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The effect of moderately halophilic bacteria supernatant on proliferation and apoptosis of cancer cells and mesenchymal stem cells

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Abstract

Many drug discoveries and developing of their applications has originated from microbial metabolites. The most efforts in development of new drugs are concerned with anti-cancer agents that cause better treatment results, less side effects, and more economical production. Several anti-tumor drugs have been recently extracted from natural microbial products. Among these various microbial diversity, Marin bacteria and Archaea have been considered as important and efficient organisms to serve as manufacturers of diverse bioactive compounds. Moderately halophilic microorganisms isolated from saline ponds and lakes of Iran show high capability for production of bioactive compounds like enzymes, dyes and anti-cancer agents. In this research, nine moderately halophilic bacteria isolates were screened to evaluate their anti-cancer agent productivity. After five days of culture on suitable mediums, supernatant samples were tested for in vitro anti-proliferative activity against Human Umbilical Vein Endothelial Cells (HUVEC) while same concentrations of supernatants were examined for evaluating of proliferative activity against Adipose-derived Mesenchymal stem cells (MSCs). Both assessments were carried out by MTT assay and double PI and DAPI staining. *GASX17*, *GBWy6* and *GBPX3* isolates just induced HUVEC cell deaths and exhibited anti-proliferative activity while R_2S_{12} not only reduced HUVEC cell proliferation but also enhanced proliferation of MSCs. R_2S_{12}, *GASX17*, *GBWy6* and *GBPX3* isolates were characterized biochemically and six hydrophilic components were detected. This research established new bioactive compounds that could be used as an effective treatment in chemotherapy.

Key words: Moderately halophilic, Anti-cancer activity, Drug discovery, HUVEC, stem cell.

Introduction

Since ancient times, combating against diseases and fatal disorders was a major effort for human being. Besides chemical synthesis of drugs and medical molecules , natural production of living organisms specially microorganism was discovered and developed since biologically derived compounds had better biocompatibility, much less side effects and more economical reasons to be manufactured (1, 2).

Based on estimation till the date 2007, just only about 0.1% of bacteria species has been identified and among these known species, few of them were screened and studied for bioactive molecule production (3). Secondary metabolites which are defined as organic compounds produced by organisms such as fuels (oil and natural gas) and medicines (hormones, antiobiotics) started a new era in biotechnology (1). Various secondary metabolites (almost 100,000) with molecular weight less than 2500 have been studied while about half of these molecules were produced by microorganisms (4). Mortality and morbidity related to cancer was located at top of death causes globally especially in developing countries (5). Although various drugs were used to prolong patient's life, because of drug resistance occurrence, low efficiency and unsolved side effect issues, efforts to find other antitumor molecules lacking of the mentioned problems, encouraged many scientists to study new anticancer agents (6). Antitumor compound for chemotherapy are mainly microbial antibiotics such as actinomycin D, the anthracyclines and doxorubicin (7). Although Taxol antitumor drug was discovered from plant metabolites for the first time, it has been also characterized in microbial metabolites recently (8). Finding and development of anti-cancer agents has a great importance in microbial biotechnology and should be categorized in 2 ways 1) To identify new antitumor agents due to increasing rate of cancer deaths, 2) To find new microorganisms which are capable of producing antitumor agent (1).

Moderate halophiles are microorganisms that can grow in saline environments with salt concentration between 3-15 percent (9). These microorganisms shows significant potential for bioactive compound production (10). For example, In china 45 moderately halophilic eubacteria strains were isolated from water of weihai solar saltern by Chen research team. Chen research team also screened them to assess their bioactive agent productivity. Twenty-three of 45 strains showed antibacterial activity against B. subtilis while just one strain inhibited the growth of E.coli. More importantly, crud extracts of 14 strains showed cytoxicity against hepatocellular carcinoma BEL-7402 cells and five strains were assessed to have IC_{50} value below 40 microgram per liter. However, none of the active agents were determined to be identified, they hoped it would be led to new discoveries (11). After two years, Streptomyces sp. Nov. WH26, was reported to display cytotoxicity activity (12).

Another bioactive molecules which have attracted many interests especially in tissue engineering, are proliferation enhancers for stem cells. Alan Mish and his

coworkers carried out Platelet-rich plasma (PRP) to affect proliferation of Mesenchymal stem cells. They showed PRP increased proliferation rates of MSC and their results led to better treatment for acute and chronic tendon disorders (13).

