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# Research Article
# Cytotoxicity of n-Butanol Extracts of *Streptomyces* Against Human Breast Cancer Cells

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## Abstract

**Background and Objectives:** Breast cancer is the second most common cancer in the world and the most frequent cancer among women. This study was conducted to investigate the anticancer activities of n-butanol extracts prepared from *Streptomyces* isolated from soils in Jordan against breast cancer MCF7 cells. **Materials and Methods:** After isolation and identification of *Streptomyces* isolates by conventional methods, n-butanol extracts were prepared from *Streptomyces* cultures. Hemolytic activity of extracts was determined. The cytotoxic effect of non-hemolytic extracts on normal MCF10A cells was evaluated by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) assay. The *in vitro* and *in vivo* selective cytotoxicity of extracts against breast cancer was estimated. The mechanism of action of extracts that exhibited *in vivo* cytotoxicity was determined. Isolates that gave *in vivo* cytotoxicity were identified by PCR amplification and sequencing. The $IC_{50}$ values of extracts were determined by non-linear regression analysis. For tumor volume inhibition ratio, one-way ANOVA was applied for statistical evaluation of data and significant differences were considered significant at $p<0.05$. **Results:** The white aerial mycelia and the production of Rectus-Flexibilis (RF) sporophores as well as soluble pigments were the most common among *Streptomyces* isolates that screened from soil samples. The non-hemolytic n-butanol crude extracts of *Streptomyces* isolates (48 isolates) were screened for their cytotoxicity against normal breast MCF10A cells. Results indicated that out of the 23 non-toxic extracts on MCF10A cells, extracts of 9 isolates showed selective *in vitro* cytotoxicity against breast cancer MCF7 cells with $IC_{50}$ values ranged from 0.68-1.64 mg $mL^{-1}$. It was found that *in vivo* inhibition of breast cancer tumor in experimental animals was significantly increased, at $\alpha = 0.05$, after treatment with extracts of 3 *Streptomyces* isolates (S7, S17 and S61). The DNA laddering (apoptosis feature) was observed in MCF7 cells treated with extracts of isolates S7 and S61. Analysis of 16S-23S rRNA gene sequence revealed that those 3 isolates have maximal identity to the genus *Streptomyces*. **Conclusion:** The result of the current study suggests that n-butanol extracts of 3 *Streptomyces* isolates have selective cytotoxicity against breast cancer MCF7 cells and 2 of the extracts induce apoptotic property in MCF7 cells.

Key words: *Streptomyces*, n-butanol, cytotoxicity, breast cancer, 16S rRNA

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Data Availability: All relevant data are within the paper and its supporting information files.

## INTRODUCTION

Cancer is undoubtedly a serious and potentially life-threatening illness. It was reported that breast cancer is the second most common cancer in the world and the most frequent cancer among women with an estimated 1.67 million new cancer cases diagnosed in 2012[1]. This represents about 12% of all new cancer cases and 25% of all cancers in women. Breast cancer ranks as the fifth cause of death from cancer overall (522,000 deaths). In Jordan, cancer is the second most frequent cause of death after heart disease[2]. It was responsible for the death of more than 10,000 Jordanians from 1996 till now. A total of 8744 new cancer cases were registered by Jordan Cancer Registry (JCR) in 2013, of these, 5416 cases (61.9%) were among Jordanians[3]. Out of new cases of cancer recorded amongst Jordanians in the 2013, 2852 cases (52.6%) were females. Breast cancer was the most common among Jordanian females, with 1040 diagnoses in 2013 (36.5% of all cancers in women)[3].

Natural products were and are still the main reservoir to investigate in search for remedies for various afflictions and cancer treatment is no exception[4-7]. Spread of cancer among human populations raises the demand to find an urgent and a safe therapy for this disease. However, efforts dedicated to screening of microbial natural products for cancer therapy is very small compared to other therapeutic natural products.

*Streptomyces* is an interesting Gram-positive soil bacterium and it is unusual among bacteria in having a complex developmental life cycle involving several morphologically distinct cell types: Spherical spore, branching hyphae that form a mycelium and aerial structures that turn into chains of spores. *Streptomyces* species are of great industrial importance because of their ability to produce many clinically useful antibiotics[8] and has been the subject of many studies in the quest for novel antibiotics[9].

It was found that several compounds isolated from *Streptomyces* were able to inhibit tumor growth. For example, Borrelidin which was isolated from *S. rochei* serves as an efficient inhibitor of lung metastasis of B16-BL6 melanoma cells[10]. Furthermore, Geldanamycin compounds isolated from *S. hygroscopicus* PNK1-3 showed strong cytotoxicity against human epidermoid carcinoma cell lines of nasopharynx and breast cancer cell lines[11]. Also, Vijayabharathi *et al.*[12] reported that *Sterptomyces* showed anticancer activity against HepG2 (hepatic carcinoma) and HeLa (cervical carcinoma) *in vitro*. In addition, Yip *et al.*[13] isolated a purified fraction from a novel strain of *Streptomyces* induced an anti-proliferative effect in MCF7 and MDA-MB-231 breast cancer cells.

