

Improvement the production of cytotoxic metabolites by *Streptomyces griseus* KJ623766

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ABSTRACT

The cell-free culture supernatant (CFCS) obtained from *Streptomyces (S.) griseus* (accession code KJ623766) fermentation, locally isolated *Streptomyces* strains from Egyptian soil sample, showed potent cytotoxic activity against *Caco2* cell line. Fermentation was carried out in a 14 L laboratory fermenter, under optimum conditions of 28 °C, 200 rpm, 5 standard liters per minute (SLPM) aeration, 2 bar airflow pressure and uncontrolled pH. After 72 h of incubation, the cell-free culture supernatant (CFCS) was collected and extracted using ethyl acetate (1:1, v/v) at pH 7.0. Using 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium-bromide (MTT) assay, the ethyl acetate extract showed potential cytotoxic activity against *Caco2* with CD_{50} of 14 $\mu\text{g/mL}$. This showed an increase in cytotoxic activity by about 1.6 folds when compared to results obtained from shake flask (CD_{50} 22 $\mu\text{g/mL}$). Production improvement of cytotoxic activity was carried out also by genetic manipulation using a dose of 4 KiloGray (KGy) of gamma radiation. Fifteen out of forty-seven mutants showed higher potential cytotoxic activities when compared to that of the wild-type strain of *S.griseus* KJ623766. Mutants G31, G44, and G45 showed the most potent cytotoxic activities where they exhibited about 7 folds increase in potential cytotoxic activity with CD_{50} of 3.2 ± 0.2 , 2.9 ± 0.1 and 3.25 ± 0.43 $\mu\text{g/mL}$, respectively.

Keywords: Bioassay-guided fractionation; cytotoxic activity; Fermentation; *Streptomyces griseus*; genetic manipulation; gamma radiation.

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1. INTRODUCTION

Cancer diseases are a major health problem in both developed and developing countries [1]. Its complexity is due to; it is not only caused by the abnormal growth of cells with the potentiality to invade different organs but also by an impaired differentiation [2]. The World Health Organization (WHO) reports that every year there are approximately 38 million new cases of non-communicable diseases (NCD) with cancer representing the second cause of NCD with 8.2 million deaths, corresponding to 22% of all NCD

in 2012 [3]. Colorectal cancer is the second highest cause of cancer occurrence and death for men and women in the United States combined. An estimated 141210 cases were diagnosed in 2011 [4].

Natural products have been the mainstay of cancer chemotherapy for the past 30 years as; there is a strong urgency to find new therapeutic strategies for the treatment of cancers, especially for those that show drug-resistance, high risk of relapse, unavailability and / or poor therapeutic strategies. For this reason, much attention is paid

to the therapeutic use of natural products, due to their high efficacy and low adverse effects [5]. These natural products include chemotherapeutic agents from plant, marine and bacterial sources[6].

Microorganisms have been the principal source of antibacterial agents and also provided some of the key drugs for cancer chemotherapy. To date, numerous bioactive compounds with profound impact on society have been reported from the genus *Streptomyces* whereby over 7000 bioactive compounds with diverse bioactivities including antimicrobial, antioxidant, anticancer and antifungal properties are identified from *Streptomyces* [7].

Bleomycin (Blenoxane), dactinomycin, mitomycin C, and the anthracyclines daunomycin and doxorubicin (Adriamycin) are notable drugs originating from Streptomycetes and used for cancer chemotherapy. All of these were introduced to the clinic before their modes of action had been determined [8].

Intensive approaches aimed at searching for more selective and novel structural agents have started to overcome the secondary effects of these compounds. so tissue culture microtiter-plate based screens were developed for the screening of novel microorganisms producing antitumor or cytotoxic agents [9]. Accordingly, this study aimed at the production of cytotoxic agents by *S. griseus* KJ623766 in shake flask, followed by extraction and determination of cytotoxic activity against *Caco2* cell line. Production improvement of cytotoxic activity by the recovered soil *Streptomyces* strain was carried out using; large-scale production in a laboratory fermenter and genetic manipulation by mutation using gamma radiation.

