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Original Article

Purification and Structure Elucidation of Potential Cytotoxic Agents from *Streptomyces parvus* KJ623765

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ABSTRACT

Fermentation of *Streptomyces (S.) parvus* (accession code KJ623765), a locally isolated *Streptomyces* strains from Egyptian soil sample, was carried out in a 14 L laboratory fermentor. The optimum fermentation conditions were; incubation temperature, 28°C, agitation, 200 rpm, aeration, 5 standard liters per minute (SLPM), 2 bar airflow pressure, uncontrolled pH. After 72 h, the cell-free culture supernatant (CFCS) was collected and extracted using ethyl acetate (1:1, v/v) at pH 7.0. The crude extract showed potential cytotoxic activities against *Caco2* and *HeLa* cancer cell lines with CD_{50} of 3.4 and 6.8 µg/ml, respectively. Bioassay guided fractionation had led to the purification of compounds FF1 (12 mg), FGH4 (35 mg) and FGH5 (42 mg) per liter of the CFCS. Based on mass, 1D and 2D NMR spectroscopic analysis, they were identified as thymidine, diadzein and genistein, respectively. Production improvement was done using gamma radiation, where fourteen mutants showed marked increase in cytotoxic activity that reached 9.4 fold increase with mutants P43 and P59. Accordingly, *S. parvus* KJ623765 can be used as an industrial isolate for the commercial production of important cytotoxic metabolites. It is the first report about production of these anti-proliferative metabolites by the respective isolate.

Keywords: Bioassay guided fractionation; Cytotoxic activity*;* diadzein; Fermentation*;* Genistein; *Streptomyces parvus;* thymidine.

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INTRODUCTION

Cancer is a pleiotropic disease." This definition, by Nancy R. Gough [1], encompasses very well the complexity of the term ''cancer" which is not only caused by the abnormal growth of cells with the potentiality to invade different organs, but also by an impaired differentiation. 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 [2]. Colorectal cancer was the second highest cause of cancer occurrence in developed countries, An estimated 141210 cases were diagnosed in 2011 in the United States combined [3]. While cervical cancer is the second most commonly diagnosed cancer and third leading cause of cancer death among females in less developed countries. There were an estimated 527600 new cervical cancer cases and 265,700 deaths worldwide in 2012 [4].

Currently, 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], may provide many of the lead structures to be used as templates for the construction of novel compounds with enhanced biological properties [7].

Streptomyces produce numerous important bioactive compounds with profound impact on society, including compounds with antimicrobial, antioxidant, anticancer and antifungal properties [8]. Until now, cytotoxic substances such as actinomycin D, mitomycin C, bleomycin and doxorubicin originating from Streptomycetes were used for cancer therapy. In addition, the National Cancer Institute (NCI) in the USA examined the anticancer effects of different plant extracts, as well as other natural products [9]. Among them, flavonoids, widely found in different parts of plants, are known as the most important group of natural anticancer compounds [10].

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 [11]. Therefore, the objective of this study aimed at large scale production of the potential cytotoxic metabolites by *S. parvus* KJ623765, followed by purification and structural elucidation of the potential active metabolites using various analytical and spectroscopic methods. In addition, an attempt for production improvement of the respective metabolites was carried out via mutation.

MATERIALS AND METHODS

Microorganisms: *S. parvus* (KJ623765) was isolated from Egyptian soil samples from previous conducted study in our Lab. The isolate is characterized by promising cytotoxic activities against different mammalian cell lines. The isolate was identified using 16S ribosomal RNA gene sequences (GenBank database submission access code, KJ623765).

The isolate was preserved onto starch nitrate agar slants slant (soluble starch 10 g , KNO_3 2 g , K_2HPO_4 1 g , NaCl 0.5 g , $MgSO_4.7H_2O$ 0.5 g , $CaCO_3$ 3 g , agar agar 20 g , $FeSO_4.7H_2O$ 0.1 g , $MnCl_2$ $4H_2O$ 0.1 g , $ZnSO_4$ $7H_2O$ 0.1 g per 1 L of distilled water) [12] at 4 °C and sub cultured every month on the same medium. For long term preservation, isolate was plated on the surface of starch nitrate agar and incubated for seven days at 28°C. After incubation, the formed spores were scalped off, suspended in stock medium (tryptone 10 g , yeast extract 5 g , glycerine 500 ml and distilled H_2O to 1L) [13] and stored at -20°C.

Cell Lines: Three 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 (*Caco*-2 cell line) and epitheliod carcinoma derived from human cervix (*HeLa* cell line). These cell lines were obtained from VACSERA, Egypt.
Caco-2 and *HeLa* cell lines were used to study the potential cytotoxic activities of isolated compounds; stock culture of these cell lines 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 were dissociated with trypsin solution (0.25% in phosphate buffer saline).

Vero cell line is a continuous non-tumourigenic when a cell passage was not prolonged [14]. It was used to detect the cytotoxic effect of the isolated compounds on non-tumourigenic cell line. It was propagated in Eagle minimum essential medium (EMEM) with Earl's balanced salt solution [15], supplemented with 10% Fetal bovine serum (FBS) and antibiotics (100IU penicillin and 100IU

streptomycin /ml) solution every 48 h, and maintained in EMEM with Earl's balanced salt solution (EBSS) supplemented with 2% FBS and antibiotics solution.