It is observed that all conventional medical treatments for cancer until now is hopeless. Therefore, alternative techniques are needed to be developed against cancer. All the efforts are dedicated to screening microbial natural products for cancer therapy are incomplete. For this reason, this research aims to examine the anticancer activities of *Streptomyces* isolates for the possible use to improve means for current cancer therapy. This study could be useful for scientists, pharmacists and doctors as they work to find new alternative therapies for cancer problem. Having a clearer understanding of how microbial metabolites may help to reconsider the current conventional research methods which are used for exploring new anticancer agents from natural sources.

## MATERIALS AND METHODS

**Collection of samples and isolation of *Streptomyces*:** A total of 150 soil samples were collected in summer 2015 from 12 locations of Jordan representing various habitats; including, Red Sea shore, Shoaib Valley, Dead Sea shore, Yarmouk river margins, Jordan river margins and Jordan desert. The bacterium *Streptomyces* was isolated according to the methods of Taddei *et al.*[14] and Dastager *et al.*[15] using Starch Casein Nitrate Agar (SCNA) medium supplemented with antifungal agents (50 mg $L^{-1}$ cyclohexamide and 50 mg $L^{-1}$ Nystatin). Inoculated SCNA plates with diluted soil were incubated at 30°C up to 3 weeks. After incubation, typically pigmented dry powdery colonies were selected from mixed plate culture and subcultured on new SCNA plates and incubated at 30°C for 3 weeks. The mass color of mature sporulating aerial mycelium and the distinctive colors of the substrate (reverse) mycelium were determined. The production of soluble pigments and the sporophore shapes were also recorded after growth on SCNA plates.

***Streptomyces* crude extracts preparation:** Streptomyces cultures, prepared in 250 mL Tryptone Soy Broth (TSB) and incubated at 30°C for 3 weeks, were centrifuged at 13,000 rpm for 10 min. The supernatant was extracted with an equal volume of n-butanol. Then, the extracts were filtered through 0.45 µm membrane syringe filter. The filtrated extracts were evaporated at 40°C in water bath. After evaporation, the remained residues were resuspended in Phosphate Buffer Saline (PBS) to achieve a concentration of 200 mg $mL^{-1}$ concentration and used for testing the hemolytic and anticancer activities.

**Hemolytic activity:** Human erythrocytes were freshly prepared and used to determine the hemolytic activity of

bacterial crude extracts. Hemolytic activity was tested on blood agar medium, composed from normal human erythrocytes (5%), by inoculating 50 µL of bacterial crude extract into each well (5 mm i.d.) prepared on blood agar plates. The type of hemolysis was determined after incubation of plates at 37°C for 48 h[16].

**Cells and culture conditions:** Two human cell lines, including; MCF10A (non-tumorigenic human breast epithelial cell line) and MCF7 (human breast adenocarcinoma cell line) were kindly supplied from Dr. Saeid Ismaeil, Faculty of Medicine/University of Jordan and used in this study. The human cell line MCF7 was used to investigate the anticancer activities of bacterial crude extracts. Whereas, MCF10A cells were used to determine the selectivity of anticancer activity produced from bacterial extracts against breast cancer MCF7 cells.

The adherent human breast cancer cell line MCF7 was grown in Dulbecco's Modified Eagle's Medium (DMEM)[17], pH 7.4, supplemented with 10% Feotal Bovine Serum (FBS), 40 µg $mL^{-1}$ gentamicin, 50 µM 2-mercaptoethanol, 10 mM N-2-hydroxyethylpiperzine-N-2-ethane sulfonic acid (HEPES), 1 mM sodium pyruvate, 100 U $mL^{-1}$ penicillin, 100 µg $mL^{-1}$ streptomycin and 2 mM L-glutamine and then they were harvested at ~70% confluence and subcultured every 48 h at 37°C in a humidified 5% $CO_2$ incubator[17]. To harvest the adherent MCF7 cells, growth medium was removed and cells were washed with PBS. To produce a cellular suspension, a cell dissociation solution made of trypsin-EDTA (1X) was added and incubated at 37°C for 5 min in a humidified 5% $CO_2$ incubator. Trypsinized cells were reseeded in fresh medium at ~$10^5$ cells $mL^{-1}$ and incubated at 37°C in a humidified 5% $CO_2$ incubator.

The adherent MCF10A cells were grown in DMEM/F12 media, pH 7.4, supplemented with 5% horse serum, 20 ng $mL^{-1}$ Epidermal Growth Factor (EGF), 10 µg $mL^{-1}$ insulin, 100 µg $mL^{-1}$ hydrocortisone, 10 ng $mL^{-1}$ cholera toxin, 100 U $mL^{-1}$ penicillin and 100 µg $mL^{-1}$ streptomycin. The cells were harvested, trypsinized and reseeded in the same manner as for MCF7 cells.

***in vitro* cytotoxicity:** For testing cytotoxicity against normal MCF10A cells, 200 µL of non-hemolytic bacterial crude extract (5 mg $mL^{-1}$ constant concentration; i.e. 2 mg $well^{-1}$) was added to 200 µL of harvested MCF10A cells in fresh medium, mixed thoroughly by pipetting and 100 µL medium was loaded into each well of 96 well micro test plate to achieve 500 µL total volume. Cells were plated at a density $4\times10^4$ cells $well^{-1}$, counted by hemocytometer. Then, the 96 well micro test plates were incubated at 37°C in a humidified 5% $CO_2$ incubator for 48 h. At the end of incubation time, the viability of cells was assessed by a cell colorimetric proliferation test called 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT)[18-19]. The MTT assay was done 48 h after inoculation of the bacterial crude extract into cell suspension. Each well of the 96 well microtest plate received 40 µL (50 µg) of MTT and incubated at 37°C for 2-4 h in a humidified 5% $CO_2$ incubator. After that, 100 µL of dimethyl sulfoxide (DMSO) was added. The optical densities were measured at 450 nm with 630 nm reference wavelength using ELISA microplate reader. Each treatment was performed in triplicate and repeated 5 times. The survival rate was determined by comparing the average of absorbance values with that in the control without extracts. The 50% inhibitory concentration ($IC_{50}$) was also determined.