2. MATERIALS AND METHODS

2.1. Microorganisms

Locally isolated *Streptomyces* strain from Egyptian soil sample, *S. griseus* KJ623766, identified using 16S ribosomal RNA gene sequences (GenBank database submission access code KJ623766). The purified colonies of

Streptomyces were stored onto starch nitrate agar slants slant (soluble starch 10 gm, KNO₃ 2 gm, K₂HPO₄ 1 gm, NaCl 0.5 gm, MgSO₄.7H₂O 0.5 gm, CaCO₃ 3 gm, agar 20 gm, FeSO₄.7H₂O 0.1 gm, MnCl₂ 4H₂O 0.1 gm, ZnSO₄ 7H₂O 0.1 gm per 1liter of distilled water) and subcultured every month on the same medium.

For long-term preservation, the isolate was plated on the surface of starch nitrate agar for seven days of incubation at 28 °C. After incubation, the formed spores were scalped off, suspended in a stock medium (tryptone 10 gm, yeast extract 5 gm, glycerine 500 ml and distilled H₂O to 1 L) and stored at -20 °C

2.2. Cell line

Two cell lines were used, Kidney epithelial cells derived from the African green monkey (*Vero* cell line, ATCC No.CCL-81), Colorectal adenocarcinoma derived from human colon (*Caco2* cell line). These cell lines were obtained from VACSERA, Egypt.

Caco-2 cell line was grown in T-75 tissue culture flasks containing 20 mL of RPMI-1640 medium with 1% antibiotic-antimycotic solution and 10% fetal bovine serum. The medium was changed at 48-h intervals and cells dissociated with trypsin solution (0.25% in phosphate buffer saline). *Vero* cell line was propagated in Eagle minimum essential medium (EMEM) with Earl's balanced salt solution [10], supplemented with 10% Fetal bovine serum (FBS) and antibiotics (100 IU penicillin and 100 IU streptomycin /ml) solution every 48 h, and maintained in EMEM with Earl's balanced salt solution (EBSS) supplemented with 2% FBS and antibiotics solution.

2.3. Production of cytotoxic agent(s) by *S. griseus* KJ623766

2.3.1. Preparation of seed culture

From stock culture medium, an aliquot was spread on the surface of the starch nitrate agar plate, incubated for seven days at 28 °C. After incubation, spores were scalped from the agar surface and suspended in 5 mL of distilled water. After homogenization, 2 mL of the resulting

spore bacterial suspension was used to inoculate 100 mL of soybean meal broth (soybean 15 gm, glucose 15 gm, NaCl 5 gm, CaCO₃ 1 gm per 1 L of distilled water in 100 mL flask) (2% v/v) contained in 1 L flask. The flask was then incubated at 28 °C and 200 rpm for 72 h. The culture obtained (seed culture) was used to inoculate the production medium [11].

2.3.2. Production of cytotoxic agent(s) in shake flasks

An aliquot (50 mL) of seed culture obtained was used to inoculate 250 mL of soybean meal broth contained in 2 L flask. The inoculated flask was then incubated at 28 °C and 200 rpm for 72 h. The culture obtained was centrifuged at 6000 RPM for 10 min using EBA20 Centrifuge (Hettich, Germany) and the cell-free culture supernatant (CFCS) obtained was collected [11].

2.4. Extraction

About 250 mL of the CFCS was extracted with ethyl acetate at the level of 1:1 (v/v) in a subsequent manner [12] and the collected organic layers were evaporated using rotavapor (Heidolph instruments GmbH and Co. Schwabach, Germany) under vacuum at 45 °C. After complete evaporation, a sample of the fraction residue was redissolved in 1.25% DMSO in tissue culture medium. The cytotoxic activity of the redissolved fraction was evaluated against *Caco2* using MTT assay.

