

## Extracted metabolite from *Streptomyces Levis* ABRIINW111 altered the gene expression in colon cancer

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### ABSTRACT

**Aim:** In this study we attempt to indicate anti-carcinogenic influence of ether extracted metabolites of *Streptomyces Levis* sp. on gene expression in colon cancer.

**Background:** Colon cancer is one of the most prevalent cancers worldwide. In recent decades, researchers have been seeking the treatment for cancer. Natural products are valuable compounds with fewer side effects in comparison to chemotherapy drugs.

**Methods:** Secondary metabolites were extracted with the inoculation of bacterial sample in Mueller Hinton Broth. MTT assay was done to evaluate the cytotoxicity effect of metabolites on SW480 cells. qRT-PCR was performed to observe effects of metabolites on Bcl-2, P53, SOX2, KLF4,  $\beta$ -Catenin, SMAD4, K-ras, BRAF genes expression in colon cancer.

**Results:** The metabolites exhibited cytotoxic effects on colon cancer in a dose/time dependent manner ( $P < 0.001$ ). After 48 h treatment, fold expression of Bcl-2, SOX2,  $\beta$ -catenin, K-ras, BRAF genes fold of expression were decreased, whereas P53, KLF4, SMAD4 genes were increased in treated cells ( $P < 0.001$ ).

**Conclusion:** These findings indicate that ether extracted metabolites of *Streptomyces Levis* ABRIINW111 have anti-carcinogenic effects on colon cancer.

**Keywords:** Colon cancer, Metabolites, oncogenes, *Streptomyces Levis* ABRIINW111.

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### Introduction

Colon cancer is a global challenge worldwide (1-3). Genetic and epigenetic alterations in the normal colonic epithelium lead to colon adenocarcinoma (4). Benign adenomatous polyp is the first step, then polyps develop into a malignant adenoma with high-grade dysplasia which subsequently transform into invasive cancer (5). Changes in genes expression is a significant prognosis factor for initiation and progression of colon

cancer which could be a biomarker for targeting colon cancer. Colon cancer is most commonly initiated by changes in the Wingless/Wnt signaling pathway. Inactivation of tumor suppressor genes and activation of oncogenes such as Bcl-2, P53, SMAD4, BRAF, K-ras, Beta-catenin, SOX2, and Klf4 lead to development of colon cancer. Some of the predominant alterations that have been demonstrated to play an important role in initiation of colon cancer include K-ras, P53; TGFBR2 and SMAD4 as elements of the TGF-p signaling pathway are involved too.

Bcl-2 family control the integrity of mitochondrial membrane. The anti-apoptotic proteins including Bcl-2, BAG, Bcl-x, Bcl-XS, Bcl-XL, Bcl-w and the pro-apoptotic proteins like Bax, Bid, Bak, Bad, NOXA, and

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PUMA are members of this family (6-10). Bcl-2 acts as an anti-apoptotic member and controls the apoptosis by several mechanisms such as releasing of ions into the cytoplasm through altering the permeability of the intracellular membranes (11, 12). P53 as a transcription factor has a suppressor activity and it is mutated in 50% of primary colon cancers (13). Expression of several pro-apoptotic genes such as Bax, NOXA, and PUMA is controlled by P53 (14-16).

Sox2 is a member of the Sox gene family, belongs to the SOX B1 subgroup. It acts to preserve development potential and encode transcription factors with a single HMG DNA-binding domain (17). The Kruppel-like factor (KLF) family of genes regulates a wide range of cellular processes such as differentiation, migration, apoptosis, proliferation, tumor formation and inflammation. KLF4 is exceeding and has observed in the gastrointestinal epithelial cells, skin, and endothelial cells in vascular system (18-21). KLF4 as a regulator of cell proliferation, induces cell cycle arrest at G1 to S phase in a p53-dependent manner by activation of p21WAF/Cip1 gene as the negative cell-cycle-regulatory cyclin-dependent kinase inhibitor (22, 23).