To determine the selective anticancer activity of non-hemolytic crude extracts against breast cancer MCF7 cells, the cytotoxicity assay was performed in the same manner as for MCF10A cells using bacterial extracts that did not exhibit cytotoxicity against MCF10A cells.

***in vivo* cytotoxicity:** A total of 30 female rats were used for each bacterial n-butanol extract that exhibited a non-hemolytic selective cytotoxicity against MCF-7 cells (i.e. cytotoxic against MCF7 cells but not against MCF10A cells). All rats were divided into 3 groups of 10 rats each. Rats in Groups I and II were induced mammary carcinogenesis by providing single subcutaneous injection in right pectoral area of 25 mg 7,12-Dimethylbenzanthracene (DMBA) in 1 mL emulsion of 0.75 mL oil and 0.25 mL normal saline to each rat[20]. During the experimental period, animals were observed daily and weighed weekly to assess their general health.

After DMBA administration, right pectoral area of all rats were followed up for the tumoral development. Palpation of mammary tumors began 1 month after animals received DMBA. The volume of every tumor was measured weekly using calipers. After 2 months of the breast cancer development, treatments with bacterial extract was started in group II (Treatment Group). In Group I: control animals received no treatment. In Groups II and III, animals were given extract (100 mg $kg^{-1}$, b.wt.) daily through an oral gavage. Groups III animals had been used as control group for the side effects of crude treatment. After 1 month of treatment, rats were sacrificed. The tumor volume inhibition ratio was calculated.

**DNA extraction and measurement of apoptosis:** DNA was extracted from breast cancer MCF7 cells as well as from MCF7 cells treated with different concentrations (100-1000 mg with

100 unit interval) of each promising bacterial extract which had *in vivo* cytotoxicity after 48 h of incubation. Cells were incubated with proteinase-K for 30 min at 37°C. Thereafter, they were washed twice with PBS and then lysed in cold lysis solution (5 m mol $L^{-1}$ of Tris, pH 7.4, 20 m mol $L^{-1}$ of EDTA, 0.5% (v/v) Triton X 100) for 20 min. Cell lysates were centrifuged at 14,000 rpm for 15 min and DNA was extracted from the aqueous phase with phenol: chloroform: isoamyl alcohol (25:24:1 (v:v:v)) as described previously[21]. DNA was precipitated with sodium acetate and 2 volumes of cold absolute ethanol. The extracted genomic DNA was electrophresized in 2% agarose gel at 60 V for 1 h using 0.5 X TBE as running buffer. After electrophoresis was completed, genomic DNA was visualized and photographed by UV Transillumination to determine the degree of apoptotic DNA fragmentation.

**Molecular characterization of cytotoxic *Streptomyces* isolates:** The DNA was extracted from *Streptomyces* isolates that their n-butanol extracts exhibited both *in vitro* and *in vivo* cytotoxicities according to the method of Perez-Roth *et al.*[22]. After that, amplification of the interspacer region16S-23S rDNA with GP1 and GP2 primers was carried out in a DNA Thermal Cycler for 35 reaction cycles as described previously[23]. The PCR products were analyzed by electrophoresis using 10 µL of each PCR sample with 2 µL of loading buffer loaded onto a 1% agarose gel. A 500 bp DNA ladder marker (Genedirex, USA) was used to estimate the approximate molecular weight of the amplified products (the predicted size is 300-400 bp). Generated bands were digitally photographed under UV light.

The sequences of the interspacer region 16S-23S rRNA gene from PCR products of *Streptomyces* isolates were determined with an Applied Biosystems model 373A DNA sequencer by using the ABI PRISM cycle sequencing kit (Macrogen, Korea). The sequences were compared with those contained within GenBank[24] by using a BLAST search[25]. Furthermore, the accession number for each sequence was kindly provided after submition to GenBank. The most closely related 16S-23S rRNA gene sequences to the isolates of this study were retrieved from the database.

**Statistical analysis of cytotoxicity:** To calculate inhibition percentage depending on the results obtained from MTT *in vitro* viability assay, the obtained absorbance values were corrected by subtracting the average absorbance of blank (medium + MTT) from average vehicle (medium + cells + MTT) absorbance and from average treatment (medium + cells + extract + MTT) absorbance. To calculate inhibition percentage of cells, the following formula was used:

$$\text{Inhibition } (\%) = \frac{A - B}{A} \times 100$$

where, A is the vehicle absorbance at 450 nm and B is the treatment absorbance at 450 nm[26].

The 50% inhibitory concentration ($IC_{50}$) was determined by comparing the average of *in vitro* mortality values of 5 bacterial extract concentrations (1.25, 2.5, 5, 7.5 and 10 mg $mL^{-1}$; i.e. 0.25, 0.5, 1, 1.5 and 2 mg $well^{-1}$, respectively) with that in the control without extract. The $IC_{50}$ values, regression equations and correlation coefficients ($R^2$) were determined by non-linear regression analysis (MS Excell, Microsoft Co., 2010). Each treatment was achieved in triplicate.