2.5. Production improvement of the cytotoxic activity of tested isolate

2.5.1. Fermentation in a laboratory fermenter

The fermentation process was carried out in 14 L CelliGen 310 bioreactor (New Brunswick Scientific, Edison, NJ, USA) with 5 L working volume of soybean meal medium. After inoculation of the fermentation medium with the prepared seed culture (5% v/v), the fermentation condition was adjusted at 28 °C incubation temperature; 200 rpm agitation speed, initial pH 7, 3 i.e 15 SLPM aeration rate and 2 bar airflow pressure. The dissolved oxygen concentration

was adjusted to obtain 100% saturation at the beginning of the run and dissolved oxygen (DO) percentage was sensed by the DO probe and monitored during the fermentation process. The fermentation process was left for 72 h during which foam was suppressed using silicon oil. The culture obtained was centrifuged at 6000 RPM for 10 min using EBA20 Centrifuge (Hettich, Germany) and the cell-free culture supernatant (CFCS) obtained was collected. The CFCS was extracted with ethyl acetate at the level of 1:1 (v/v) in the subsequent manner [12] and the collected organic layers were evaporated using rotavapor (Heidolph instruments GmbH and Co. Schwabach, Germany) under vacuum at 45 °C. After complete evaporation, a sample of the fraction residue was redissolved in 1.25% DMSO in tissue culture medium. The cytotoxic activity of the redissolved fraction was evaluated against *Caco2* using MTT assay.

2.5.2. Mutagenesis by treatment with gamma radiation

Mutagenesis by treatment with gamma radiation was carried out according to Khaliq *et al.* [13] with some modifications; *Streptomyces* isolate was cultured in soybean meal broth at 28 °C, 200 rpm for 72 h to yield a count of 10⁷ CFU/mL (determined using viable count technique). About 5 ml aliquots of the three days aged culture, contained in 20 mL transparent sterile glass vials, were exposed to increasing doses of gamma-irradiation (3, 4 and 5 kGy) inside the irradiation chamber of gamma cell 220 equipment (National Center for Radiation Researches and Technology, Atomic Energy Authority, Nasr City, Cairo, Egypt). A ⁶⁰Co source from Indian gamma cell that provides a dose rate of 1.43 KGy/h at the time of the experiment was used as a radiation source.

The dose of 4 KGy gamma radiation resulted in 3 log kill (99.9%) as determined by counting the survivors [14]. After mutagenesis, the irradiated cell suspension was suitably diluted, plated on starch nitrate agar and incubated at 28 °C for 7 days. The resultant colonies were

randomly selected, isolated and purified on starch nitrate slants. The isolated colonies obtained after exposure to gamma radiation were screened for their productivities of cytotoxic agents using 25 mL soybean meal medium contained in 250 mL shake flasks and under optimum conditions of fermentation (28 °C, 200 RPM and 2% v/v inoculum size from seed culture for 72 h).

After fermentation, the CFCS was extracted using an equal volume of ethyl acetate as described before, the organic layer evaporated under vacuum at 45 °C. The residue obtained was tested for cytotoxic activity against *Caco2* cell line, from which the CD₅₀ of each mutant was determined and compared to that of the wild-type *Streptomyces* strain.

2.6. Statistical analysis

MTT assay measurements for all mutants that showed higher potential cytotoxic activities than wild-type strain were conducted in triplicates, thus, the results reported in this part represent respective average values ± standard deviation. Data sets were analyzed using Graph Pad program (Graph Pad Software Inc., USA).

2.7. Cytotoxicity assay using MTT method

MTT assay was carried out as described by Saliba *et al.* [15] with some modifications; 100 µL of the crude extract (1mg in 5% DMSO and tissue culture medium) was added to the well that contains 100 µL of tissue culture medium, followed by two-fold serial dilution. Twelve dilutions were used for each crude extract to calculate CD₅₀ for each. Control wells contained two aliquots of 100 µL of ethyl acetate extract of soybean meal medium (1 mg dissolved in 5% DMSO and tissue culture medium) and 100 µL of tissue culture medium, followed by two-fold serial dilutions.

