Azorine enters the cytosol of mcf-7 cells and migrates to the nucleus, increasing the intracellular levels of p53 and Bax thereby, consequently, initiating the release of mitochondrial cytochrome C into the cytosol, which activates caspase and initiates apoptosis and results in significant tumor breakdown (Punj V et al, 2004). Rhamnolipid is another metabolite of Pseudomonas aeruginosa. This metabolite consists of two main types of mono and di-Rhamnolipid. di-Rhamnolipid has anti-proliferation activity against human breast cancer (Thanomuub B et al, 2007). Rhamnolipid has also different effects on the growth of keratinocytes and human fibroblasts. Rhamnolipid is easily isolated by culture and is produced by hydrophobic and hydrophobic substrates such as carbohydrates, hydrocarbons, herbal oils, or industrial waste (Stipcevic T et al, 2005). In China, the chemical structure and biological activity of biosurfactants produced by 4808 ml of Pseudomonas aeruginosa were measured. In this research, the main components of the biosurfactant were identified as two types of Rhamnolipids and Dirhamnolipids. Diramololipids at a minimum concentration of one microgram showed antiproliferative activity against human breast cancer cells (Agodi A et al, 2007). The effect of dipeptide cytotoxicity on Pseudomonas aeruginosa PAO1 in apoptosis of human cancer cell lines was investigated. In this study, the combined effect of CDP consisting of the (L-pro-L-try), (L-pro-L-value), and (L-pro-L-phase) cycles isolated from Pseudomonas aeruginosa was investigated on cancer cells. It was shown that these compounds can have an inhibitory effect of 50% (Bailey FA et al, 2007).

Considering the rising issue of antibiotic resistance in bacteria, systemic toxicity and drug resistance in chemotherapy, the discovery of new bioactive compounds is essential. So far, a number of studies have been conducted on the effects of produced microbial metabolites, especially by Pseudomonas strains on cancer cells. Therefore, in this study, considering the stability of antimicrobial metabolite produced by Pseudomonas sp.UW4 against the lipase enzyme, its anti-cancer effect was measured by the rate of proliferation and apoptosis of the HT29 cell.

2.Materials and methods

Preparation of Pseudomonas sp. Samples UW4

UW4

In this study, soil isolated Pseudomonas sp. UW4 that was able to produce antimicrobial metabolites was used. For this separation, 70 soil samples from different regions of Isfahan province were collected from depth of 10 to 15 cm along the roots of the plant. To investigate the potential for producing antimicrobial metabolism against pathogenic microbes, agar diffusion method was used. In order to isolate the produced antimicrobial metabolites, the 24-hour culture of bacteria in TSB medium was used, the cultures were centrifuged at 12000 g for 10 minutes, then the cells were isolated from supernatant and supernatant of cells were used (Pei AY et al, 2010). In the next step, using pasteurized pipettes, sterile wells (with 5 mm diameter) were created on a Muller Hinton Agar culture medium. Subsequently, each of the Escherichia coli bacteria (PTCC 1399), Bacillus cereus (PTCC 1154) and Staphylococcus aureus (PTCC 1189) was added to 3 ml of Broth BHI medium to obtain an opacity equivalent to 0.5 McFarland, then they were cultured on Muller Hinton agar solid culture medium. Then, 100 µl of each supernatant was added to each well and finally, the plates were incubated for 24 hours at 37 ° C. After 24 hours, the diameter of the growth inhibition zone for bacteria and the presence or absence of antimicrobial activity were measured using a millimeter ruler (Mulet M et al, 2015; Peix A et al, 2010). Then, the sample with the best growth inhibition zone was used to isolate antimicrobial metabolites. It was centrifuged at 18000 rpm for 10 minutes. Then, 2cc of the metabolites was poured into the lyophilized vials and then was put into the lyophilizer for 7 hours and then they were dried. Prepared samples were freezed at -20 ° C until the experiments.

Evaluation of antimicrobial sustainability of Pseudomonas sp.UW4 against the lipase enzyme

Was dissolved in 1 mg/ml. Then 100 microliters of antimicrobial metabolite with 100 micro liters of the enzyme was mixed and incubated at 30 ° C for 3 hours. The sample was then placed into a well in nutrient-agar medium stained with Staphylococcus aureus and incubated at 37 ° C. After 24 hours, the effect of enzyme on antimicrobial activity of metabolite was investigated using the size of the inhibition zone of the wells (antimicrobial metabolite solution without enzyme was also used as control) (Innis MA et al, 1990).