Because little is still known about bioactive profiles of moderately halophiles and also high biodiversity of moderately halophiles in Iran was reported, in present study, We evaluated effects of nine moderately halophiles' supernatant on proliferation of HUVEC cancer cell line and Mesenchymal Stem Cells. We used MTT assay, PI (Propidium iodide) and DAPI (4',6-diamidino-2-phenylindole) staining for measurement of proliferation rates. Preliminary chemical investigation by TLC chromatography also was carried out.

Materials and methods

Inoculum development and supernatant preparation

Nine moderately halophile strains were deposited at Extremophile's Lab at University of Tehran Table 1. We preferred to use abbreviations instead of Strain names.

After receiving them, for preparation of inoculum, SWN agar medium was used for three of strains while for others MH agar medium culture was used.MH medium includes 20.25 gr/lit NaCl, 1.75 gr/lit $MgCl_2$ 2.4 gr/lit $MgCl_2$ 0.09 gr/lit $CaCl_2$ 0.015 $NaHCO_3$ 0.0065 gr/lit NaBr, 5 gr/lit proteose peptone, 10 gr/lit yeast extract, 1 gr/lit Glucose and 15 gr/lit agar. $CaCl_2$ solution autoclaved separately and PH of main solution was adjusted about 7 by Tris solution. SWN medium includes 20 gr/lit NaCl, 3 gr/lit $MgCl_2$ 6 H_2O 0.5 gr/lit $CaCl_2$ 5 gr/lit $MgSO_4$ 7 H_2O, 5 gr/lit proteose from meat, 1 gr/lit yeast extract, 2 gr/lit meat extract and 15 gr/lit agar.PH of solution before being autoclaved was adjusted about 7 by Tris solution.

After 3 days after that strains grew on agar mediums, 10 mL inoculum solutions for each strain were prepared. Their concentration were adjusted about 0.01 OD by spectrophotometry, then they were added to 140 ml of mediums in 500 ml Erlenmeyer flask. 2 additional flasks of SWN and MH mediums as bacterial medium controls were prepared technically. Eleven flasks were shacked for 5 days at 140 rpm at 34 centigrade temperature by (BIOSHACKER).

Each flask was centrifuged at 12000 rpm for 30 mins and after separation of supernatant (cell free part of solution), each supernatant was filtered by (ORANGE) filters to become sterilized. Supernatant solutions were stored in refrigerator at 4 c temperature.

Cell line and culture conditions

Human umbilical vein endothelial cells (HUVEC) and and Mesenchymal stem cells(MSCs) were received from Stem Cell Technology Research Center in Tehran. For both cell lines' culture, universal DMEM supplemented with 10% FBS medium was used.

MTT assay

50 microliter of prepared supernatant was added to each well in 24-well plates to adjust the supernatant concentration at 10 % while MSCs were seeded at initial density of 2000 per well.(450 microliter of DMEM containing MSCs were added to each before.)In 24, 72 and 188 hour after culture, MTT assay method was used to assess the viability of cells.at each point MTT reagents (50 mg/ml in DMEM) was added to each well and then plates located in incubation at 37 c for 3.5 hours. Supernatant was removed and 200 microliter DMSO was added to each well. At 570 nanometer by spectrophotometer optical density was read. Same procedure was carried out for HUVEC cells.

PI and DAPI double staining using fluorescent microscopy

Fluorescent Microscope was used and adjusted to study of cell deaths of HUVEC cell's treatment with each supernatant after 24 h. PI (Propidium iodide) staining and DAPI (4',6-diamidino-2-phenylindole) staining was used. Both viable and death cells show Blue fluorescent while just death cells show red fluorescent. DAPI reagents were added after PI staining step to each sample. Cell death percentage was measured by comparing of double staining methods together.

Chemical analysis by TLC chromatography

Ethyl acetate and also 50% ethyl acetate mixed with 50% ethanol were mobile solvents. We used commercialized silica gel sheets to separate components. For detection of spots,2 UV lamps were carried out. One of them emitted in 254 nanometer wavelength and the other one emitted 360 nanometer wavelength.

Results

Effect of supernatant on proliferation of MSCs

Optical density for studying effects of supernatants on MSCs proliferation has been shown in Figure 1.