For *in vivo* study, the tumor volume inhibition ratio (%) was calculated by the following formula:

$$\text{Inhibition } (\%) = \frac{A - B}{A} \times 100$$

where, A is the average tumor volume of the control group (Group I) and B is the average tumor volume of the treated group (Group II)[26]. All data were expressed as the Mean±Standard Deviation (SD).

**Statistical analysis of in vivo tumor inhibition:** For statistical evaluation of data for tumor volume inhibition ratio, one-way ANOVA (Tukey's studentized range) was applied using the program IBM SPSS statistics 19.0 for Windows[27]. Significant differences were considered significant at $p<0.05$.

## RESULTS

**Phenotypic and microscopic characterization of isolates:** It was found that 127 bacterial colonies isolated from the screened soil samples were belonged to the genus *Streptomyces* (Table 1). *Streptomyces* isolates were classified

Table 1: Isolation of *Streptomyces* from Jordanian soils

| Location | No. of samples | No. of bacterial colonies (No. of *Streptomyces*) |
|---|---|---|
| Ajloun | 14 | 44 (9) |
| Amman | 19 | 93 (19) |
| Aqaba | 12 | 47 (9) |
| Balqa | 25 | 106 (25) |
| Irbid | 25 | 113 (34) |
| Jerash | 8 | 32 (8) |
| Karak | 7 | 17 (4) |
| Ma'an | 8 | 18 (3) |
| Madaba | 10 | 29 (7) |
| Mafraq | 7 | 25 (2) |
| Tafilah | 8 | 24 (5) |
| Zarqa | 7 | 26 (2) |
| Total | 150 | 574 (127) |

Table 2: Classification of *Streptomyces* isolates based on morphological and cultural characteristics

| | Color series of aerial mycelium | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| *Streptomyces* isolates | Black | Brown | Gray | Green | Orange | Pink | Red | White | Yellow | Total (%) |
| No. of isolates | 2 | 9 | 17 | 38 | 3 | 2 | 2 | 49 | 5 | 127 |
| Pigment production | | | | | | | | | | |
| Melanin | 2 | 9 | 5 | 10 | 0 | 0 | 0 | 16 | 2 | 44 (34.65) |
| Substrate | 2 | 9 | 5 | 38 | 0 | 0 | 2 | 16 | 5 | 77 (60.63) |
| Soluble | 2 | 9 | 14 | 38 | 0 | 0 | 1 | 36 | 5 | 105 (82.68) |
| *Sporophore morphology* | | | | | | | | | | |
| Rectus-Flexibilis | 2 | 4 | 11 | 34 | 2 | 0 | 2 | 34 | 5 | 94 (74.02) |
| Monoverticillate | 0 | 2 | 2 | 2 | 0 | 2 | 0 | 10 | 0 | 18 (14.17) |
| Biverticillate | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 4 (3.15) |
| Spiral | 0 | 1 | 4 | 2 | 1 | 0 | 0 | 3 | 0 | 11 (8.66) |

Table 3: Hemolytic activity of n-butanol extracts of *Streptomyces* isolates against human erythrocytes

| | Type of hemolysis[a] | | |
|---|---|---|---|
| Color series (Aerial Mycelium) | α | β | γ |
| Black | 1 | 0 | 1 |
| Brown | 2 | 2 | 5 |
| Gray | 5 | 7 | 5 |
| Green | 7 | 14 | 17 |
| Orange | 0 | 2 | 1 |
| Pink | 0 | 1 | 1 |
| Red | 0 | 1 | 1 |
| White | 11 | 23 | 15 |
| Yellow | 2 | 1 | 2 |
| Total | 28 | 51 | 48 |

[a]Type of hemolysis: β; complete hemolysis, α: Partial hemolysis, γ: No hemolysis

according to the color of aerial mycelium into 9 color series (Table 2). The white color series were the most common among the isolates followed by the green color series. Production of melanin, substrate and soluble pigments was observed (Table 2). It was found thta 44 isolates were melanin producers, 77 isolates were able to produce substrate pigments and most of the isolates (82.68%) gave soluble pigments. The isolates that displayed orange and pink aerial mycelia did not produce substrate and soluble pigments. According to the shape of sporophores, the isolates were grouped into four groups (Rectus-Flexibilis (RF), Monoverticillate (MV), Biverticillate (BIV) and Spiral (S)) (Table 2). About three fourths of isolates (74%) produced RF sporophores. Whereas, it was found that only 2 isolates produced BIV sporophores.

**Hemolytic activities:** The *Streptomyces* crude extracts were tested for their hemolytic activity against human erythrocytes (Table 3). About one-third of the isolates (48 isolates; 37.8%) were non-hemolytic (γ-type). The remaining 79 isolates exhibited either partial hemolysis (α-type) or complete hemolysis (β-type).

***In vitro* cytotoxicity:** Non-hemolytic *Streptomyces* isolates (48 isolates) were screened for their cytotoxicity against normal MCF10A cell line (Table 4). To determine the degree of cytotoxicity of *Streptomyces* extracts, MTT assay was used. As illustrated in Table 4, 23 non-hemolytic extracts exhibited no (-) to low (+) cytotoxic activity against normal MCF10A cells. Whereas, the remaining non-hemolytic extracts were found to exhibit cytotoxicity against MCF10A cells ranging from moderate (++) to very high (++++). All extracts of isolates that displayed black, brown and pink aerial mycelia color were cytotoxic to MCF10A cells.