PRODUCTION OF CYTOTOXIC AGENTS BY *S. PARVUS* KJ623765

Preparation of seed culture: From stock culture medium, an aliquot was spread on the surface of starch nitrate agar plate, incubated for seven days at 28ºC. After incubation, spores were scalped from the agar surface and suspended in 5 ml distilled water. After homogenization, 2 ml of the resulting spore bacterial suspension was used to inoculate 100 ml of soybean meal broth (soybean 15 g , glucose 15 g , NaCl 5 g , $CaCO_3$ 1 g per 1 L of distilled water) (2% v/v) contained in 1 L flask. The flask was then incubated at 28ºC and 200 rpm for 72 h. The culture obtained (seed culture) was used to inoculate the production medium [16].

Fermentation in a laboratory fermentor: 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, intial pH 7, 3 vvm 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. 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 (Hettrich, Germany) and the CFCS was collected.

EXTRACTION OF CYTOTOXIC AGENT(S)

About 1 L of CFCS of the tested isolate was extracted with ethyl acetate at the level of 1:1 (v/v) in subsequent manner [17] and the collected organic layer was evaporated using rotavapour (Heidolph instruments G bH and Co. Schwabach, Germany) under vacuum at 45°C. After complete evaporation, sample of the crude extract (yellowish brown) was redissolved in 1.25% DMSO in tissue culture medium. The cytotoxic activity of the redissolved fraction was evaluated against *Caco-2* and *HeLa* cancer cell lines using MTT assay.

CYTOTOXICITY ASSAY USING MTT METHOD

MTT assay was carried out as described by Saliba *et. al* (2002) [18] with some modifications; 100 µl of the tested crude extract or compound (1mg of the crude extract or compound dissolved in 5% DMSO in tissue culture medium) was added to the well that contain 100 µl of tissue culture medium, followed by two fold serial dilution. A total of 12 dilutions were used for each crude extract or compound to calculate CD_{50} for each. Control wells contained two aliquots of 100 µl of ethyl acetate extract of soybean meal medium (1 mg dissolved in 5% DMSO in 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 CO_2 incubator, wells were washed with PBS, followed by incubation with 100 µl MTT solution (1 mg/ml) per each well for 1 hr 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 compound were collected and their absorbance was measured at 540 nm using differential wave length of 630 nm using Platos R496 Microplate reader AMD diagnostics, Graz, Austria. Control wells were similarly treated. The percentage cytotoxicity was calculated by the following formula [19].

Cytotoxicity% = 1- {A_{540} of test culture / A_{540} of control culture} ×100.

ISOLATION OF CYTOTOXIC AGENT(S)

The ethyl acetate crude extract of the CFCS was evaporated under vacuum at 45°C using rotavapour and the yellowish brown residue obtained (1.6 g) was subjected to medium pressure chromatographic separations using flash column (Combiflash® with Retrieve® fraction collector, USA). About 1.6 g of residue was dissolved in 50 ml of chloroform, and then introduced into the column via dry load using 4 g silica. According to Atta (2009) [20] with some modifications; elution started with $CHCl_3$: MeOH (100%:0%) gradient to yield 30 fractions.

Elution was monitored using TLC (normal phase silica gel precoated plates F254, Merck, Germany) with UV-detection and spraying with vanillin sulphuric acid spray reagent and the similar fractions were pooled together. The cytotoxic activities of the obtained fractions were determined using MTT assay. Fractions showed potent cytotoxic activities where further purified using preparative HPLC (Knauer, Germany) on Kromasil ODS preparative column (10 mm× 250 mm) at flow rates 4 ml/min and UV detection at 254.

CYTOTOXIC ACTIVITIES OF ISOLATED COMPOUNDS

The potential cytotoxic activities of isolated compounds were determined using MTT assay method. Cytotoxicity assay was carried out against *Caco-2* and *HeLa* cell lines, as an example of cancer cell lines, and also against *Vero* cell line which is non tumoregenic cell line. The potential cytotoxic activity of each isolated compound was expressed in terms of CD_{50}.

STRUCTURE ELUCIDATION OF THE ISOLATED COMPOUNDS

Nuclear magnetic resonance (NMR):Compounds were dissolved in 0.2 ml of the suitable deteurated solvent and then transferred in 3 mm NMR tube. The number of scans were determined according to the amount of the analyzed sample but generally 128 scans were done for proton experiments, 8000 scans for APT experiments, 80 scans for HMBC experiments and 16 scans for COSY experiments. ^{1}H NMR was determined at 400.1 MHz and APT NMR was determined at 100 MHz.

LC-ESI-MS

HPLC analysis coupled with an ESI mass spectrometer as a detector, this allowed simultaneous isolation of the compounds and determination of the molecular weight of the isolated peaks [21].

PRODUCTION IMPROVEMENT OF THE CYTOTOXIC AGENTS BY THE TEST ISOLATE

Mutagenesis by treatment with gamma radiation: Mutagenesis by treatment with gamma radiation was carried out according to Khaliq *et al.* (2009) [22] with some modifications; *Streptomyces* isolate was cultured in soybean meal broth at 28°C, 200 rpm for 72 h to yield a count of 10^6 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 ^{60}Co source from Indian gamma cell that provide dose rate of 1.43 KGy/h at the time of the experiment was used as radiation source.

Screening of the selected colonies for their potential cytotoxic activities: The dose of 4 KGy gamma radiation resulted in 3 log kill (99.9%) as determined by counting the survivors [23]. 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 (28ºC, 200 rpm and 2% v/v inoculum size from seed culture for 72 h).

After fermentation, the CFCS of each mutant was extracted using equal volume of ethyl acetate as described before, the organic layer evaporated under vacuum at 45ºC. The crude residue obtained was tested for cytotoxic activity against *Caco-2* cell line, from which the CD_{50} of each mutant was determined and compared to that of the wild type *Streptomyces* strain as previously determined.