Determination of anti-cancer property of Pseudomonas sp. UW4 derived metabolite by investigating HT29 cell line proliferation and apoptosis

Cultivating bacteria

At first, a pure culture of Pseudomonas sp.uw4 bacteria was transferred to TSB medium and placed in a shaker incubator at an optimal temperature of growth by 250 rpm. After 72 hours, the microbial culture was centrifuged and the cells were isolated from the supernatant and sterilized by using a 0.22µm sterile filter.

Cell culture:

Primary culture of HT29 cell line

The HT29 colon cancer cell line was obtained from the cell bank of the Pasteur research center of Iran. After melting the cells in a 25 ml flask, they were cultured in RPMI1640 medium containing 10% cow's embryo serum. Flasks were incubated in a CO2 incubator containing 95% air, 5% carbon dioxide, 95% relative humidity and 37 ° C temperature. Cultured cells were examined with a reverse microscope every day. The culture medium in each flask was changed every 48 hours.

Cell culture in 96 well plate

In order to perform cell culture, cells were first trypsinized and then they were centrifuged and counted, and then they were transferred to 96 well plates so that there were 5,000 cells in each house. In the next step, 100 µl of culture medium was added and the 96 well plate was placed in a 5% CO 2 incubator.

Preparation of MTT solution

In order to prepare MTT solution, 5 milligrams of MTT was dissolved in 1 cc of PBS.

MTT test

Apoptosis test using Annexine kit

Annexin staining protocol

V-Fitc annexin is used to determining the quantitative percentage of cells in a population under apoptosis. This substance moves from the inner surface of the membrane to the outer surface of the membrane in ps apoptotic cells of a phospholipid membrane, so ps is exposed to the external environment. V-FITC annexin is a protein-dependent phospholipid-binding Ca2+ that has a high affinity for ps and is useful for detecting apoptotic cells with ps which is in the external environment. PI is a standard flowcitometric survival test and is used to detect living cells from non-living cells. Living cells with full membranes are impervious to PI, while the membrane of dead cells is permeable to PI. Cells with A positive and PI negative are in the early stages of apoptosis. Cells with A positive and PI positive are in the final stages of apoptosis, under the necrosis or are dying. Cells with both A and PI negative result for staining are viable and not measurable for being apoptotic.

Annexin test method

3.Results

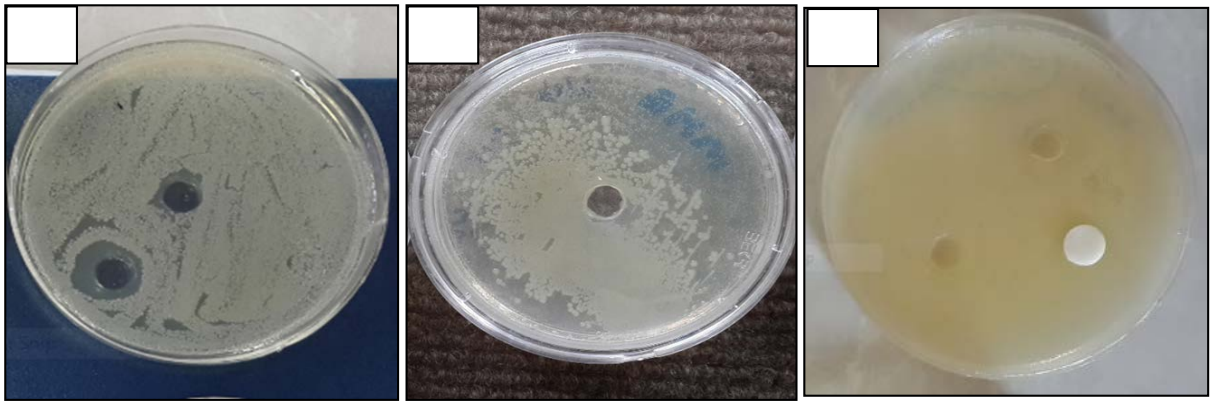
Evaluation of Pseudomonas sp. Uw4 isolate antimicrobial metabolites

The results obtained at this stage on the basis of WDA method indicated that Pseudomonas sp. UW4 was able to produce antimicrobial metabolites against Staphylococcus aureus (PTCC 1189).

