Isolating sorbicilin-producing fungi from Darband cave and evaluating the sorbicilin biomedical applications

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Abstract
To evaluate the potential of fungi from Darband cave, Semnan, Iran, for valuable antibacterial and anticancer agents, molecular screening was done against polyketides (PKS); the source for numerous diverse secondary metabolites. Fungi were isolated from soil and sludge. The antibacterial activity of the isolates was studied against indicator bacteria by well diffusion agar method, and analyzed by PCR for PKS genes. The positive strains were compared for toxicity against indicators and A549 cells. Production of antibacterial agents was investigated in 26 days followed by partial purification of the agents. Thin layer chromatography (TLC) and High Performance Liquid Chromatography (HPLC) analyses were done to reveal the nature of the toxin(s). The isolates exhibited the antimicrobial activity against *Staphylococcus aureus*, *Bacillus cereus*, and *Bacillus subtilis*. Three isolates selected for further studies based on the yellow culture broth, the presence of PKS, and antibacterial activity. The strains were identified as *Penicillium chrysogenum* using 18s rDNA analyzing. The yellow culture, 87% identity of the PKS 12/ PKS 13, the results of HPLC, and the toxic effects against certain bacteria and A549 cells confirmed the production of the sorbicillinoid in *P. chrysogenum* strains; DDFCC170 and DDFCC186. The partially-purified antibacterial-anticancer agents named as AnBa170 and AnBa186 exhibited bactericide effect on *S. aureus* and *B. cereus*. These compounds killed A549 cells with an IC₅₀ value of 0.25 µM and 0.22 µM respectively. AnBa170 and AnBa186 are attractive for pharmaceutical industries. Based on the literature, the cytotoxicity of sorbicillinoids against tumor cells and bacteria is related to their oxidation capacity.

Key words – Antibacterial – *Penicillium chrysogenum* – PKS – Sorbicillin

Introduction
Microorganisms have progressively been considered for the production of valuable drugs and chemicals (Lam 2007, Park et al. 2019). Antibiotics, antifungals, anti-virus, and anti-tumors are the most known classes of microbial by-products introduced in pharmaceutical industries (Rolain et al. 2016). However, the adaptation of microorganisms for industries as well as the retaining stability of the active molecules has been a problem. On the other hand, growing rate of cancer and antimicrobial resistance, raises greater concern to either develop or discover new drugs with new features. To overcome this problem and generate industrially viable strains for bio-based active molecule production, today’s metabolic engineering, genome sequencing, and synthetic biology have brought together (Chae et al. 2017, Guzmán-Chávez et al. 2018).
Native strains can improve quantitatively and qualitatively the production of certain bioactive molecule based on naturally occurred mutations. Discovering new native resources for either known bio-compound or new molecules is performed by molecular screening against the key gene(s) in the biosynthesis pathway (Pye et al. 2017). Polyketides and non-ribosomal peptides (NRPs) synthetases are a category of the enzymes in bacteria, filamentous fungi, and plants, that biosynthesize a diverse family of bioactive molecules. Antibiotics (erythromycin A, rifamycin S, penicillin G, vancomycin and bacitracin), antifungals (amphotericin B), anti-cancer drugs (doxorubicin, epothilone), anti-parasites (avermectin), cholesterol-lowering agents (lovastatin), and immunosuppressants (rapamycin, cyclosporine, and siderophores) have been reported as the most important human medicine composed by PKS and or NRPs enzymes (Fischbach 2009).

This project aimed to discover new antibiotics and or new producer strains. A cultural collection of fungi from the Darband cave (the north of Dasht Desert, Semnan, Iran) was screened against PKS genes and their antibacterial activity. Two *Penicillium* strains were characterized as sorbicillinoid maker. *Penicillium* was the first fungal species introduced for production of β-lactam antibiotic; penicillin, albeit it’s potential for construction of secondary metabolites has been still investigated (Houbraken et al. 2012). The production of penicillin G, rococoumulin, melagrin, and xanthohycytin X in 15 different strains of *Penicillium chrysogenum* were documented from indoor environments (Scott et al. 2004). The marine *P. chrysogenum* strain was introduced as sorbicillinoid producer (Nicoletti & Trincone 2016). More than 40 compounds were identified from the culture broth of industrial strains of *Penicillium* (Salo et al. 2016). *Penicillium* NRRL1951 is a strain specified for sorbicillinoid production. This strain does not make any other classes of β-lactam bioactive molecules.