Table 1. Strain names.

Abbreviation	Strain name
BG7	*Paracoccus aestuarii*
R_2S12	*Bacillus mojavensis IFO15718*
GAWy5	*Marinobacter hydrocarbonoclasticus MBIC1303*
R3A11	*Italomanoy fontilapidosi 5CR*
Gasx17	*Idiomarina Zobelli KMM 231 (T)*
GBPx3	*Vibrio ordalii ATCC 33509 (T)*
GBWy1	*Bacillus horikoshii DSM 8719 (T)*
GAAy6	*Halomonas boliviensis LC1 (T)*
GBwy6	*Halomonas andesensis LC6 (T)*

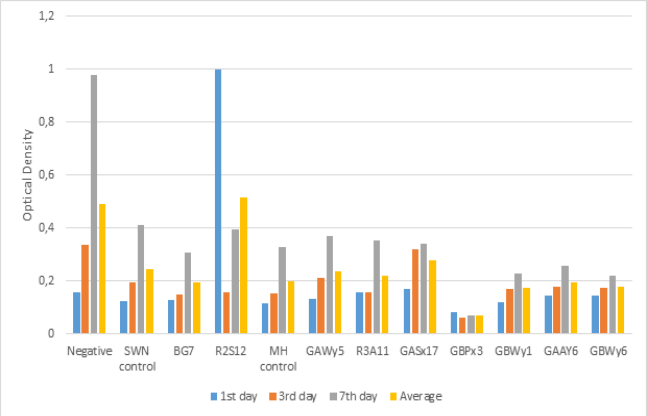

Figure 1. OD of MSC's samples after treatment. Yellow column displays average OD of each sample for 3 days experiment.

Comparative histogram shows the results and also average OD. As seen in the figure, R2S12 affected the cell proliferation in a more positive manner in comparison to that observed by other isolates. Comparison of the viable cells on days 3 and 7 in different groups, showed that all isolates except R2S12 decreased the rate of proliferation.

MTT assay for anticancer activity

Optical density of samples on 3 days of MTT assay and average of ODs for each sample has been mentioned in yellow in figure 2. Death percentage of each supernatant has been compared against its medium control (negative control) in figure 3. MTT assay showed that in addition to R2S12, GASX17, GBWy6 and GBPX3 decreased the viability and proliferation rate of cells during 7 days of study.

PI and DAPI double staining

After 24 h of treatment by supernatants, for each sample we calculated death percentage by comparison of PI stained death cells against all cells which have been stained by DAPI as blue objects. Figure 4 shows double stained samples and ratio of cell death percentages against medium controls was displayed in Figure 5. These data confirmed the anticancer activity of isolates observed in MTT assay.

TLC chromatography analysis

Chosen supernatants that shows anticancer activity, have been characterized by TLC chromatography. Figure 6 shows the results. Left image was detected under

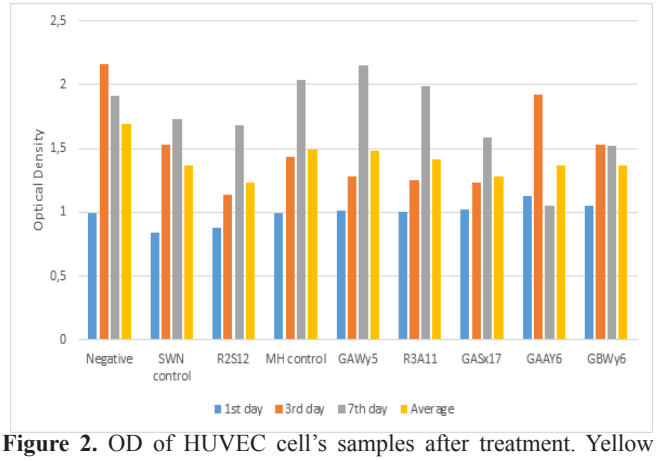

Figure 2. OD of HUVEC cell's samples after treatment. Yellow column displays average OD of each sample for 3 days experiment.