$\beta$ -catenin is one of the elements of the APC/ $\beta$ -catenin/TCF/Lef pathway and its expression is increased by activation of the Wnt signaling pathway. It plays a main role in cancers such as melanoma, and gastric cancer (24-26).

SMAD4, mutated mostly in colon cancers, belongs to the SMAD family of genes and acts as a tumor suppressor gene. In the transforming growth factor-p (TGF-p) signaling pathway, SMAD4 codes cytoplasmic mediators (27, 28).

K-ras is one of the important elements in the Ras/MAPK signaling pathway. This signaling pathway, by inducing the synthesis of cyclin D1, plays a key role in apoptosis, differentiation and cell proliferation. Mutation of the K-ras as a proto-oncogene activates this pathway, which is found in 36% of colorectal cancers (29-33). Three RAF genes that are regulated by binding to RAS, mediate the RAS-induced cellular response to growth signals by encoding cytoplasmic serine-threonine kinases. BRAF is one of the three known RAF genes that have resulted from gene duplication (30).

The SW480 cell line is obtained from the colon adenocarcinoma with moderate level of differentiation. Previous studies have illustrated that SW480 cell line displays most of the genetic changes which are seen in aggressive colon cancers, including a K-ras mutation (34), p53 mutation (35), loss of the DCC gene on chromosome 18 (36).

*Streptomyces sp* as the largest genus among actinomycets, produces a wide range of important secondary metabolites, including antimicrobial and anticancer (37). For example, Rapamycin – isolated from the soil bacteria *Streptomyces hygroscopicus* - has revealed anticancer activity (38-40). Recent studies are focused on microbial natural products as the most promising source for developing better antibiotics (41). In our screening program for producing bioactive compounds, the diethyl ether extracted from *Streptomyces Levis ABRINW111* has shown strong activity against colon cancer cells (unpublished data).

One of best methods for cancer therapy is using natural products. They act as anti-cancer agents without serious side effects. They can induce apoptosis and change genes expression in cancer cells (42). Because of these advantages, metabolites as natural products can be a good choice for cancer therapy. In this study we evaluated the *Streptomyces Levis ABRINW111* metabolites effect on the pro-apoptotic, anti-apoptotic and several oncogenes to understand how these metabolites could be effective products in cancer therapy.

## Methods

*Streptomyces Levis ABRINW111* was purchased from the Department of Microbial Biotechnology, AREEO, Tabriz, Iran. Metabolites were extracted as described, bacteria was cultured in Nutrient agar medium (Sigma /70148) at 29 °C for 7 days. loop full of bacteria was inoculated into 25 ml of Mueller Hinton Broth (Sigma /70192) and incubated while agitating on shaker incubator set at 70 rpm at 29 °C for 36 h (43). As previously described, we used spectrophotometrical reading and chose turbidity 620 nm, 0.08 O.D, as an appropriate concentration for inoculation(43). After fermentation time, 1 ml of pre-culture was used to inoculate 1,000-ml Erlenmeyer flasks; each contained 150 ml of fresh Mueller Hinton Broth medium. The

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fermentation was carried out at 29 °C for 7 days on shaker incubator set at 70 rpm, centrifuged at 4000 rpm for 20 minutes. The Cell free filtrate was mixed with equal volume of Diethyl ether (1:1 V/V) shaken for 1 h at 175 rpm, extracted by Diethyl ether (100921/Merck), using separating funnel. Finally, the obtained organic extract was concentrated at room temperature until 0.01 gr reddish brown crude extract obtained; the resulting extract was kept at 44 °C until used (43). Also, *Streptomyces Levis ABRINW111* metabolites fractions were analyzed by HPLC method (44). Metabolites were dissolved in final concentrations of 100, 500, 1000, 2000, 5000 ng/ml in DMSO (43, 45).

#### Cell culture and MTT assay

SW480, a human colon cancer cell line, was obtained from Pasteur Institute (Tehran, Iran). Cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 1% penicillin and streptomycin in 5% CO<sub>2</sub> at 37 °C.