The 23 *Streptomyces* extracts that showed no to low cytotoxicities against MCF10A cells were screened for their selective cytotoxicity against breast cancer MCF7 cells (Table 5). It was found that 14 extracts produced no to low cytotoxicity against MCF7 cells. Whereas, 9 extracts, which had no to low cytotoxic effects against MCF10A cells, showed high to very high selective cytotoxicity against MCF7 cells. These selective isolates distributed in three color series including gray (3 isolates), green (2 isolates) and white (4 isolates) color series (Table 5). Seven of them produced RF sporophores (Table 6), whereas isolates S20 and S46 produced MV and BIV sporophores, respectively.

As shown in Table 6, the $IC_{50}$ values of the 9 cytotoxic *Streptomyces* crude extracts against breast cancer MCF7 cells ranged from 0.68-1.64 mg $mL^{-1}$. It was observed that isolate S46, which belonged to white color series and produced BIV sporophores, showed the highest significant cytotoxic effect against MCF7 cells. According to the $IC_{50}$ values, MCF7 cells were more susceptible to the cytotoxic crude extracts of *Streptomyces* isolates that produced white aerial mycelia.

***in vivo* cytotoxicity:** When the tumor volume reached to about 200 $mm^3$, treatment with nine *Streptomyces* crude extracts, which had *in vitro* selective cytotoxic activities against breast cancer MCF7 cells, started in Group II of rats. Control group (Group I) received no treatment. The tumor volume inhibition ratio was calculated for each crude. As shown in Table 7, only crude extracts of three *Streptomyces* isolates (S7, S17 and S61) appeared to induce significant

Table 4: *In vitro* cytotoxicity of non-hemolytic n-butanol extracts of *Streptomyces* isolates against normal human breast MCF10A cells

| | Cytotoxicity degree against MCF10A[a] | | | | | |
|---|---|---|---|---|---|---|
| Color series (Aerial Mycelium) | - | ± | + | ++ | +++ | ++++ |
| Black | 0 | 0 | 0 | 1 | 0 | 0 |
| Brown | 0 | 0 | 0 | 3 | 1 | 1 |
| Gray | 1 | 1 | 1 | 2 | 0 | 0 |
| Green | 1 | 3 | 6 | 0 | 2 | 5 |
| Orange | 0 | 0 | 1 | 0 | 0 | 0 |
| Pink | 0 | 0 | 0 | 0 | 0 | 1 |
| Red | 0 | 0 | 1 | 0 | 0 | 0 |
| White | 5 | 0 | 2 | 0 | 1 | 7 |
| Yellow | 1 | 0 | 0 | 0 | 1 | 0 |
| Total | 8 | 4 | 11 | 6 | 5 | 14 |

[a]Degree of cytotoxicity was graded on the basis of the relative value of absorbance to the vehicle: ++++, very high (<0.1), +++: High (0.1-<0.4), ++: Moderate (0.4-<0.7), +: Low (0.7-<0.9), ±: Very low (0.9-<0.95), -: Non-toxic (≥0.95)

Table 5: *In vitro* selective cytotoxicity of non-hemolytic n-butanol extracts of *Streptomyces* isolates against human breast cancer MCF7 cells

| Color series | Cytotoxicity Degree against MCF7[a] | | | | | |
|---|---|---|---|---|---|---|
| (Aerial Mycelium) | - | ± | + | ++ | +++ | ++++ |
| Black | 0 | 0 | 0 | 0 | 0 | 0 |
| Brown | 0 | 0 | 0 | 0 | 0 | 0 |
| Gray | 0 | 0 | 0 | 0 | 3 | 0 |
| Green | 0 | 2 | 6 | 0 | 2 | 0 |
| Orange | 0 | 0 | 1 | 0 | 0 | 0 |
| Pink | 0 | 0 | 0 | 0 | 0 | 0 |
| Red | 0 | 1 | 0 | 0 | 0 | 0 |
| White | 1 | 1 | 1 | 0 | 1 | 3 |
| Yellow | 0 | 0 | 1 | 0 | 0 | 0 |
| Total | 1 | 4 | 9 | 0 | 6 | 3 |

[a]Isolates that exhibited no to low cytotoxicity against normal breast MCF10A cells were examined for their cytotoxicity against breast cancer MCF7 cells. The degree of cytotoxicity was graded on the basis of the relative value of absorbance to the vehicle: ++++: Very high (<0.1); +++: High (0.1-<0.4); ++: Moderate (0.4-<0.7); +: Low (0.7-<0.9); ±: Very low (0.9-<0.95), -: Non-toxic (≥0.95)