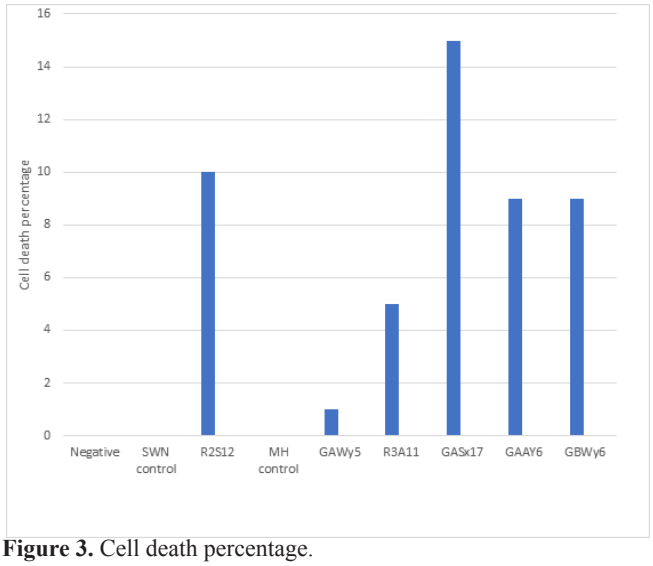

Figure 3. Cell death percentage.

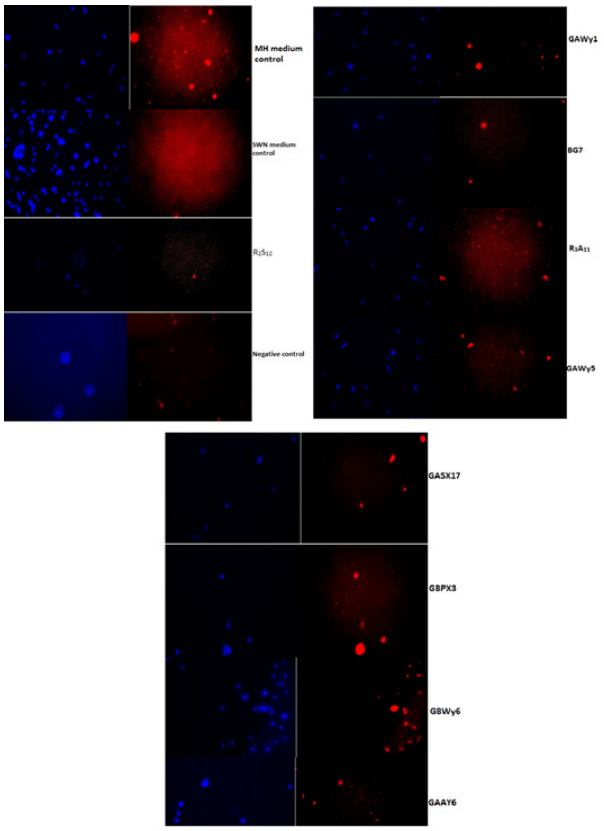

Figure 4. Left picture (with blue objects) was obtained by DAPI staining while right one(with red objects) was obtained by PI staining.

254 nanometer UV light while right image was detected under 360 nanometer UV light.

Discussion

Because of drug resistance and other systemic problems found in alternative anticancer drugs, discovery of new antitumor agents has urged massive researches on studying new drugs. Microorganisms, as one of the most important sources of medical molecules have been evaluated for anti-proliferative activity. Phonnok and his coworkers in Thailand studied almost 400 microbial extracts against apoptosis-inducing and anticancer activity for 4 cell lines. They exhibited that 20 samples has varying anticancer activity while just 4 of them could be the potent ones to be carried out as antitumor drug

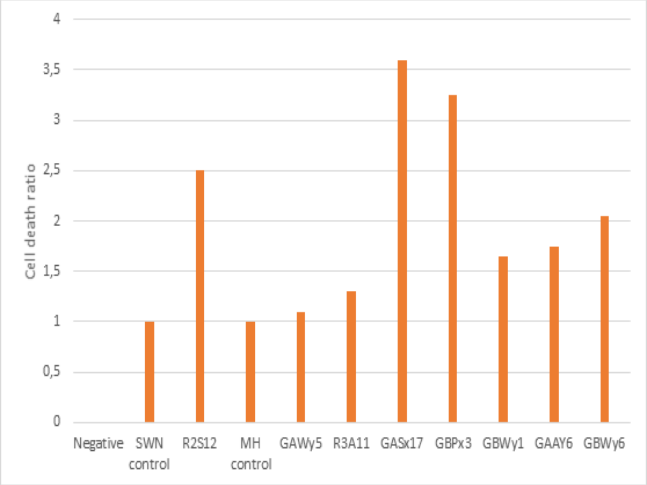

Figure 5. Ratio of cell deaths to bacterial medium controls.