Currently, sorbicillinoids are known as valuable pharmaceutical molecules (Harned & Volp 2011). Sorbicillinoids are a large family of hexaketide metabolites, including more than 90 highly oxygenated molecules produced by *Aspergillus*, *Penicillium*, *Streptomyces*, *Trichoderma* and *Verticillium* species (Salo et al. 2016). The first sorbicillinoid was isolated from *Penicillium nutatum* about 60 years ago. Up to 2016, 90 sorbicillinoids were reported with certain antibacterial, antifungal, antiviral, antioxidant and anticancer activity (Meng et al. 2016). They control the cytopathic effect induced by HIV-1 and influenza virus A (H1N1) (Nicoletti & Trincone 2016). Moreover, bisvertinol and bisvertinolone showed activity against HL-60 cells and fungi. Oxosorbicillinol and dihydrosorbicillinol were found to be toxic for *Staphylococcus aureus* and *Bacillus subtilis* (Guzmán-Chávez et al. 2017). Taken together, sorbicillinoids have received growing attention for biomedicine and biotechnology based industries. Therefore, in this project we considered the potential of sorbicillinoid production in *Penicillium* strains from the culture collection of fungi isolated from Darband cave, Mahdishahr Semnan (35°44′55.7"N, 53°21′09.3"E). No study has been performed on the biome of this region up to our knowledge. Based on the literature there is a chance to obtain bioactive molecules with specific function from unknown ecosystems (Cordell 2005).

**Materials & Methods**

**Soil sampling and isolation of fungi**

The soil samples were aseptically collected at a depth of 20 cm from Darband cave, Mahdishahr Semnan (35°44′55.7"N, 53°21′09.3"E) in November 2016. 25 samples were immediately taken to the laboratory and stored at 4°C. The soil samples were suspended (1g/10 ml) in normal saline for 2 h. 100 μl of the samples were cultured on Yeast Extract–Peptone–Dextrose (YPD), Potato Dextrose Agar (PDA) and Sabouraud Dextrose Agar (SDA) media followed by incubation at 37°C and 28°C for 14 days. The fungi colonies were purified by successive subculturing on YPD, PDA and SDA media. The pure cultures were maintained in Dasht Desert Fungi Culture Collection, DDFCC.

**Screening for PKS genes**

Fungi and yeasts from DDFCC were studied for the presence of PKS (Polyketide Synthase) genes. The DNA from fungi were extracted according to the standard protocol (Al-Samarrai &
Schmid 2000), and further analyzed with PKSI universal degenerated primer pairs DKF; GTG CCG GTN CCR TGNGY TC / DKR; GCG ATG GAY CCN CARCARYG (Chiu et al. 2001, Miller et al. 2012). The PCR reaction initiated with 96°C for 3 min, 35 cycles of 96°C for 30 s, 50°C for 40s (DKF, DKR), and ended with a final extension of 72°C for 10 min. The PCR products were sequenced by Macrogen Company (South Korea) and analyzed using NCBI-BLAST services. The candidates were considered for ITS1-4 conserved DNA using universal primers (Gao et al. 2008, Smolik et al. 2010).

Evaluation of antimicrobial activity

The antimicrobial activity of the isolates was tested by agar diffusion method against 9 indicator bacteria; Escherichia coli (ATCC 25922), Shigella (sp), Staphylococcus aureus (ATCC25923), Bacillus subtilis (ATCC 12711), Bacillus cereus (PTCC1015), Proteus (sp), Pseudomonas aeruginosa (ATCC 27853) and Klebsiella pneumonia (ATCC13883). The indicator bacteria were cultured in BHI medium and incubated at 37°C for 16 h. An equal amount of 100 μl of the inoculated culture in growth phase were seeded on nutrient agar.