Table 6: Median inhibitory concentration of selectively cytotoxic n-butanol extracts of *Streptomyces* isolates against breast cancer MCF7 cells

| | | pigments | | | |
|---|---|---|---|---|---|
| Isolate | Color series | Substrate | Soluble | Sporophore morphology[a] | IC50[b] (mg mL$^{-1}$) |
| S7 | Gray | - | Brown | RF-Straight | 1.21 (1.03-1.36) |
| S9 | Gray | - | Brown | RF-Fascicled | 1.06 (0.94-1.23) |
| S10 | Gray | - | Yellow | RF-Straight | 1.64 (1.38-1.90) |
| S17 | Green | Brown | Brown | RF-Flexous | 1.40 (1.27-1.56) |
| S20 | Green | Brown | Brown | MV | 1.34 (1.24-1.64) |
| S44 | White | - | Pink | RF-Flexous | 0.94 (0.83-1.14) |
| S46 | White | - | Pink | BIV | 0.68 (0.53-0.80) |
| S47 | White | - | Pink | RF-Fascicled | 1.48 (1.25-1.73) |
| S61 | White | - | Orange | RF-Fascicled | 0.94 (0.82-1.11) |

[a]RF: Rectus-Flexibilis, MV: Monoverticillate no spirals, BIV: Biverticillate no spirals, [b]$IC_{50}$: Median inhibitory concentration. Confidence limits for the mean are given in parentheses and they are expressed in terms of a confidence coefficient with 95% interval

Table 7: Tumor volume inhibition ratio for selectively cytotoxic n-butanol extracts of *Streptomyces* isolates

| | Tumor volume inhibition ratio[a] (%) after (weeks) | | | | |
|---|---|---|---|---|---|
| Isolate | 0 | 1 | 2 | 3 | 4 |
| S7 | 0$^{a}$ | 18.3±2.1$^{b}$ | 26.1±4.3$^{c}$ | 32.4±3.7$^{c}$ | 43.5±5.1$^{d}$ |
| S9 | 0$^{a}$ | 6.7±2.9$^{b}$ | 8.1±3.2$^{b}$ | 8.5±3.0$^{b}$ | 9.3±3.2$^{b}$ |
| S10 | 0$^{a}$ | 0$^{a}$ | 2.1±1.6$^{b}$ | 4.3±2.2$^{bc}$ | 6.2±1.9$^{c}$ |
| S17 | 0$^{a}$ | 16.7±1.8$^{b}$ | 31.3±3.9$^{c}$ | 40.3±4.6$^{d}$ | 61.2±6.8$^{e}$ |
| S20 | 0$^{a}$ | 0$^{a}$ | 0$^{a}$ | 2.1±1.7$^{b}$ | 5.5±3.5$^{b}$ |
| S44 | 0$^{a}$ | 1.3±0.4$^{b}$ | 3.8±2.1$^{bc}$ | 7.2±4.1$^{cd}$ | 9.9±5.1$^{d}$ |
| S46 | 0$^{a}$ | 0$^{a}$ | 0$^{a}$ | 2.9±1.1$^{b}$ | 5.6±2.9$^{b}$ |
| S47 | 0$^{a}$ | 0$^{a}$ | 0$^{a}$ | 3.2±0.9$^{b}$ | 4.1±1.7$^{b}$ |
| S61 | 0$^{a}$ | 14.3±2.6$^{b}$ | 20.1±5.3$^{bc}$ | 27.9±3.9$^{c}$ | 39.8±4.2$^{d}$ |

[a]Inhibtion ratio was represented as Means±SD. Means±SD within column followed by the same letter are not significantly different (Tukey's studentized range test: $\alpha = 0.05$)

Table 8: Molecular characterization of *in vivo* cytotoxic *Streptomyces* isolates

| Isolate | Sequence: GenBank Accession No. | Sequence: No. of nucleotides[a] | Closest phylogenetic relative[b] | Score | E-value | Gaps (%) | Identity (%)[c] |
|---|---|---|---|---|---|---|---|
| S7 | KY711432 | 430 | *S. fulvissimus* DSM 40593 (CP005080) | 278 | 6e-118 | 1 | 80 |
| S17 | KY711433 | 428 | *Streptomyces* sp. SirexAA-E (CP002993) | 268 | 6e-68 | 1 | 80 |
| S61 | KY711434 | 451 | *S. griseus* subsp. *griseus* NBRC 13350 (AP009493) | 435 | 1e-70 | 2 | 95 |

[a]Number of 16S-23S rRNA gene nucleotides used for the alignment, [b]GenBank acession number was provided between parentheses, [c]Percentage identity with the 16S-23S rRNA gene sequence of the closest phylogenetic relative of *Streptomyces*

inhibition of breast cancer tumor in experimental animals. After 4 weeks of treatment, the mean tumor volume inhibition ratio of crudes of isolates S7, S17 and S61 in treated group (Group II) was 43.5±5.1, 61.2±6.8 and 39.8±4.2% in that order compared with the untreated animals in Group I (Table 7). Whereas, extracts of the remaining isolates showed weak inhibitory effect (less than 10% mean tumor volume inhibition). No signs of toxicity (weight loss, ruffled fur and behavioral changes) were observed in any of the treated animals in Group II compared with that in Group III.

**Mechanism of action:** Agarose gel electrophoresis was performed to detect if the cytotoxic *Streptomyces* crude extracts induced DNA laddering (apoptotic feature) or DNA smearing (necrotic feature). DNA laddering was observed in response to the treatment of MCF7 cells with n-butanol extract prepared from *Streptomyces* isolates S7 and S61 (Fig. 1) suggesting that these extacts induced apoptosis in breast cancer MCF7 cells. Whereas, the cytotoxic extracts of the remaining *Streptomyces* isolate (S17) at different concentrations did not induce apoptotic effect.