In the plates containing Escherichia coli and Bacillus cereus pathogenic bacteria, growth inhibition zone was observed.

Table 1: Antimicrobial effect of Pseudomonas sp. Uw4 isolate metabolites (per milliliters)

Diameter of the growth inhibition zone of the microbial strains metabolites (in millimeters)	
Staphylococcus aureus	11
Bacillus cereus	-
Escherichia coli	-



*** Insensitivity**

Figure 5: Antimicrobial activity of supernatant derived from Pseudomonas sp. UW4. A) zone of inhibition in the plate containing Staphylococcus aureus, B) absence of inhibition zone in E. coli-containing plate; C)absence of inhibition zone in Bacillus cereus bacteria plate.

Evaluation of Antimicrobial Sustainability of Pseudomonas sp. UW4 against the Lipase Enzyme

Results obtained at this stage -based on the WDA method- indicated that the lipase enzyme was not effective on Pseudomonas sp.UW4 antimicrobial metabolite.

Culture of Colon Cancer Cells

Cells of HT29 line in different concentrations of Pseudomonas sp.The UW4 supernatant (5, 10 and 20 mg / ml) were cultured at 24 (Fig. 6), 48 (Fig. 7) and 72 hours (Fig. 8). The difference in reduction in treatment and control cells group are shown statistically.

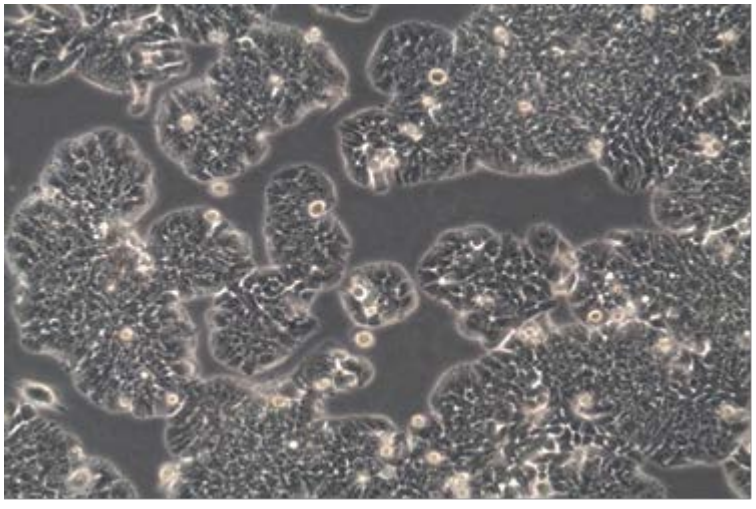


Figure 6: Effect of 5 mg/ml concentration of Pseudomonas sp.UW4 metabolite on HT29 cell line

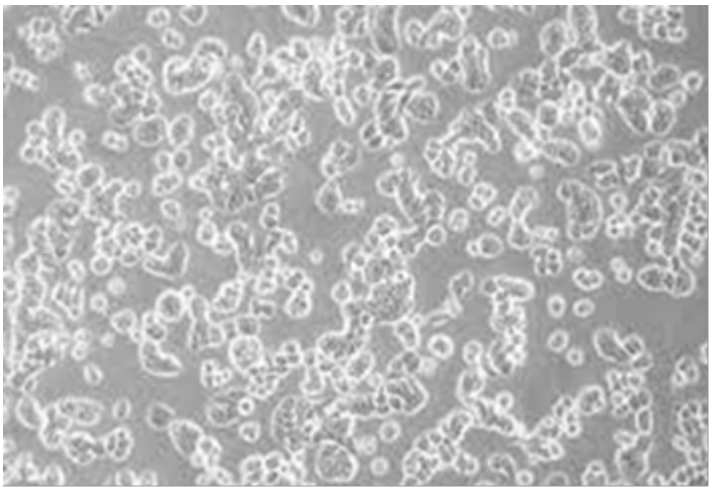


Figure 7: Effect of 10 mg/ml concentration of Pseudomonas sp.UW4 metabolite on HT29 cell line