After 24 h incubation period at 37 °C in the CO₂ incubator, wells were washed with PBS, followed by incubation with 100 µL MTT solution (1 mg/mL) per each well for 1 h at 37 °C. Supernatants were then removed by decantation and the cells were treated with 100 µL DMSO per each well to dissolve formazan

crystals formed in the viable metabolically active cells. Elutes of the 12 wells of each tested crude extract were collected and their absorbance was measured at 540 nm using a different wavelength of 630 nm using Plato's R496 Microplate reader AMD diagnostics, Graz, Austria. Control wells were similarly treated. The percentage of cytotoxicity was calculated by the following formula [16].

$$\text{Cytotoxicity\%} = 1 - \{A_{540} \text{ of test culture} / A_{540} \text{ of control culture}\} \times 100$$

3. RESULTS AND DISCUSSION

3.1. Identification of the selected *Streptomyces* isolates

The selected *Streptomyces* isolate previously identified in a study conducted in our lab, using 16S rRNA gene sequence analysis [17]. The alignment pattern and scores obtained accounts for 100% identity to *S. griseus* and its 16S rRNA gene sequences were submitted to the GenBank database under accession code KJ623766.

3.2. Preparation of crude extract of cytotoxic agents produced by the tested isolate

3.2.1. Production of cytotoxic agents by the tested isolate

The fermentation process was carried out in a 2 L shake flask as described before. After three days incubation period, the culture (250 ml) was collected, centrifuged at 6000 rpm for 10 min. The collected supernatant (reddish brown color) was evaluated for cytotoxic activity and results showed promising cytotoxic activity (data not shown).

3.2.2. Large-scale production of cytotoxic agents by the tested isolates

The fermentation process was carried out using 14 L fermenter as described before. After three days incubation period, the culture (2 L) was collected from fermentor vessel, centrifuged at 6000 rpm for 10 min. The collected supernatant (reddish brown color) was evaluated

for cytotoxic activity and results showed promising cytotoxic activity (data not shown).

3.2.3. Extraction of cytotoxic agents from the CFCS

Maskey *et al.* [18] studied the anti-cancer and antibacterial trioxacarcins with high anti-malaria activity from a marine *streptomycete*. They utilized ethyl acetate as an organic solvent for extraction of trioxacarcins from the fermentation broth of *Streptomyces*. Also, Ahmed *et al.* [19] extracted the antimicrobial agent isolated from *Streptomyces violachromogenes* (isolate no. YA118) using ethyl acetate at pH3.

Atta *et al.* [12] carried out their work for the biosynthesis of antifungal substance that demonstrated inhibitory effects against pathogenic fungi from *Streptomyces albidoflavus*, 143. The active metabolite was extracted using ethyl acetate (1:1, v/v) at pH 7.0. In a previous study conducted in our lab, results obtained from extraction of the CFCS of *S. griseus* using different organic solvents, matched with all the previous results in the literature. Where ethyl acetate was shown as an excellent solvent for extraction of cytotoxic agents produced by the tested isolate at pH 7. Therefore in the present study extraction was done using ethyl acetate (1:1, v/v) at pH 7.0.

As described by Atta *et al.* (2011) [12], in brief; after the fermentation process, the CFCS was extracted by using Ethyl acetate at the level of 1:1 (v/v) in a subsequent manner. The organic phase was collected and evaporated under reduced pressure using a rotary evaporator to yield a reddish-brown solid residue (0.2 mg for shake flasks and 1.5 g for laboratory fermenter). A sample of each fraction residue was redissolved in 1.25% DMSO in tissue culture medium to evaluate the cytotoxic activities against *Caco2* cancer cell line and *Vero* cell line using MTT assay.

For shake flasks, the obtained results showed potent cytotoxic activity against *Caco2* and *Vero* cell lines with CD_{50} of 22 $\mu\text{g}/\text{mL}$ and 74 $\mu\text{g}/\text{mL}$, respectively. In the case of laboratory fermenter,

higher cytotoxic activities were observed with CD_{50} of 14 $\mu\text{g}/\text{mL}$ and 68 $\mu\text{g}/\text{mL}$, respectively (Fig. 1).