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 instat program (Graph Pad Software Inc., USA).

RESULTS

Identification of the selected *streptomyces* isolate: The selected *Streptomyces* isolate was previously identified as described by Kavitha *et al.* (2010) [24] using 16S ribosomal RNA gene sequence analysis. The alignment pattern and scores obtained accounts for 100% identity *to Streptomyces parvus* and the16S rRNA gene sequences was submitted to the GenBank database under accession code, KJ623765.

PREPARATION OF CRUDE EXTRACT OF CYTOTOXIC AGENT(S) PRODUCED BY THE TESTED ISOLATE

Large scale production of cytotoxic agents by the tested isolate: The fermentation process was carried out using 14 L fermentor 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 (yellowish color) was evaluated for the cytotoxic activity and the obtained results showed promising cytotoxic activities (data not shown).

Extraction of cytotoxic agents from CFCS: As described by Atta (2011) [17], in brief; after fermentation process, the CFCS (1 L) was extracted by using Ethyl acetate at the level of 1:1 (v/v) in subsequent manner. The organic phase was collected, and evaporated under reduced pressure using rotary evaporator to yield 1.65 g of dark

yellow solid residue. Sample of this fraction residue was redissolved in 1.25% DMSO in tissue culture medium to evaluate the cytotoxic activities against *Caco-2* and *HeLa* cancer cell lines using MTT assay. The results showed potential cytotoxic activities against both cell lines with higher activity against *Caco-2* (CD_{50} 3.4 µg/ml) than *HeLa* cell line (CD_{50} 6.8 µg/ml).

ISOLATION AND PURIFICATION OF CYTOTOXIC AGENTS

After extraction process, 1.6 g of the yellowish brown residue obtained was subjected to medium pressure chromatographic separations using Combiflash® with Retrieve® fraction collector, U.S.A and 30 fractions were eluted. Elution was monitored using TLC (normal phase silica gel precoated plates F254, Merck, Germany) with UV-detection and spraying with vanillin sulphuric acid spray reagent. Similar fractions were pooled together to yield 16 major fractions (FA-FP). The potential cytotoxic activity of each fraction against *Caco-2* and *HeLa* cell lines using MTT assay was determined. Results revealed that; Fractions coded FF, FG and FH showed the most potent cytotoxic activities (Fig 1).

Fraction FF showed the most potent cytotoxic activity against *Caco-2* and *HeLa* cell lines with CD_{50} of 0.632 µg/ml and 0.88 µg/ml respectively, followed by fraction FG (with CD_{50} of 3.31 µg/ml and 5.44 µg/ml against *Caco-2* and *HeLa* cell lines respectively) and fraction FH (CD_{50} 6.28 µg/ml and 7.84 µg/ml against *Caco2* and *HeLa* cell lines, respectively). The three fractions (FF, FG and FH) were subjected to TLC using CHCL3: MeOH (8:2) as mobile phase. Results revealed that, fractions FG and FH nearly similar to each other and therefore, were pooled together to yield fraction FGH.

Further purification using preparative HPLC (Knauer, Germany) on Kromasil ODS preparative column (10 mm× 250 mm) at flow rates 4 ml/min and UV detection at 254 was carried out to isolate and purify compounds showed potential cytotoxic activities in fractions FF and FGH. Results revealed that, fraction FF yielded one pure compound which coded FF1 (Fig 2), while fraction FGH yielded two pure compounds coded FGH4 and FGH5 (Fig 3).

CYTOTOXIC ACTIVITIES OF ISOLATED COMPOUNDS

Compounds FF1, FGH4 and FGH5 were subjected to MTT assay to measure potential cytotoxic activity of each compound against *Vero, Caco-2* and *HeLa* cell lines. Results showed that; the three isolated compounds FF1, FGH4 and FGH5 possess potent cytotoxic activities against *Caco-2* and *HeLa* cell lines and much lower cytotoxic activities were observed against *Vero* cell line (Table 1).

IDENTIFICATION OF ISOLATED COMPOUNDS

Compound FF1: Compound FF1 (12 mg) was isolated as white powder, ^{1}H NMR analysis revealed typical pattern of pyrimidine ribosides with one singlet aromatic proton at δ_H 7.83 (*s*, 1H) and one methyl group at δ_H 1.9 (*s*, 3H) together with the evidence for the deoxyriboside with the anomoric proton H-1' at δ_H 6.30 (*m*, 1H) and the other hydroxylated protons of the sugar in the region from δH 3.76-4.42. The structure was confirmed through the APT spectrum coupled with 2D NMR data, where the COSY spectrum showed strong correlations between protons H-1' at δ_H 6.30 (*m*, 1H) and 2' at δ_H 2.19-2.27 (*m*, 2H) which in turn showed strong correlations with H-3' at 4.42 (*m*, 1H) which is coupled with H-4' at 3.92 (*m*, 1H), these correlations confirmed the deoxysugar framework of the molecule. On the other hand HMBC correlations of compound FF1 confirmed the connectivity of the sugar to the pyrimidine moiety where it revealed strong correlations between H-1' δ_H 6.30 (*m*, 1H) and C-6 at δ_C 136.8 together proton H-6 at δ_H 7.83 (*s*, 1H) and the protons of the 5-CH_3 at δ_H 1.9 (*s*, 3H) showed correlations with C-4 at δ_C 164.9, finally the 5-CH_3 protons showed correlations with C-5 and C-6 at δ_C 110.2 and 136.8, respectively. 1D and 2D NMR data strongly agree with those reported in the literature for thymidine [25], thus compound FF1 (Table 2) is unambiguously identified as the pyrimidine nucleoside thymidine (Fig 4).