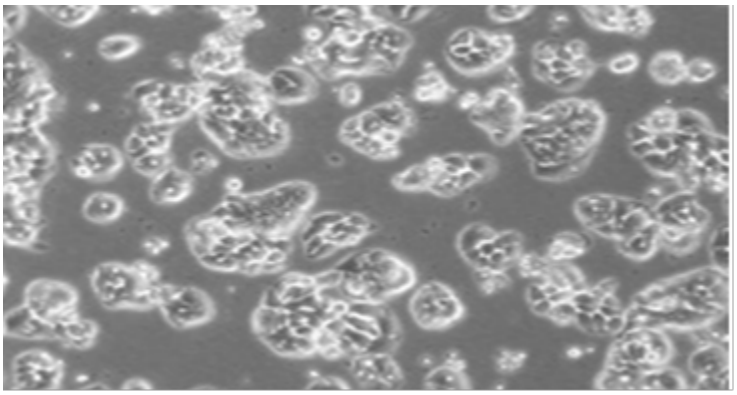


Figure 7: Effect of 10 mg / ml concentration of Pseudomonas sp.UW4 metabolite on HT29 cell line

Light absorption by ELISA results

Light density of 3 plates after 24, 48 and 72 hours of incubation was read by the ELISA device at 570 nm. The results are as follows:

(Getting inhibitory in the first 24 hours)

1. Results of optical density of 24-hour treatment of HT29 cells with produced metabolites

The optical density results are shown in Table 2 and IC50 can be observed in the chart.

Chart 1: IC50 results at 24 h

Table 2: optical density of 24-hour treatment of HT29 Cells with the metabolite (at 570 nm wavelength)

Density	Repetition 1	Repetition 2	Repetition 3
5 mg/ml	0.72	0.682	0.65
10 mg/ml	0.475	0.495	0.466
20 mg/ml	0.231	0.209	0.210
Control	0.858	0.886	0.881

2. Results of optical density of 48-hour treatment of HT29 cells with produced metabolites

The optical density results are shown in Table 3 and IC50 can be observed in chart 2.

Table 3: optical density of 48-hour treatment of HT29 Cells with the metabolite (at 570 nm wavelength)

Density	Repetition 1	Repetition 2	Repetition 3
5 mg/ml	0 98m	0.79	0.73
10 mg/ml	0.630	0.650	0.600
20 mg/ml	0.374	0.260	0.350
Control	0 980	0.960	0.960