According to the screening program of American National Cancer Institute (NCI), a crude extract is generally considered to have *in vitro* cytotoxic activity if the CD_{50} value is $\leq 30\mu\text{g}/\text{ml}$. Therefore; *S. griseus* KJ623766 ethyl acetate extract showed potential cytotoxic activities against *Caco2* cell lines which indicate the presence of compounds with higher selectivity to human colorectal adenocarcinoma in the ethyl acetate extract. While weak cytotoxic activity against *Vero* cell line, non-tumourigenic when a cell passage was not prolonged [20]. These results indicate the presence of compounds with promising anticancer activity.

In bioprocesses, microorganisms are part of the chemistry, and they are susceptible to changes in the microorganism's living environment such as temperature, dissolved oxygen, pH, and others. Both aeration and agitation were kept constant at 3 vvm and 200 rpm, respectively during fermentation in laboratory fermenter, and played a significant role in increasing the productivity of the process; as both cannot be controlled in shake flask.

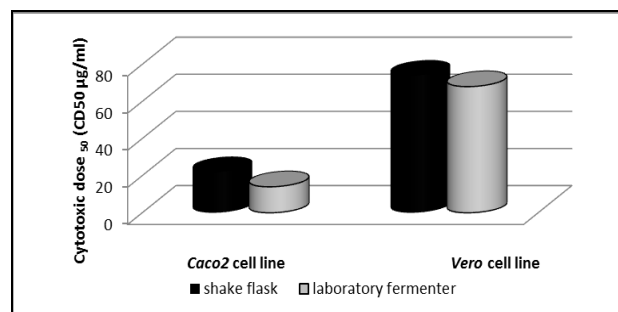


Fig. 1 potential cytotoxic activities of crude extracts obtained from *S. griseus* KJ623766 fermentation in shake flask and laboratory fermenter against *Caco2* and *Vero* cell lines

3.3. Improvement of the production of cytotoxic metabolites using gamma radiation

Random mutagenesis and fermentation screening have been reported as an effective way

to improve the productivity of industrial microbial cultures [21]. The most effective way to broaden the spectrum of base pair substitutions for yield improvement is to develop a protocol for AT-CG transversions. So far, no chemical mutagen has been reported that can induce AT to CG transversions [22]. However, AT-CG transversions were found in cells treated with ^{60}Co gamma rays [23]. So treatment with ^{60}Co gamma rays was used to improve the productivity of the cytotoxic metabolites by the studied isolate.

This was carried out by isolation of mutants resistant to 4 KGy doses of gamma mutation, the dose that caused 99.99% kill. The collected colonies obtained after mutagenesis were screened for potential cytotoxic activities. As illustrated in table 1, the collected colonies exhibited lower, equal or higher cytotoxic activities as compared to that of the wild-type strain of each isolate. Results revealed that; about 32% of collected mutants showed an increase in potential cytotoxic activities.

Table 1 Screening of collected mutants of *S.griseus* KJ623766 for their potential cytotoxic activities

Isolate	No. of collected colonies	No. of colonies showing potential cytotoxic activities			Percentage of colonies with higher cytotoxic activities
		lower	Equal	Higher	
<i>S.griseus</i> KJ623766	47	22	10	15	32%

After mutagenesis, 47 colonies were randomly selected and mutants isolated were tested for potential cytotoxic activities against *CaCO2* cell line using MTT assay.

Twenty-two mutants showed lower potential cytotoxic activities (Fig. 2); ten mutants exhibited no detectable changes in the potential cytotoxic activities (Fig. 3) and fifteen mutants showed higher potential cytotoxic activities (Fig. 4) when compared to that of the wild-type strain of *S.griseus* KJ623766.

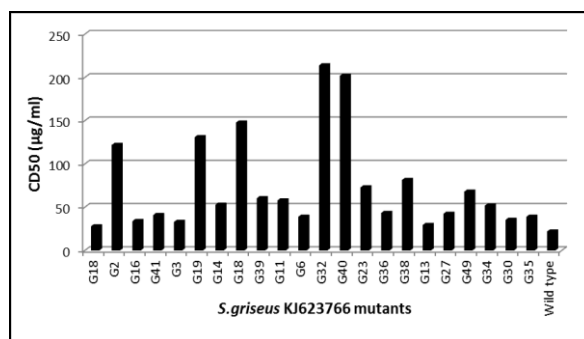


Fig 2 *S.griseus* KJ623766 mutants showed a decrease in potential cytotoxic activities against *CaCO2* cell line when compared to that of the wild-type strain