Compound FGH5: Compound FGH5 (42mg) was isolated as white amorphous powder. . ESI-MS revealed pseudomolecular ion peak at m/z 268.99 $(M-H)^-$ (Fig 5) corresponding to molecular formula $C_{15}H_{10}O_5$. Interpretation of the ^{1}H NMR spectrum of FGH5 revealed typical pattern of isoflavonoid aglycone derivatives with distinct singlet signal at δH 8.07 (s, 1H) characteristic for H-2 of the isoflavonoids in addition to the presence of the para-disubstituted aromatic system with two sets of ortho coupled protons at δH 7.39 (d, 8.1, 2H) and 6.86 (d, 8.1, 2H) corresponding to H-2', 6' and H-3', 5', respectively. Finally the presence of two Meta coupled protons at δH 6.35 (d, 2.1, 1H) corresponding to H-6 and δH 6.23 (d, 2.1, 1H) corresponding to H-8. The APT spectrum of FGH5 revealed the presence of thirteen carbon signals classified as five methine signals and eight quaternary signals. The 1H and APT spectra of FGH5 (table 3) were identical to those of the isoflavonoid aglycone genistein in the literature [26], thus compound FGH5 was unambiguously identified as genistein (Fig 6).

Compound FGH4: Compound FGH4 (35 mg) was isolated as white amorphous powder. Interpretation of the ^{1}H NMR spectrum of compound FGH4 showed great similarity to that of compound FGH5, with the typical pattern of isoflavonoid aglycone derivatives with distinct singlet signal at δ_H 8.22 (s, 1H) characteristic for H-2 of the isoflavonoids. Whereas in compound FGH4 the pattern of the aromatic protons in ring A showed a distinct ABX system rather than the meta coupled pattern in compound FGH5 as revealed by the ortho coupled proton H-5 at δ_H 8.16 (*d*, 8.9, 1H) and the ortho-meta coupled proton H-6 at δ_H 7.23 (*dd*, 8.9, 2.1, 1H). In addition to meta coupled proton at H-8 δ_H 7.27 (*d*, 2.1, 1H), the presence of the para-disubustituted aromatic system with two sets of ortho coupled protons at δH 7.40 (d, 8.6, 2H) and 6.87 (d, 8.6, 2H) had been conserved. COSY correlations had revealed all the proton connectivities of compound FGH4. The APT spectrum and ^{1}H NMR spectrum of compound FGH4 (Table 4) were identical to those of the isoflavonoid aglycone diadzein in the literature [27], thus compound 3 was unambiguously identified as diadzein (Fig 7).

Compound(s) isolated from soybean meal medium: Isoflavonoids are found to be main phytochemicals of many plants, especially representatives of the Legume family such as soybean [27]. So 1 L of soybean meal medium was extracted under the same conditions of the CFCS extraction. After evaporation of the oraganic layer (ethyl acetate) under vacuum, a solide yellow residue was obtained. Results obtained from HPLC chromatogram (Fig 8) indicate the presence of one major compound coded CC1. Compound CC1 was isolated (4.5 mg) as amorphous solid, the proton and carbon NMR showed great similarity to those of genistein (compound FGH5) with the preservation of the main nucleus. The main difference was the appearance of the glucose signals with the anomeric proton H-1" at δH 5.07 (d, 6.5, 1H) and C-1" at 100.2 (Table 5) suggesting that compound CC1 is the common glucoside genistin (Fig 9) as revealed by the similarity to the literature data of genistin [27].

PRODUCTION IMPROVEMENT OF THE CYTOTOXIC AGENTS

An attempt was made to improve the productivity of the cytotoxic agents by the studied isolate. This was carried out by isolation of mutants resistant to 4 KGy dose of gamma mutation, the dose that caused 99.99% kill. The collected colonies obtained after mutagenesis, were screened for potential cytotoxic activities, and results showed that; fourteen out of fourty two mutants (about 33%) showed increase in potential cytotoxic activities. when compared to that of the wild type strain (CD_{50} 7.87 µg/ml), Mutants P24, P46, P48 and P52 exhibited about 2.6 folds increases in potential cytotoxic activity (with CD_{50} of 3.57 ±0.24, 2.6 ±0.28, 3.16 ±0.28 and 2.8 ±0.35 µg/ml respectively). Mutants P31, P41, P47 and P60 exhibited about 5.5 folds increase in potential cytotoxic activity (with CD_{50} of 1.38 ±0.25, 1.3 ±0.24, 1.19 ±0.195 and 1.39 ±0.04 µg/ml, respectively). Finally mutants P43 and P59 showed marked increase in cytotoxic activites where they exhibited about 9.4 folds increase in potential cytotoxic activity (with CD_{50} of 0.666 ±0.124 and 0.68 ±0.21 µg/ml, respectively) (Fig 10).

DISCUSSION

The screening program of American National Cancer Institute (NCI) stated that; a crude extract is generally considered to have in *vitro* cytotoxic activity if the CD_{50} value is ≤ 30µg/ml; accordingly, the crude extract obtained from the CFCS of *S. parvus* KJ623766 showed potent cytotoxic activities against *Caco-2* and *HeLa* cell lines. Bioassay guided fractionation of the crude extract using different chromatographic techniques had led to the purification of three cytotoxic agents. Based on mass, 1D and 2D NMR spectroscopic analysis, the cytotoxic agents were identified as thymidine, diadzein and genistein.