For MTT assay, 1×10<sup>4</sup> SW40 cells were seeded per well in 96-well micro plates with 100µl of culture medium containing RPMI 1640 medium supplemented with 10% FBS, 1% penicillin and streptomycin in 5% CO<sub>2</sub> at 37 °C and incubated for 24 h. Metabolites were diluted in culture medium with less than 0/1% DMSO (Dimethyl Sulfoxide) and various concentrations of bacterial metabolites (100, 500, 1000, 2000 and 5000 ng/ml) were incubated in 5% CO<sub>2</sub> at 37 °C for 24, 48 and 72 h. Untreated cells served as control. After incubation time, supernatant was carefully replaced with 20 µL of MTT reagent (M6494/ Sigma) {3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (5mg/ml), incubated in 5% CO<sub>2</sub> at 37 °C for 4 h and 100 µL of DMSO was subsequently added to dissolve the appeared colored formazan crystals. The optical density was measured at 570 nm with reference wavelength 630 nm by micro plate Elisa reader (Biotek ELx 808, USA).

#### Real time PCR

The 1×10<sup>6</sup> cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 1% penicillin and streptomycin in 5% CO<sub>2</sub> at 37 °C. After 24 h, supernatant was removed and cells were treated in 1000 ng/ml of metabolites and incubated in 5% CO<sub>2</sub> at 37 °C for 48 h. Thereafter, the cells were harvested by using Trypsin-EDTA solution (Sigma, T4049) and collected via centrifugation in 1000 g for 5 minutes. RNA

extraction from the harvested cells was performed using RNX plus kit (RN7713C, Sina clon, IRAN). Briefly, 1 ml ice cold RNX™ –PLUS solution was added to the harvested cells in 2ml microtubes. Samples were vortexed for 10 seconds and incubated 10 minutes at room temperature (RN7713C, Sina clon, IRAN). 200µl chloroform was added to the samples and resuspended, the samples were then incubated on ice for 5 min. Samples were centrifuged at 12000 rpm at 4 °C for 15 min. The aqueous phase was transferred to new RNase-free 1.5 ml tube, and an equal volume of Isopropanol was added to the solution, gently mixed and incubated on ice for 15 min. The mixture was centrifuged at 12000 rpm at 4 °C for 15 min. Supernatant was discarded and 1 ml of 75% Ethanol was added to the mix, briefly vortexed to dislodge the pellet and then centrifuged at 4 °C for 8 min at 7500 rpm. The supernatant was discarded and the pellet was allowed to dry at room temperature for a few minutes. Pellet was dissolved in 30 µl of DEPC treated water. To help dissolve the pellet, the tube was placed in a 55-60 °C water bath for 10 min. A NanoDrop 2000c spectrophotometer was employed for concentration and OD measurements. Samples with acceptable OD 260/280 and 260/230 values (~1.8 - 2) were subjected to cDNA synthesis.

The single stranded cDNA was synthesized by using cDNA synthesis kit (K-2261-6, Bioneer, Korea) according to manufacturer's instructions. Briefly, 5µg of RNA was added to cDNA synthesis tube in a final volume of 20 µl DEPC-treated water. The cDNA synthesis tube was placed in a 60 °C water bath for 1 h and finally, it was placed in a 95 °C water bath for 5 min.

qRT-PCR was performed by using the SYBR Green master mix real-time PCR kit (75675 500 RXN ebioscience, USA) according to the manufacturer's instructions. Briefly, 7 µl of SYBR Green Master Mix PCR, 0.35 µl forward and reverse primers from a 4µmol stock, 0.7 µl of diluted cDNA template and 5.95 µl of DEPC treated water were added to tube. qRT-PCR was done as follows: initial denaturation at 95 °C for 3 min, 40 cycles of denaturation at 95 °C for 15 sec, annealing at 60 °C for 60 sec and elongation at 72 °C for 5 min. The GAPDH (endogenous housekeeping gene) gene was used as an internal control. Quantitative real-time PCR was performed with Rotor-Gene 6000(version: 1.7) to determine CT values and the

threshold was adjusted to 0.1 (inside the exponential phase). Delta CT values were calculated in relation to GAPDH CT values by the  $2^{-\Delta\Delta Ct}$  method, in which  $\Delta Ct$  represents the difference between the CT value of target genes and the CT value of GAPDH(46).