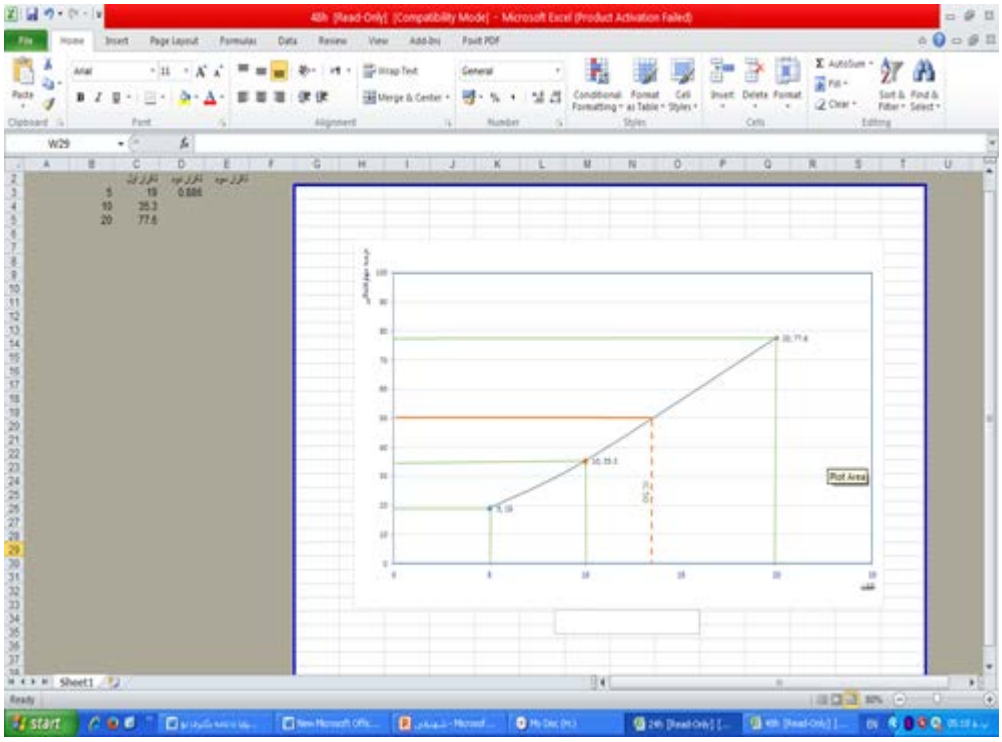


Chart 2: IC50 amount in 48h treatment.
(getting inhibitory un the first 48 hours)

3. Results of optical density of 72-hour treatment of HT29 cells with produced metabolites

The optical density results are shown in Table 4 and IC50 can be observed in chart 3.

Table 4: optical density of 72-hour treatment of HT29 Cells with the metabolite (at 570 nm wavelength)

Density	Repetition 1	Repetition 2	Repetition 3
5 mg/ml	0.248	0.257	0.228
10 mg/ml	0.220	0.210	0.215
20 mg/ml	0.175	0.186	0.172
Control	0.473	0.420	0.412

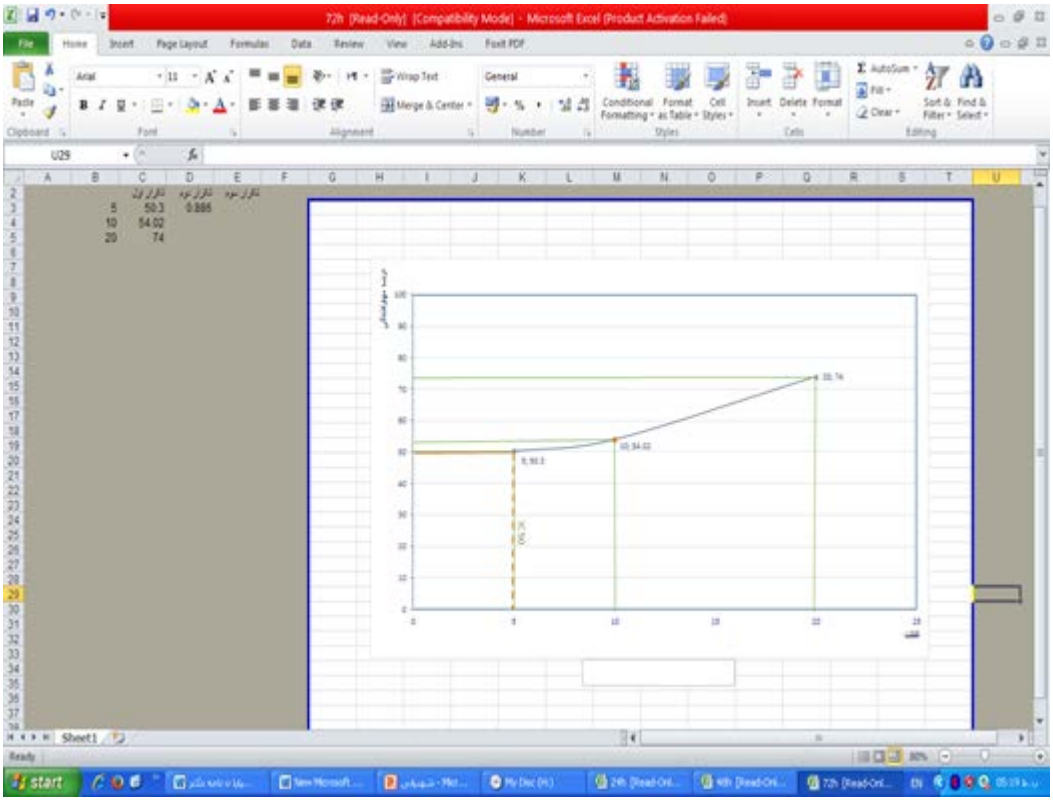


Chart 3: IC50 amount in 72h treatment.