Isoflavonoids are found to be main phytochemicals of many plants, especially representatives of the Legume family such as soybean. they ,especially genistein and daidzein, exhibited additionally anticancer activities [28], where they were used as potent agents in both prophylaxis and treatment of cancer and various other chronic diseases [26]. Generally, the total isoflavone content in the soybean was approximately 2.5 mg/g, and 97–98% of the entire soy isoflavone was in glycosidation form [27]. So, flavonoids and flavonoid intermediates seemed to be restricted to plants, but several studies reported their biosynthesis by *Streptomyces* species. Álvarez-Álvarez *et al.* (2015) [29] reported for the first time naringenin, a flavonoid intermediate used as an antioxidant, biosynthesis by *S. clavuligerus.* Others who have worked in this area have shown that microbial biotransformations of isoflavanones and flavanones are very common among bacteria and fungi. For example;, *Streptomyces griseus*, has been more intensively studied, and shown to produce genistein from genistin by biotransformation through the action of β-glucosidase [30].

So in order to demonstrate that the genistein and diadzein found in the crude extract described were not simply extracted from the media (soybean meal

broth), An experiment was performed as described by Hessler *et al.* (1997) [30] with some modifications. Where inoculated and un-inoculated soybean based broths were extracted following 72 hours of shaking incubation at 28°C. Results from HPLC analyses indicate that peaks for genistein and diadzein were not present in the crude extract obtained from the un-inoculated broth; instead HPLC analyses showed an early-eluting peak at approximately 3.25 min which stands for genistin (glycosidic form of genistein). The fermentation was therefore, determined to be an essential part of the process for genistein and diadzein productions from soybean meal medium. But the question was how the genistein and diadzein might be formed. To answer this question; Wang *et al.* (1994) [31] compared the amount of isoflavoniod aglycone produced after fermentation by the total amount of isoflavoniod glycosides actually present in the soybean production medium. Since the isoflavone content in the soybean was approximately 2.5 mg/g and 97–98% of the entire soy isoflavone was in glycosidation form [31]. Therefore, the total isoflavone content present in the production medium in this study was 37.5 mg/L and the amount of genistein and diadzein isolated were 42 mg and 35 mg, respectively. So this result shows the possibility of the biosynthesis of isoflavonoids by *S. parvus* KJ623765, but this does not rule out the possibility of the biotranformation process in our experiment. Results of the cytotoxic activities of genistein and diadzein were matched with those obtained by various authors [2,26]. They explained the anticancer activity of genistein against human colon cancer. The cytotoxic activity of genistein has been shown to be due to targeting the PI_3K/Akt pathway. Downstream, genistein inhibits EGF induced FOXO3 disassociation from p53 (mut), which further promotes FOXO3 activity and leads to increased expression of the p27kip1 cell cycle inhibitor, which inhibits proliferation in colon cancer cells. The activity of genistein against *HeLa* cell line may be due to inhibition of the expression of VEGF and VEGF receptors and Down-regulation of the expression of miR-27a which lead to induction of cell cycle arrest [2].

The third compound was unambiguously identified as the pyrimidine nucleoside thymidine. It is uncommon for thymidine to be released outside the living cells, but similar results was shown by Stadecker *et al*. (1997) [32], Where they observed the presence of an inhibitor of DNA synthesis and cell proliferation in macrophage supernatants. High concentrations of thymidine selectively kill certain human tumor cells in *vitro* [33]. Lockshin and his co-workers [34] studied the cytotoxicity and perturbations of the deoxyribonucleoside triphosphate pools caused by thymidine, in thymidine sensitive and resistant human tumor cells. Where they found that; incubation with 1 mM thymidine reduced cell viability by more than 90% in three sensitive cell lines. Mutagenesis; increases the potential cytotoxic activity of fourteen out of forty two variants (about 33%) when compared to that of the wild type strain. This increase in cytotoxic activity may be due to either an increase in production of genistein and diadzein by mutants, or due to the increase in efficiency of biotransformation, due to increase in β-glucosidase, of the total isoflavoniods present in soybean meal medium.

CONCLUSION

S. parvus KJ623765 can be used as an industrial strain for the commercial production of important cytotoxic metabolites, the isoflavonoids, genistein and diadzein. It is the first report about production of these anti-proliferative metabolites by the respective soil bacterium.

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Table 1: potential cytotoxic activities of compounds isolated from *S. parvus* KJ623765 CFCS extract against different cell lines.

Tested compound	Cytotoxic activity (CD_{50}) against different cell lines (µg/ml)		
	Caco2	*HeLa*	*Vero*
FF1	0.69	0.77	18
FGH4	6.59	8.83	58
FGH5	4.78	5.58	48.5

Table 2: NMR data of compound FF1, Chemical shifts are expressed in δH values (ppm) from internal TMS. Coupling constants in parentheses are given in J in Hz.

Position	δ_H (CD_3OD, 400 MHz, *J* in Hz)	δ_C (CD_3OD, 100 MHz)
1	-	-
2		151.1 (C)
3	-	-
4	-	164.9 (C)
5	-	110.2 (C)
5-CH_3	1.9 (*s*, 3H)	11.0 (CH_3)
6	7.83 (*s*, 1H)	136.8 (CH)
1'	6.30 (*m*, 1H)	84.8 (CH)
2'	2.19-2.27 (*m*, 2H)	39.9 (CH_2)
3'	4.42 (*m*, 1H)	87.4 (CH)
4'	3.92 (*m*, 1H)	70.8 (CH)
5'a	3.76 (*m*, 1H)	61.3 (CH_2)
5'b	3.81 (*m*, 1H)	

Table 3: NMR data of compound FGH5, chemical shifts are expressed in δH values (ppm) from internal TMS. Coupling constants in parentheses are given in J in Hz.