The fungi isolates were cultured in YPD medium, incubated at 30°C for 10-30 days, and pelleted at 8000 rpm for 10 minutes. 130 μl of the free cell supernatants (FCS) was loaded in the 8 mm wells and incubated at 37°C for 16 h. The halo zones were measured (Rajalakshmi & Mahesh 2014).

Kinetics of antimicrobial agent’s production

The positive strain for PKS genes; DDFCC186, DDFCC170, and DDFCC114 were subjected to a time course test. The cultures were examined for their antibacterial activity using the disk diffusion method within 26 days on a step of 48 h.

Extraction of antimicrobial agents

The isolates DDFCC186, DDFCC170, and DDFCC114 were fermented in YPD at the optimum time. The culture media were filtered and decanted with ethyl acetate (EtOAc) (2:1v/v) two times for 1 h. The organic phase containing the metabolites was separated and dried at 25°C. The residues were dissolved in Tris buffer (0.01 M), sterilized by filter membrane (0.45 μm) and stored at 4°C (Hemashenpagam 2011), named as AnBa (Anti-Bacterial agent).

Minimum inhibitory concentration (MIC)/Minimum bactericidal concentration (MBC)

The antibacterial activity of the candidates was determined by minimum inhibitory – concentration. The FCS and the EtOAc-extracted were tested against B. subtilis, B. cereus and S. aureus. 150 μl of FCS and or EtOAc-extracted were serially diluted and loaded in 96 wells plate containing 150μl nutrient broth. The indicators cultures (0.5 McFarland, 5μl) were seeded in the wells. The inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were calculated in terms of volumetric volume (μl /ml) after 16-18 h at 37°C (Corral et al. 2018).

Antibacterial activity in comparison to antibiotics and hemolytic activity

The antibacterial activity of DDFCC186, DDFCC114, and DDFCC170 against indicator bacteria (B. subtilis, B. cereus and S. aureus) was compared with antibiotics. The indicators with 0.5 McFarland were cultured on nutrient agar. The antibiotic discs of erythromycin (15 mg), chloramphenicol (30 mg), tetracycline (30 mg), ampicillin (10 mg), amoxicillin (25 mg), and gentamicin (10 mg) were placed on the medium where 100μl of FCS and EtOAc-extracted loaded in the wells, for 24 h. The halo-zones were measured after 24 h incubation at 30°C. DDFCC186, DDFCC114, and DDFCC170 were cultured on the sheep blood agar for 18 h at 37°C to evaluate

Chemical analyses

Thin-layer chromatography (TLC) was performed on silica gel 60. Rf values were measured applying 10% MeOH in CH₂Cl₂ (Maskey et al. 2005).
A Knauer Smartline HPLC instrument (Berlin, Germany) equipped with a quaternary HPLC pump, a UV-VIS detector (D-14163 model), and a C18 Eurospher-100 (5 μm particle, 250 mm × 4.6 mm). Samples and standard solution were filtered by hydrophilic PTFE membrane (0.45 μm) and injected in a volume of 20 µL. The chromatography data were processed using ChromGate software (version 3.1). The flow rate of 1 ml/min was applied for column elution and peaks monitored at 227 nm.

The mobile phase was a gradient of acetonitrile: 0.02% acidic water 90:10 to 25:75 in 15 min, followed by 25:75 back to 80:20 in 40 min, then to acetonitrile in (45 -50) min. The method was ended by acetonitrile: 0.02% acidic water 10:90 to 55 min and maintained for 60 min. The monitoring wavelength was 227 nm. The compounds were analyzed on the retention time and chromatogram matching with the standard.