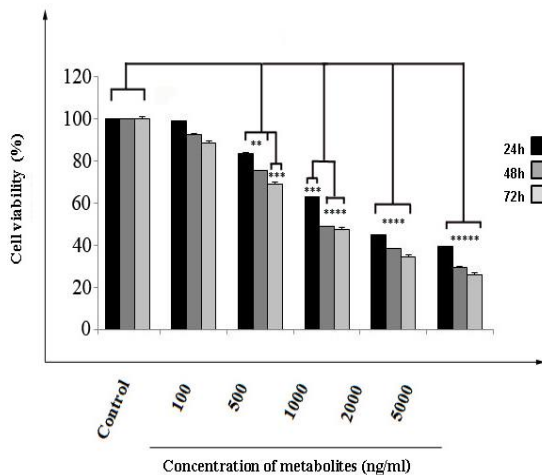
**Statistical analysis**

Each experiment was carried out in triplicate. All data are expressed as means  $\pm$  SD. One-way analysis of variance (ANOVA) was performed with the Dennett’s test, using software Graph Pad Prism 6. Significant differences were shown by “\*”, “\*\*”, “\*\*\*” and “\*\*\*\*” respectively for 0.05, 0.01, 0.001 and 0.0001significance levels.

**Results**

***Streptomyces Levis ABRIINW111* killed SW480 colon cancer cells.**

MTT assay was performed for evaluating the cytotoxicity and cell viability of SW480 colon cancer following incubation with *Streptomyces Levis ABRIINW111*. Metabolites inhibited cell growth and reduced viability based on the dose and time dependency. Cells were treated for 24, 48 and 72 h use in final concentration of 100, 500, 1000, 2000, 5000 ng/ml metabolites. Viability decreased significantly to 63.27, 48.95 and 47.58 in 1000 ng/ml after 24, 48 and 72 h respectfully. Concentration of 1000 ng/ml was chosen as IC50 value (Figure 1).



**Figure 1.** Cell cytotoxicity was examined by MTT assay. SW480 cells were incubated with indicated concentrations of *Streptomyces levis* metabolites for 24, 48 and 72 h. Cell growth was proportional to absorbance at a wave length of 570-630 nm. Values are expressed relative to the control and as mean  $\pm$  SD of three independent experiments (P < 0.001).

***Streptomyces Levis ABRIINW111* altered genes expression in colon cancer cells.**

We treated SW480 cells with metabolites using 1000 ng/ml in final concentration for 48 h. qRT-PCR was performed for harvested cells to evaluate Bcl-2, P53, SOX2, KLF4,  $\beta$ -Catenin, SMAD4, K-ras, BRAF fold expression. P53 gene expression – pro-apoptotic gene involved in apoptosis and cell cycle- increased 3-fold in treated cells (Figure 2A). Also, BCL-2 gene expression – anti-apoptotic gene- was significantly decreased to 0.1 fold of expression in treated cells (Figure 2B).

KLF4 – tumor suppressor - gene expression was significantly increased to 4.5 fold of expression in treated cells (Figure 2C) and also, SOX2 gene expression- oncogenic gene - was significantly decreased to 0.4 fold of expression in treated cells(Figure 2D).