Position	δ_H (DMSO-*d6*, 400 MHz, *J* in Hz)	δ_C(DMSO-*d6*, 100 MHz)
2	8.07 (s, 1H)	154.3 (CH)
3		121.7 (C)
4		180.1 (C)
4a		104.7 (C)
5	12.95 (s, 5-OH)	162.4 (C)
6	6.35 (d, 2.1, 1H)	99.5 (CH)
7		165.1 (C)
8	6.23 (d, 2.1, 1H)	94.17 (CH)
8a		158.0 (C)
1’		122.7 (C)
2’,6’	7.39 (d, 8.1, 2H)	130.6 (CH)
3’,5’	6.86 (d, 8.1, 2H)	115.5 (CH)
4’		157.8 (C)

Table 4: NMR data of compound FGH4, Chemical shifts are expressed in δH values (ppm) from internal TMS. Coupling constants in parentheses are given in J in Hz

Position	δ_H (CD_3OD, 400 MHz, *J* in Hz)	δ_C (CD_3OD, 100 MHz)
2	8.22 (*s*, 1H)	153.3 (CH)
3		124.8 (C)
4		176.68 (C)
4a		108.4 (C)
5	8.16 (*d*, 8.9, 1H)	127.0 (CH)
6	7.23 (*dd*, 8.9, 2.1, 1H)	115.6 (CH)
7	-	165.1 (C)
8	7.27 (*d*, 2.1, 1H)	103.5 (CH)
8a	-	162.1(C)
1’	-	122.6 (C)
2’,6’	7.40 (*d*, 8.5, 2H)	130.0(CH)
3’,5’	6.87 (*d*, 8.5, 2H)	114.9 (CH)
4’	-	157.4 (C)

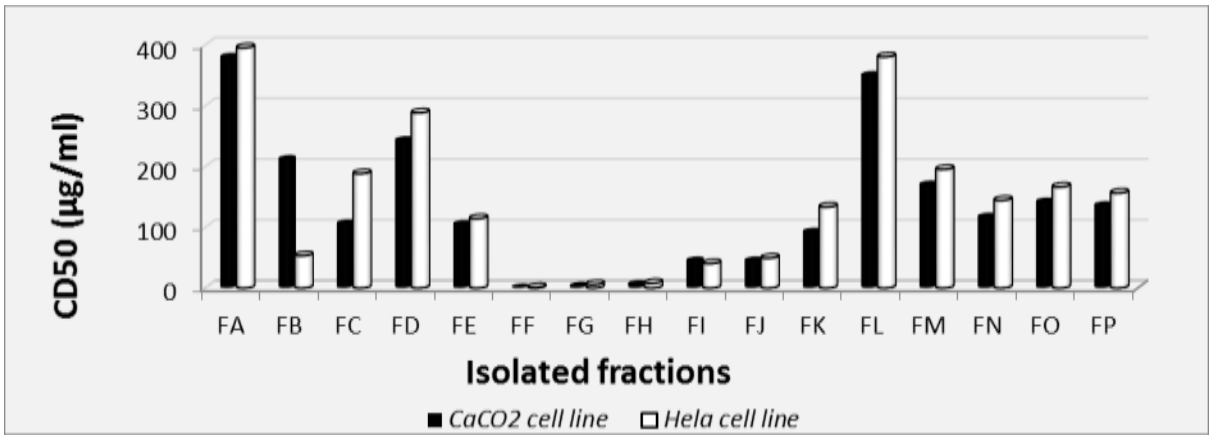

Figure 1: potential cytotoxic activities of collected fractions (FA-FP), isolated from the CFCS of *S. parvus* KJ623765, against *Caco2* and *HeLa* cell lines.