Cell viability assay
The cell line A549 (human lung carcinoma) was grown in DMEM/F12 medium (Biowest) supplemented with 10% fetal bovine serum (FBS-Biosera). Cells were maintained in humidified, 5% CO₂ atmosphere at 37°C. The cytotoxic potential of the AnBa170, AnBa186, and AnBa114 was evaluated using MTT assay. Cells (2×10⁴ cells/well) were seeded in the supplemented medium in 96-well culture plates and were incubated in a humidified atmosphere with 5% CO₂, 37°C, for 24 h. The medium was replaced with fresh medium supplemented with AnBa170, AnBa186, and AnBa114, in a concentration of 1/2 and standard sorbicillin at 80, 40, 20 µM, followed by incubating at 5% CO₂, 37°C, for 24 h. To avoid the possible oxidative activity of the sorbicillin compound, the wells gently washed with 200 µl of PBS (Phosphate buffer saline). Every well was subjected to 20 µL of MTT in water (5 mg/ml) and incubated again for 4 h at 5% CO₂, 37°C. Then, 100 µL of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. The spectrophotometer analysis was done at 570 nm using a scanning microplate reader.

Statistical analysis
The data were tested for normality by SPSS version 22 and were then analyzed for the traits using analysis of variance and Duncan’s multiple range test (P<0.05).

Results

Soil sampling and isolation of fungi
A total of 100 fungi were isolated from soil and sludge samples in Darband cave. The screening was performed using three media of YPD, PDA and SDA at 28°C and 37°C. 58.4% of the isolates were obtained at 28°C, and 41.67% grown at 37°C. Of the isolates, 49.1% were grown on YPD agar, 48.3% on PDA and 1.6% on SDA medium. The antimicrobial activity of the FCS was investigated against indicator bacteria by the agar diffusion method. 26 isolates showed antimicrobial activity. The FCS from isolates did not show any effect on P. aeruginosa and E. coli, while were effective against S. aureus (16 isolates), B. cereus (11 isolates), B. subtilis (5 isolates) and Proteus (1 isolate).

Molecular screening against PKS genes
The PKS gene amplicons were obtained with DKF /DKR primer pairs, respectively. The expected fragments of 700-800 bp for PKS were detected in 22 isolates.

Evaluation of the antimicrobial activity of the isolates
Fig. 1 shows the antibacterial activity of some isolates against the indicators. The existence of PKS genes in companion with the largest antibacterial spectrum made DDFCC170 as the candidate fungi for further analysis. DDFCC170 inhibited the three indicators, B. cereus, B. subtilis, and S. aureus. The growth medium for DDFCC170 obviously differed by the yellow color which probably given by carotenoid pigment. Based on the result from the agar diffusion method, producing of
sorbicillin was supposed. The antibacterial spectrum of DDFCC186 was similar to DDFCC170 as appeared in Fig. 1.

The DDFCC114 was examined because of specific antibacterial activity against *S. aureus*. In the current study, three *Penicillium* (sp) isolates making the culture medium yellow were detected.

![Fig. 1 – Antimicrobial activity of the FCS against indicator bacteria by agar diffusion method.](image)

**Molecular identification**

The blue to blue-green conidia, yellow pigment, morphology and microscopic features and molecular identification of DDFCC170, DDFCC186, and DDFCC114 confirmed that they are *P. chrysogenum*. The blastn alignment of the 700 ITS1-ITS4 bp DNA fragments showed 99% similarity to *P. chrysogenum* for all the three strains.

**Kinetics of antimicrobial agent production**

The production of antimicrobial agents was assayed against indicators by disk diffusion agar for 26 days (Fig. 2). Production of AnBa186 was on the 13th day, which reached the highest level on the 17th day. AnBa170 was obtained on the 15th day exhibited the largest inhibition zone (35mm) against *S. aureus*. The antimicrobial compound of the DDFCC114, AnBa114, produced within 15th days followed by an obvious reduction (Fig. 2).

**MIC/MBC evaluation**

FCS of DDFCC170, DDFCC186, and DDFCC114 were extracted by EtOAc and the residue dissolved in Tris buffer. The extracts were evaluated for their antibacterial activity in comparison to FCS. The results showed that the EtOAc-extracts were more effective than the FCS against *B. cereus* and *S. aureus*.