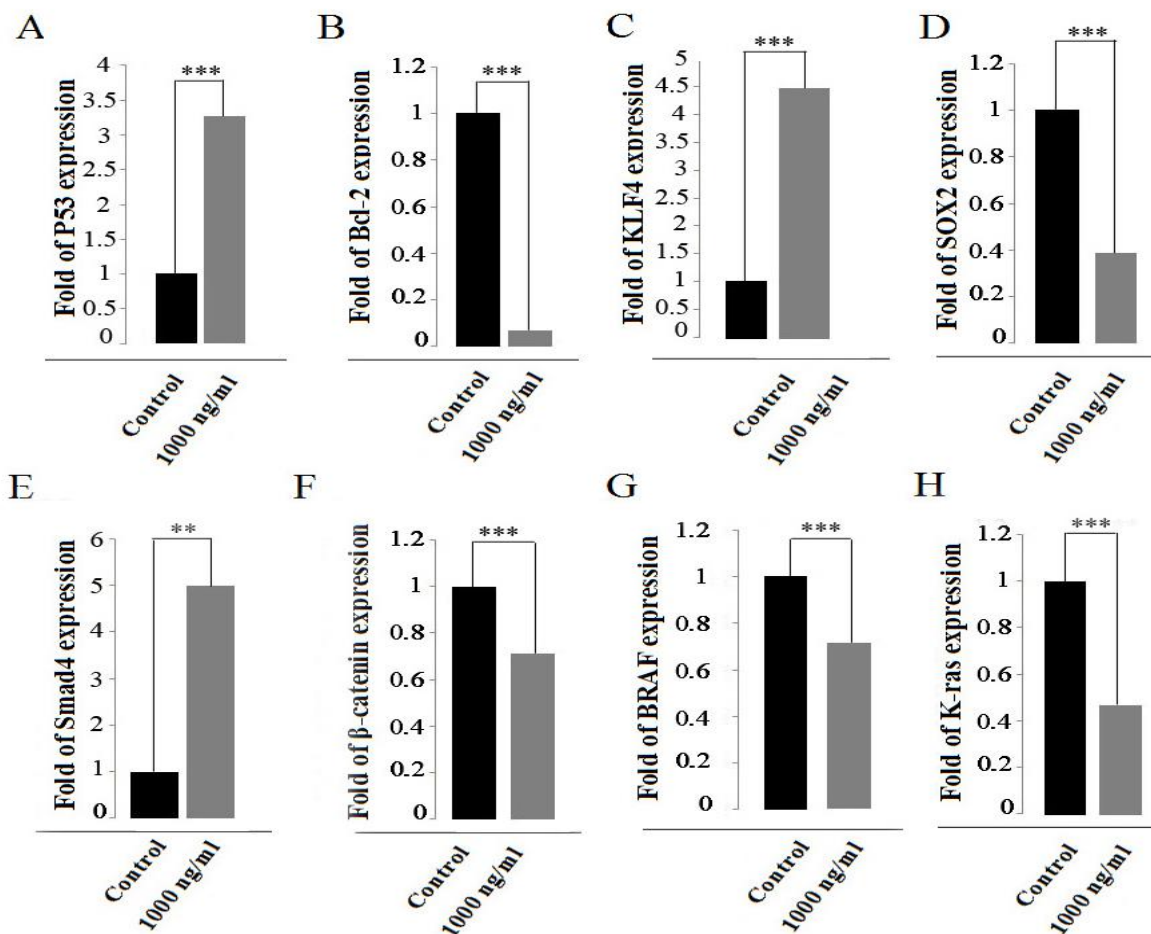
SMAD4 gene expression - tumor suppressor- was significantly increased 5 fold of expression in treated cells (Figure2E) and  $\beta$ -catenin gene expression – proto oncogene gene - was significantly decreased to 0.6 fold of expression in treated cells (Figure 2F).

BRAF gene expression – proto-oncogene -was significantly decreased to 0.7 fold of expression in treated cells (Figure2G) and K-ras gene expression – the most common mutated gene in ras family- was significantly decreased to 0.4 fold of expression in treated cells (Figure 2H).