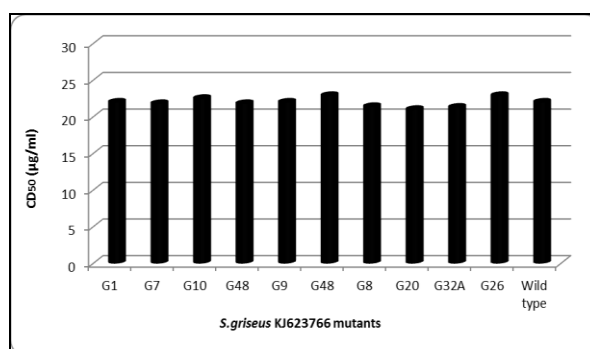


Fig 3 *S.griseus* KJ623766 mutants showed no detectable change in potential cytotoxic activities against *CaCO2* cell line when compared to that of the wild-type strain

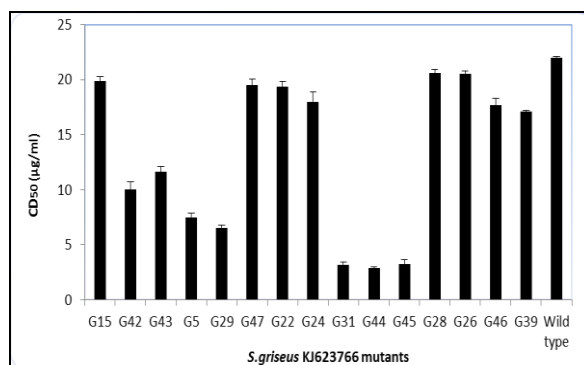


Fig 4 *S.griseus* KJ623766 mutants showed an increase in potential cytotoxic activities against *CaCO2* cell line when compared to that of the wild-type strain

Results revealed that; mutants coded G2, G18, G19, G32 and G40 exhibited a dramatic reduction in cytotoxic activities (with CD_{50} of 122, 148, 131, 214 and 202 $\mu\text{g/ml}$, respectively) when compared to that of the wild-type strain (CD_{50} 22 $\mu\text{g/ml}$). Mutants coded G42 and G43

showed about 2 folds increases in potential cytotoxic activity with CD_{50} of 10.06 ± 0.66 and 11.6 ± 0.51 $\mu\text{g/ml}$, respectively. Mutants G5 and G29 exhibited about 3 folds increase in potential cytotoxic activities with CD_{50} of 7.46 ± 0.44 and 6.51 ± 0.25 $\mu\text{g/ml}$, respectively. Finally mutants G31, G44 and G45 showed most potent cytotoxic activities where they exhibited about 7 folds increase in potential cytotoxic activity with CD_{50} of 3.2 ± 0.2 , 2.9 ± 0.1 and 3.25 ± 0.43 $\mu\text{g/ml}$, respectively. The fifteen isolates that showed an increase in potential cytotoxic activities were sub-classified into three groups; group showed potent increase in activity (G31, G44, and G45), group showed moderate increase in activity (G42, G43, G5 and G29) and group showed mild increase in activity (G15, G47, G22, G24, G28, G26, G46, and G39). One way analysis of variance (ANOVA) for these three groups and the wild-type strain showed a p-value of < 0.0001 , so results were considered extremely significant.

4. Conclusion

S. griseus KJ623766 CFCS contain metabolites with promising anticancer activity, as it showed high selectivity to human colorectal adenocarcinoma, while weak cytotoxic activity against *Vero* cell line was observed. Improvement of the production of the produced cytotoxic metabolites was achieved by large-scale production in a laboratory fermenter as well as mutagenesis using gamma radiation. Further studies are required for isolation and structure elucidation of the cytotoxic metabolites produced by a wild-type strain of *S. griseus* KJ623766, as well as those produced by mutants showed an increase in potential cytotoxic activities.

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