The effect of supernatant on apoptosis rate of HT29 cells

In order to determine the apoptosis rate V-FITC annexin test was used. Cells were exposed to different supernatant concentrations for 24, 48 and 72 hours. The effect of different concentrations of supernatant in HT29 cell line on apoptosis was measured at 24, 48 and 72 hours after treatment. What statistical test is used to obtain this conclusion ???

(Figures 9, 10 and 11)

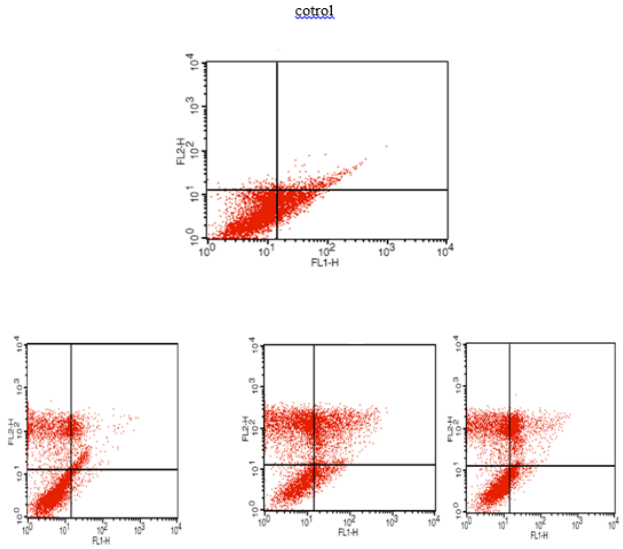


Figure 9: Effects of 5, 10 and 20 mg / ml concentrations of Pseudomonas sp. UW4 metabolites on apoptosis of HT29 cells after 24 hours.

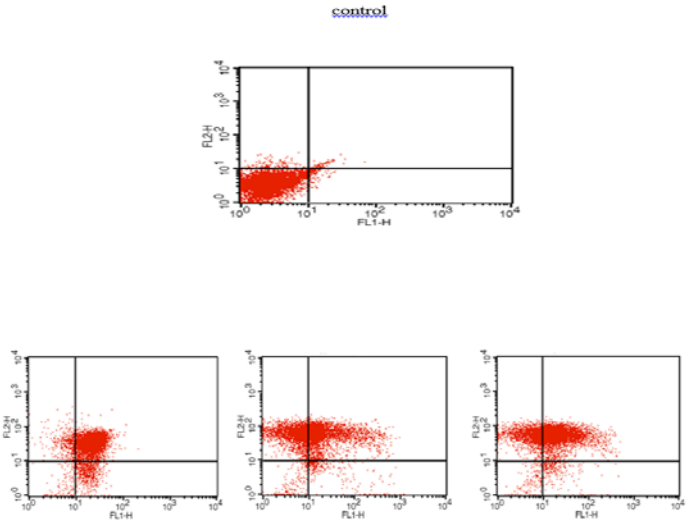


Figure 10: Effects of 5, 10 and 20 mg/ml concentrations of Pseudomonas sp. UW4 metabolites on apoptosis of HT29 cells after 48 hours.

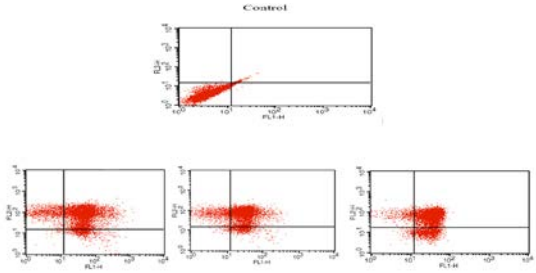


Figure 11: Effects of 5, 10 and 20 mg/ml concentrations of Pseudomonas sp. UW4 metabolites on apoptosis of HT29 cells after 72 hours.

4.Discussion and conclusion

In the present study, antimicrobial and anticancer activity of Pseudomonas sp. UW4 metabolites isolated from Isfahan soil on a number of pathogenic microbes and HT29 colon cancer cell line was studied. The results indicated that Pseudomonas sp. UW4 isolate is able to produce antimicrobial metabolite against Staphylococcus aureus (PTCC 1189).

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