AnBa170, AnBa 186 showed the highest antibacterial activity against *S. aureus* (31.2 and 62.5 µl/ml) and *B. cereus* (62.5µl/ml) and DDFCC114 against *S. aureus* with the MBC value of 62.5 µl/ml.

**The antibiogram and hemolytic activity assay**

The antibiogram assay revealed that the FCS of DDFCC186 was a weak antibacterial in comparison to all the tested antibiotics. The EtOAc-purified and FCS of DDFCC170 with a diameter of 37 mm and 32 mm were the strong killers for *S. aureus* (Fig. 3). The AnBa114 with a halo diameter of 35.5 mm specifically inhibited *S. aureus*. None of the FCS and EtOAc-extraction from
DDFCC170, DDFCC186, and DDFCC114 showed hemolytic activity, indicating that they do not destruct cells.

**Fig. 2** – The production of antimicrobials agents assayed against indicators for 26 days

**Fig. 3** – Antimicrobial activity of FCS in comparison to the antimicrobial effect of known antibiotics

**Chemical analyses**

AnBa170 and AnBa186 were analyzed by TLC. The bands appeared with exposing to UV in the range of 254 nm. The band with the Rf of 0.9 value was detected for AnBa170 and AnBa186 as well as for sorbicillin.

The standard chromatogram of sorbicillin was measured using HPLC method (Fig. 4). The chromatogram of standard sorbicillin at 227 nm showed a peak (41.8 min) that were identified in the chromatogram of AnBa170 as retention time (Rt) = 41.3 min. The peak was similarly detected in AnBa186 (41.6 min). The peaks were confirmed by the alignment of sorbicillin, AnBa170 and AnBa186 chromatograms (Fig. 4). At the same retention time, no peak was detected for AnBa114.

The HPLC and TLC analyses of the DDFCC 170 and DDFCC 186 partially- purified bioactive molecules proved the existence of sorbicillinoid molecules. The HPLC chromatogram of each extract showed the peak similar to the standard of sorbicillin.
Cell viability assay

The inhibitory effects of IC50 values are presented in Fig. 5. AnBa170 showed prominent cytotoxic activity against A549 cell line with a value for IC50 as 0.25 µM in 24 h. Same experiment with AnBa186 resulted in IC50= 0.22 µM in 24 h. The standard of sorbicillin was used as a positive control showing a strong cytotoxic effect on A549 cells.

Fig. 4 – The standard chromatogram of sorbicillin in comparison to the chromatogram of AnaBa 170 and AnaBa186. The reference peak corresponding to standard sorbicillin represented by “S”

Fig. 5 – Cytotoxic effects of sorbicillin, AnaBa 170 and AnBa186 on A549 cell

Discussion

In this study, the fungal isolates from Darband cave were screened for a distinguished antibacterial activity. Among the isolates, three *P. chrysogenum* strains were introduced here with the notable antibacterial activities against *S. aureus, B. subtilis*, and *B. cereus*. Their yellow culture
media made the isolates as much as interesting to be further chemically and microbiologically analyzed.

Two strains out of three, *P. chrysogenum* DDFCC170 and *P. chrysogenum* DDFCC186, showed no effect on the Gram-negative bacteria (lipopolysaccharide membrane) suggesting the hydrophilic properties for the antimicrobial agents from DDFCC170 and DDFCC186. The indicators *S. aureus*, *B. subtilis*, and *B. cereus* were similarly inhibited by DDFCC170 and DDFCC186 indicating the existence of some other antibacterial agents excluding penicillin derivatives. To obtain the bio-active molecules, as the polarity of the antimicrobial agent was unknown, EtOAc was used to separate both the polar and nonpolar compounds from the aqueous phase. EtOAc due to its chemical and biological properties, has a moderate polarity and minimal toxicity in the extraction of many biological and polar compounds (Altemimi et al. 2017). Ghanbari et al., partially-purified the FCS of four *Penicillium* isolates with EtOAc and examined the presence of bioactive compounds by GC-MS (Ghanbari et al. 2014). EtOAc-purified extraction showed optimum antimicrobial activity in the investigation done by Luca et al. (2019).