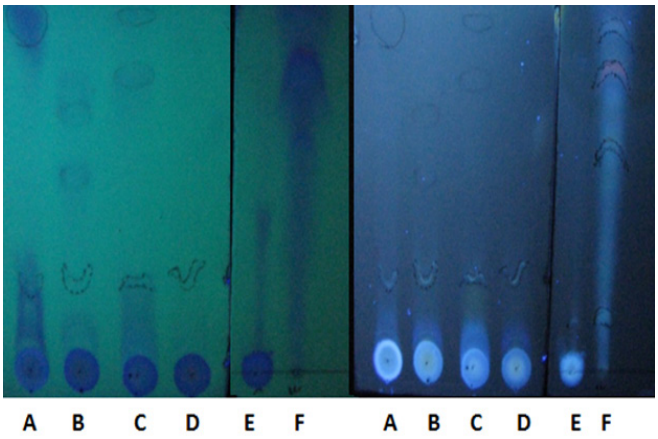

Figure 6. A: GBPX3, B: GASX17, C: GBWY6, D: MH medium control, E: SWN medium control: R_2S_{12}.

agents (14). From diverse habitats in India 60 microbial isolates were studied and it has been proven that 3 strains produced antitumor agents against cancer cell lines .Lie et all tested 45 moderately halophilic bacteria for their antibacterial and anticancer activity and their results displayed anticancer activity of 14 strains (12).

In our study, we examined anti-proliferative activity of 9 supernatant samples from moderately halophilic bacteria -isolated from various saline locations in Iran -against HUVEC cells and also we examined proliferation-inducing activity against adipose-derived MSCs. Based on results from MTT assay for cytotoxicity against cells in Fig 2, the average optical density between 3 days of the assessment has been calculated and all samples, even bacterial mediums, caused cell deaths in comparison with HUVEC cells without supernatant (Negative control). So the cell death percentage for each sample should be calculated in ratio of its bacterial medium control (Fig 3).GASX17,R_2S_{12},GBWy6 supernatants shows highest anti-proliferative activity among others.

MTT assay results for proliferation-inducing activity against adipose-derived MSCs were evaluated in a same way both in procedures and statistical analysis. R_2S_{12} shows potent capability to enhance proliferation of MSCs. It must be considered that because of negative effects caused by bacterial mediums, we just compared average columns to the negative control and R_2S_{12} increased cell proliferation about 5 percent.

From pictures taken by fluorescent microscope after double PI and DAPI staining, we could obtain death percentages for each samples and after calculation of ratio of cell death percentages against bacterial medium controls, GASX17, GBPX3 and R_2S_{12} shows highest cell death ratio among other results(Fig 5)

After re-evaluating of Figure 3 that shows Optical Density of samples, there is a significant increase of OD from 1st day to 7th day for GBWy6, GASX17, GBPX3 and $R_2S_{12\ and}$ it is proven these samples had anticancer activity based on previous assessments. This phenomenon might be because of anticancer agents' consumption before 24 hours.

Evaluation of supernatants by TLC chromatography is a preliminary way to predict and characterize biochemical features of anticancer agents so GASX17, GBPX3, R_2S_{12} and GBWY6 supernatants as proven results were chosen to be assessed. We used 100 % ethyl acetate at first but there were no spots technically. Since 100% ethyl acetate and 50% ethanol were used as solvent mixtures, the higher spots appear, the more hydrophilic they might be. There were detected almost 9 spots on chromatogram that based on their bacterial medium controls and their height from plate. Totally there were 6 hydrophilic components.R_2S_{12} supernatant had 5 components that just two of them were different from other samples. These are probable components that enhanced proliferation of adipose-derived MSCs.

We hope to find out more about anticancer agent molecules by more studies on of chemical and biological tests. Even though these strains produce current antitumor drugs, this research can conduct more attentions to discovery of bioactive compounds by moderately halophilic bacteria and we showed potential of halophiles for application in medicine and biotechnology.

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