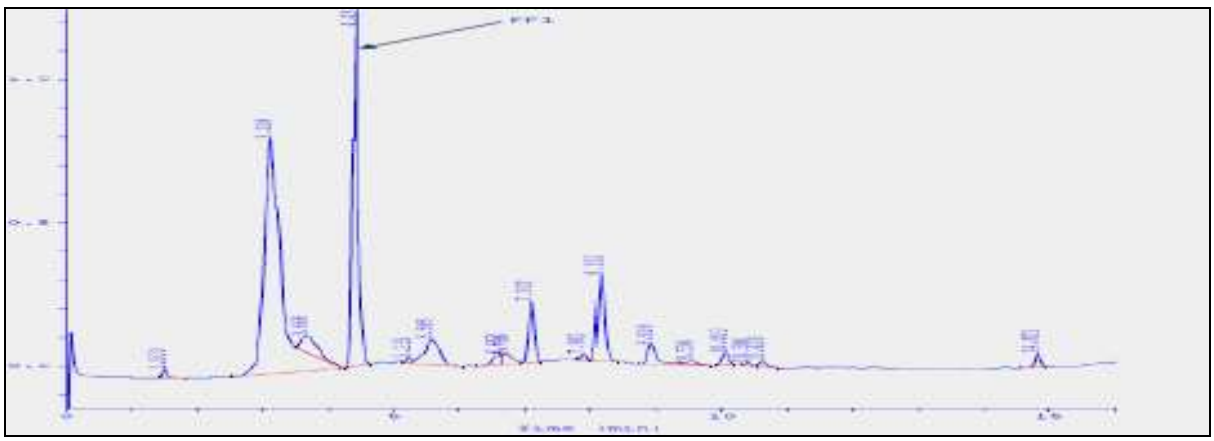

Figure 2: HPLC chromatogram of fraction FF

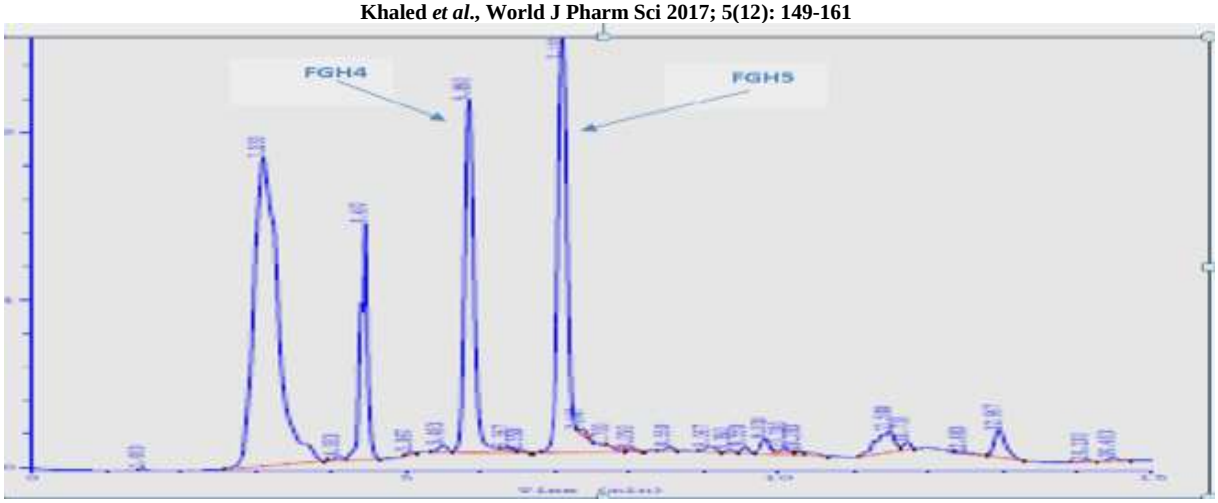

Figure 3: HPLC chromatogram of fraction FGH.

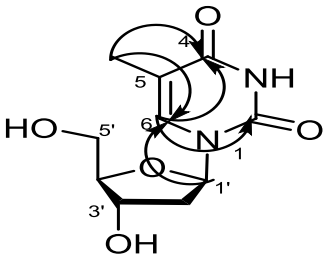

Figure 4: Structure of thymidine (⟶ HMBC correlations).

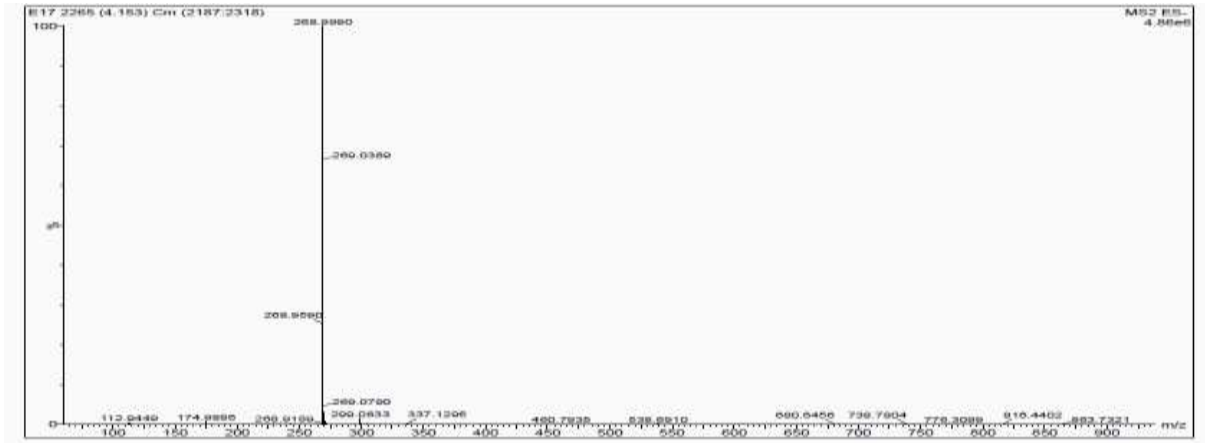

Figure 5: ESI-MS of isolated compound FGH5

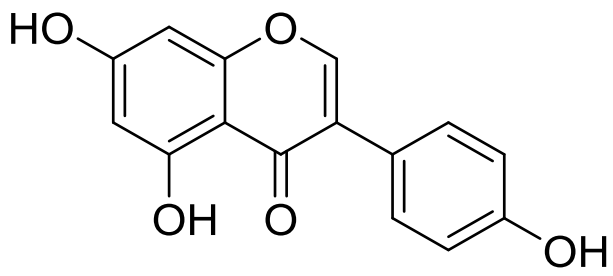

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Figure 6: Chemical structure of genistein