Considering the cytotoxicity effects beside yellow color in culture media, and literature (Guzmán-Chávez et al. 2017), DDFCC 170 was a candidate for production of sorbicillinoid compounds. Sorbicillinoids are bioactive molecules with a variety of bio-features effects and growing attention for biomedicine and biotechnology based industries. The first sorbicillinoid known from *Penicillium notatum* identified as a contaminant in the production of penicillin, the molecules are biosynthesized by the oxidative dimerization of a hexaketide molecule (Salo et al. 2016), therefore, molecular screening based on PKS genes was a useful method (Mohanty et al. 2016).

The result of sequencing for PKS I gene was evaluated for DDFCC170 against NCBI genebank. The blastn for DDFCC170 showed a similarity of 87% to PKS12/PKS13 (polyketide synthase) genes in *P. chrysogenum* leading to 72% identity at the amino acid level (blastx).

The putative sorbicillinoid gene cluster of industrial *P. chrysogenum* strains include two PKS genes; sorA, Pc21 g05080, and sorB, Pc21 g05070 (Salo et al. 2016). The molecular analyses of the 760 bp PKS gene fragment from DDFCC170 revealed 87% identity to sorA. Consequently, DDFCC170 was considered as a candidate for production of sorbicillinoids. Despite of *Trichoderma* is known as the industrial sorbicillinoid producer, currently the marine *P. chrysogenum* strain was introduced with the active sorbicillinoid biosynthesis pathway (Li et al. 2018). Based on the literature, producing the valuable fungi secondary metabolites; sorbicillinoid compounds in these three strains was assumed (Guo et al. 2013). Unlike the improved strain, Wisconsin, producing sorbicillinoids has been shown in the native isolates of *P. chrysogenum* (Guzmán-Chávez et al. 2017). Therefore, it seems that the biosynthesis pathway for penicillin and sorbicillin are not switched on at the same strain.

The HPLC and TLC analyses of the DDFCC 170 and 186 partially-purified bioactive molecules proved the existence of sorbicillinoid molecules. The HPLC chromatogram of each extract showed the peak similar to the standard of sorbicillin. The antibacterial activity of sorbicillinoid compounds against *B. cereus*, *B. subtilis*, *S. aureus* was shown previously although, it has been known as an anticancer drug (Maskey et al. 2005).

DDFCC114 with the lowest intensity of yellow color in the media harbored the most efficient antibacterial agent only against *S. aureus*. None of the other indicators were limited subjecting to the antibacterial agent from DDFCC114. Therefore, DDFCC114 considered as a penicillin derivative producer via the known metabolic pathway in *P. chrysogenum* (Viggiano et al. 2018). Harned et al. reviewed the mechanism of cytotoxic activity of the sorbicillinoid compounds. They documented these compounds as the antioxidant agent with a broad range of activity. In many papers, sorbicillinoids have been introduced as free radical scavenger (Harned & Volp 2011).

**Conclusion**

Taken together our results supported the idea that DDFCC186, DDFCC114, and DDFCC170 are three *Penicillium chrysogenum* strains, which the well-known penicillin and sorbicillinoid...
biosynthesis pathways may not be activated at the same rate in every strain. Two strains DDFCC170 and DDFCC186 produce sorbicillin compound to be toxic for A549 cell line and *B. subtilis, B. cereus,* and *S. aureus.* Based on our knowledge this is the first report on terrestrial *P. chrysogenum.*

**Ethical Issues**
Whole blood was taken from healthy volunteers with informed consent. The study was conducted according to ethical principles of the Declaration of Helsinki 1964 and its later amendments and comparable ethical standards.

**Conflict of interest**
The authors declare that they have no conflict of interest.

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