**Discussion**

Actinomycets, especially *Streptomyces* sp., the most important source for bioactive compounds are gram positive bacteria found in fresh water, plants surface, marine and terrestrial environments. The exploration of new bioactive compounds has led to the discovery of a new strain which can produce novel useful bioactive compounds (38, 47, 48). In this study, we focused on anti-cancer activity of diethyl ether extracted compounds of *Streptomyces Levis* ABRIINW111 on colon cancer. We showed that diethyl ether extracted compounds have an effect on Bcl-2, P53, SOX2, KLF4,  $\beta$ -catenin, Smad4, K-ras and BRAF genes expression. P53 is a tumor suppressor and by controlling cell cycle progression, apoptosis and by inhibiting angiogenesis is able to maintain genomic stability.

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**Figure 2.** Effect of *S. levis* ABRIINW111 secondary metabolites on gene expression of p53 (\*\*P<0.0001) (A), Bcl-2 (\*\*P<0.0001) (B), KLF4 (\*\*P<0.0001) (C), SOX2 (\*\*P<0.0001) (D) SMAD4 (\*\*P<0.001) (E), β-catenin (\*\*P<0.0001) (F), BRAF (\*\*P<0.001) (G), K-ras (\*\*P<0.0001) (H) gene expression.

Also, studies revealed that the Bcl-2 family control the apoptosis by activation of Bax or inhibition of Bcl-2. P53 expression can inhibit Bcl-2 and Bcl-XL expression (13, 49-51). Our result showed that over expression of P53 in treated colon cancer cells with extracted metabolites could downregulate Bcl-2 as an anti-apoptotic, so it could induce apoptosis in P53 dependent pathways.

SOX2 as a member of the SOX gene family is expressed in human colon cancer. High expression of SOX2 is correlated with a poor prognosis, relapse, and lower survival of patients with colon cancer (52, 53). In the other hand, studies reported that Klf4 as a tumor suppressor plays key roles during the differentiation, proliferation and apoptosis (54-59).

There is strong evidence that in colonic adenomas and carcinomas reduction of protein and mRNA level of Klf4 is observed in comparison with normal colonic tissues (60). Our result showed that after 48 h, extracted metabolites could decrease the expression of SOX2, whereas the fold expression of KLF4 was increased.

All of the tumors exhibited increased β-catenin protein compared with normal tissues. It was demonstrated that with the stimulation of epithelial cells through epidermal growth factor (EGF), β- and γ-catenin become tyrosine-phosphorylated. Additionally, a direct association of β-catenin with the EGF-receptor (EGF-R) was shown in vitro (61).

TGF- $\beta$  signaling pathway transmits growth inhibitory signals from the cell surface to the nucleus and Smad4/Dpc4 is a key element of TGF- $\beta$  signaling pathway. Mutations in SMAD4 have been reported in human pancreatic and colorectal tumors (27, 28, 62). In this study, after 48 h treatment, fold expression of  $\beta$ -catenin was decreased and fold expression of SMAD4 was increased, significantly.

K-ras, a member of the RAS family of genes, is one of the most noticeable proto-oncogenes in colon cancer. The activated K-ras activates BRAF as a primary downstream target protein. BRAF, serine- threonine protein kinase, acts as a mediator of the K-ras signal toward the downstream effectors such as Mitogen-activated protein (MAP) to increase cell proliferation. Thus, alteration of K-ras seems to promote colon-cancer formation (63, 64).

Here we showed that K-ras and BRAF fold expression were decreased in colon cancer cells treated with extracted metabolites. These findings show that the crude extracted metabolites have anti-proliferative activity and can inhibit cancer cells proliferation.

In summary, we have demonstrated that diethyl ether extracted metabolites of *Streptomyces Levis* ABRIINW111 have anti-carcinogenic effects on colon cancer and can alter anti-apoptotic, pro-apoptotic and oncogenes genes expression in treated cells. Also, extracted metabolites as natural products can be a good choice for cancer therapy but more studies are required to characterize the exact structure of metabolites and validate the clinical significance of our findings.

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## Conflict of interests

The authors declare that they have no conflict of interest.

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