**Molecular characterization of isolates:** Based on conventional classification, it was found that isolate S7 produced gray aerial mycelia, RF-Straight sporophores and brown soluble pigment, isolate S17 produced green aerial mycelia, RF-Flexous sporophores and brown substrate and soluble pigments and isolate S61 produced white aerial mycelia, RF-Fascicled sporophores and orange soluble pigment (Table 6). To confirm the classification of *Streptomyces* isolates that exhibited *in vitro* and *in vivo* cytotoxicities against MCF7 cells, genomic DNA was extracted from S7, S17 and S61 isolates. The 16S-23S rRNA gene sequence was analyzed by amplification with GP1 and GP2 primers. The amplified genomic DNA of the isolates produced a single PCR band of about 500 bp in size (Fig. 2). The obtained 16S-23S rRNA gene sequences were aligned by BLAST alignment of GenBank sequences. Based on BLAST alignment, the three isolates were allocated to the phylum Actinobacteria that contains the genus *Streptomyces* with 80-95% identity (Table 8). Moreover, the

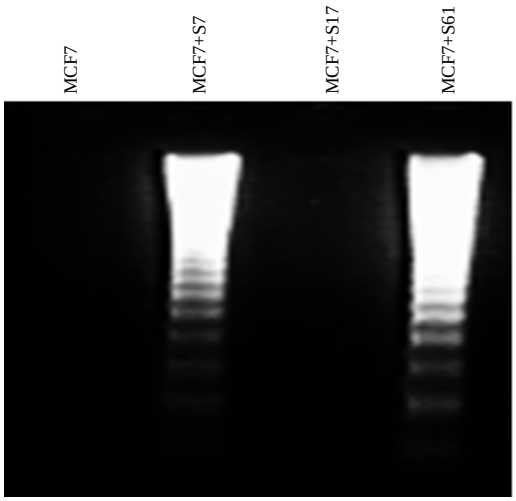


Fig. 1: Agarose gel (2%) electrophoresis of DNA fragmentation after exposure of MCF7 cells to n-butanol extracts of *Streptomyces* isolates S7, S17 and S61. A slight fragmentation of DNA was demonstrated after treatment of MCF7 cells with S7 and S61 extracts

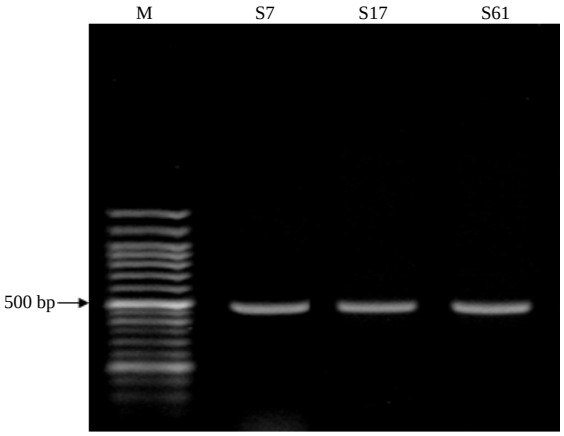


Fig. 2: Agarose gel (1%) electrophoresis of PCR amplification of 16S-23S rRNA gene fragments with forward GP1 and reverse GP2 primers of *Streptomyces* isolates S7, S17 and S61. Lane M: 50 bp DNA ladder marker (Genedirex, USA). Lanes of isolates S7, S17 and S61 show amplicons (about 500 bp) derived from 16S-23S rRNA gene

sequences were closely related to the 16S-23S rRNA gene sequence of *Streptomyces* retrieved from GenBank database. The sequences of the 3 isolates were submitted to GenBank and the GenBank accession numbers KY711432, KY711433 and KY711434 were assigned to the obtained sequences of *Streptomyces* isolates S7, S17 and S61, respectively.

## DISCUSSION

The current study was initiated to determine the anticancer activity of n-butanol extracts of Actinomycetes, in particular local *Streptomyces* isolates, against breast cancer MCF7 cells in attempt to find isolates with novel or promising anticancer activities. Actinomycetes are well known and successfully exploited as a source of secondary metabolites. There are over 23,000 known microbial secondary metabolites, 42% of which are produced by Actinobacteria, 42% by fungi and 16% by other bacteria[28]. Since the discovery of actinomycin, actinobacteria have been found to produce many commercially important bioactive compounds and antitumor agents[10] in addition to enzymes of industrial interest[29-30]. It has been estimated that approximately two-third of the thousands of naturally occurring antibiotics have been isolated from actinomycetes[31-32].

In the present study, the occurrence of local *Streptomyces* was investigated in the collected soil samples and 127 *Streptomyces* isolates were recovered. The isolates were classified based on the aerial mycelium color into 9 color series; isolates with white color series appeared the most common (49 isolates). This finding is in agreement with Msameh[33]. It was observed that majority of *Streptomyces* isolates were able to produce RF sporophores. This is in agreement with previous studies[34-36] which demonstrated that sporophores of RF-type were the most common among Jordanian isolates.