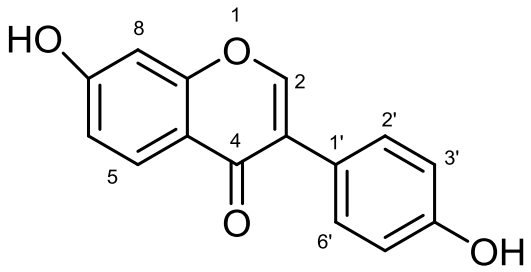

Figure 7: chemical structure of diadzein

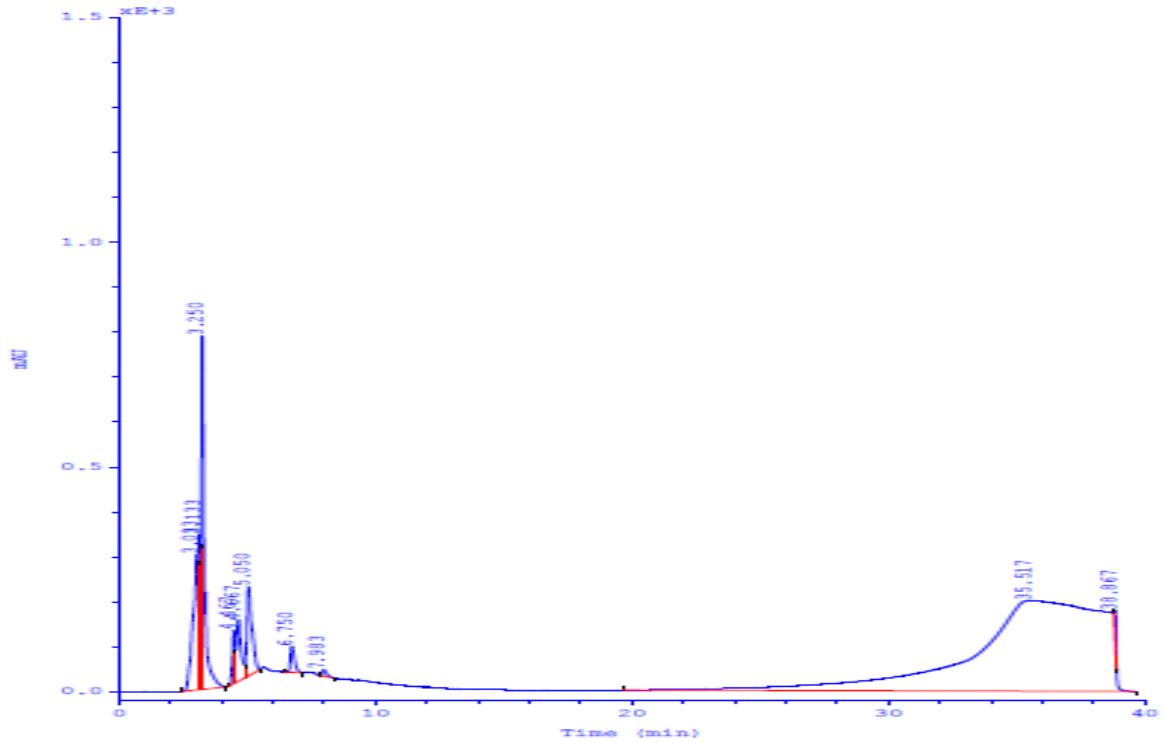

Figure 8: HPLC chromatogram of soybean meal extract.

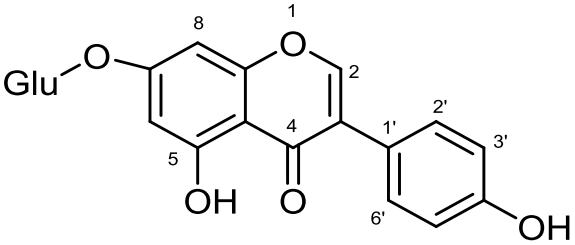

Figure 9: chemical structure of genistin

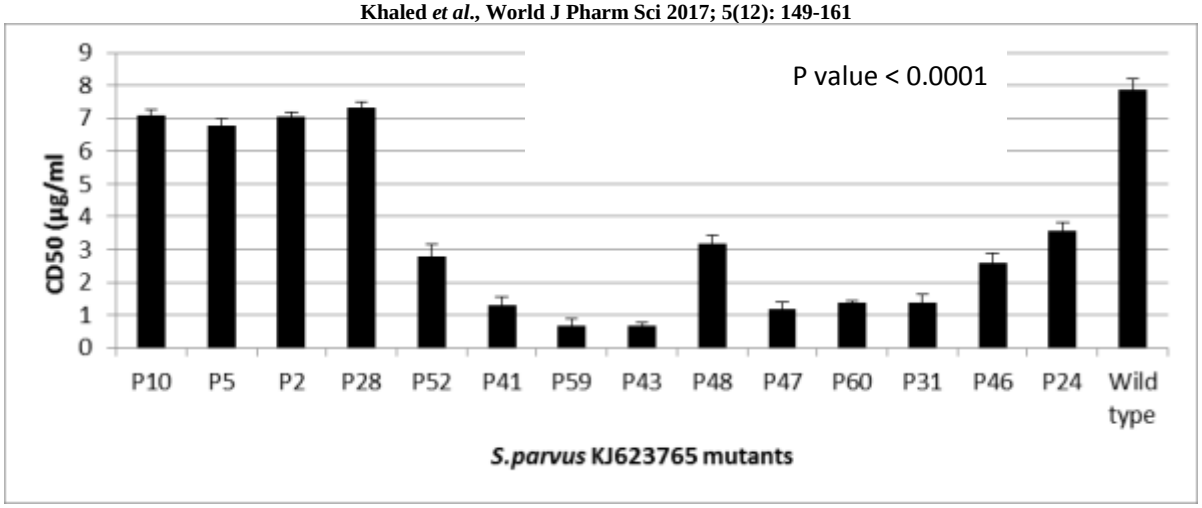

Figure 10: *S.parvus* KJ623765 mutants showed increase in potential cytotoxic activities against *CaCO2* cell line compared to that of the wild type strain.

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