In earlier studies, several metabolites isolated from *Streptomyces* species exhibited anticancer activity. For example, retamycin assists in the treatment of human leukemia[37], migrastatin inhibits metastasis of several types of tumor cells[38-39], geldanamycin has strong cytotoxicity against human epidermoid carcinoma of nasopharynx and breast cancer[11] and borrelidin has an inhibitory effect on lung cancer metastasis[10]. Furthermore, chromomycin SA and 1-(1H-indol-3-yl)-propane-1,2,3-triol[40] as well as kosinostatin[41], isolated recently from *Streptomyces*, were found to show anticancer activity against MCF7 cells. In this study, it was found that certain non-hemolytic n-butanol extracts of *Streptomyces* isolates had selective cytotoxic activity against human breast cancer MCF7 cells. Out of the screened *Streptomyces* isolates in this work, non-hemolytic n-butanol extracts of nine *Streptomyces* isolates exhibited selective *in vitro* cytotoxicity against MCF7 cells. It was demonstrated that the cytotoxic extracts were obtained from isolates belonging to different phenotypic and microscopic characters. So, non-hemolytic and selective cytotoxic compounds can be found in a variety of *Streptomyces* species. The anticancer activity in cytotoxic isolates was not attributable to the induced hemolysis in view of the fact that they showed no hemolytic activity against human erythrocytes.

It was found that only 3 isolates (S7, S17 and S61) exhibited *in vivo* antitumor activity. Tumor volume inhibition ratio differences between the treatment and control groups were all significant and no signs of toxicity were observed in any of the treated animals. Moreover, it was observed that isolates S7 and S61 induced apoptosis in treated breast cancer MCF7 cells. Whereas, S17 isolate did not induce apoptosis in treated cells. Therefore, these 3 n-butanol extracts of *Streptomyces* isolates which exhibited selective *in vitro* cytotoxicity against breast cancer cells by discrimination between MCF7 cancer cells and normal MCF10A cells, killing the former cells specifically and produced a promising *in vivo* cytotoxicity might be used in the future in therapies of breast cancer.

Abraham[42] demonstrated that n-butanol extracts of *Streptomyces* gave cytotoxic activity on PC12 and HeLa cancer cell lines. Furthermore, it was revealed that n-butanol extracts of *Streptomyces* exhibited the highest antibacterial activity[43]. Therefore, n-butanol was chosen as the most efficient solvent for extracting the desired active compounds. However in this study, none of the n-butanol extracts of *Streptomyces* isolates that exhibited *in vitro* cytotoxicity against MCF7 cells gave antimicrobial activity against 11 test bacteria and 7 test fungi (data not shown). Therefore, the inhibitory effect of n-butanol extracts on MCF7 cells might be due to novel antitumor agents contained in the prepared extracts but not due to antimicrobial agents.

The preliminary identification of the 3 isolates that exhibited *in vivo* cytotoxicity against breast cancer MCF7 cells by conventional methods was further identified by comparing the 16S-23S rRNA gene sequences of the isolates with those in the GenBank. As a result, the sequences of the 3 isolates (S7, S17 and S61) showed sequence identity to the phylum Actinobacteria and close relatedness to the genus *Streptomyces* with 80-95% identity (Table 8). In general, identity below 97% 16S rRNA gene sequence of unknown isolate with nearest relative is indicative of novel species or new type strain. Nevertheless, the globally adapted cut-off value for genus *Streptomyces* is 83.5% 16S rRNA gene

sequence identity[44]. However, it was reported that the lowest interspecies 16S rRNA gene sequence similarity to the genus *Streptomyces* is 78%[45]. Therefore, these isolates can be assigned to the genus *Streptomyces*. Considering sequence analysis using 16S rRNA gene, it was revealed that the isolates could be allocated into different *Streptomyces* species. However, this needs to be further confirmed by fatty acid analysis, protein profiling, DNA hybridization and other techniques because in *Streptomyces* systematic sequences of only about 180 species (out of more than 570 species) are available and deposited in public databases[46].

It was reported that yellow soluble pigment produced from *Streptomyces* sp. SFA5 has inhibitory activity against breast cancer MCF7 cells[47]. It was observed that all *in vitro* cytotoxic *Streptomyces* isolates produced soluble pigments with various colors; brown from isolates S7, S9, S17 and S20, yellow from isolate S10, pink from isolates S44, S46 and S47 and orange from isolate S61 (Table 6). Therefore, *in vitro* cytotoxicity produced from n-butanol extracts of these isolates on MCF7 breast cancer cell line could be attributed to the produced soluble pigments.

The finding of this study strongly suggests the possible occurrence of some *Streptomyces* isolates in soils that naturally produce selectively cytotoxic agents against breast cancer cells. This may lead to the use of these agents for medicinal and pharmaceutical purposes including treatment of breast cancer and possibly other types of cancer. Although n-butanol extracts from *Streptomyces* bacterium exhibit potent *in vitro* and *in vivo* anticancer activity against breast cancer MCF7 cell line, further analysis using chromatography techniques such as GC, HPLC and HPTLC is required to determine the chemical structure of biologically active constituent found in crude extracts.

## CONCLUSION

This study demonstrated that there is a potent anticancer effect of n-butanol extracts prepared from *Streptomyces* on the growth of human breast cancer MCF7 cells. Moreover, significant tumor volume inhibition ratios of 3 *Streptomyces* extracts (2 of them have apoptotic effects) were revealed in the experimental animals. The results of this study illustrated that anticancer agents can be screened from natural microbial products and they be used in medical and pharmaceutical therapies of cancer.

## SIGNIFICANCE STATEMENT

This study showed that natural microbial byproducts can be used as an alternative source for screening of anticancer agents. Moreover, the results of this study will be useful in medical and pharmaceutical applications including development and/or improvement of current therapies used in treatment of breast cancer.

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