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Original Research Article

Isolation of membrane-active fraction of *Streptomyces* spp. from soil

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Abstract

Purpose: *To isolate and characterize the membrane-active antimicrobial fraction and isolate metabolite produced by Streptomyces in soil samples from Iran*
Methods: *More than 60 Actinomycete strains were isolated from soil samples in Iran. A total number of 16 strains were studied using antimicrobial assay against Escherichia coli, Candida albicans and Staphylococcus aureus. Among these, three strains produced membrane-active metabolites based on artificial vesicle assay. Extracts of Streptomyces culture were obtained using ethyl acetate fractionation. Antimicrobial activity was evaluated by broth microdilution assay. Among these active extracts, one metabolite was isolated. Further fractionation and purification strategies were applied to finally identify the isolated metabolite using appropriate spectroscopic methods including thin layer chromatography (TLC), preparative thin-layer chromatography (PTLC), high performance liquid chromatography (HPLC), nuclear magnetic resonance (NMR) and liquid chromatography–mass spectrometry (LC-MS).*
Results: *Three strains isolated from the soil samples, namely, strains 0811, 08346 and 08317 showed the highest antifungal and antimicrobial activity in Tryptic Soy Broth (TSB) and International Streptomyces Projects 2 (ISP2) medium in the range of 46.8 to 62.5 µg/ml. Strain 08346 was selected for further chemical profiling based on TLC pattern and membrane activity. It yielded a purified compound which was determined to be a novel aromatic amino alkyne, named Sourin.*
Conclusion: *Streptomyces-produced 08346 strain demonstrates good antimicrobial activities against bacteria and yeasts, suggesting its potential as an antimicrobial membrane-active agent.*

Keywords: *Actinomycetes, Secondary metabolites, Streptomycetes, Membrane-active agent*

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INTRODUCTION

Microorganisms have been the most favorable resource of antibiotics in the past and will remain a main source of new bioactive natural products in the future. Almost 50 % of bioactive compounds are obtained from Actinomycetes [1].

Actinomycetes have high pharmacological interest and commercial value as they produce secondary metabolites with a diverse bioactivity.

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More than 7,600 compounds are produced by *Streptomyces* species [2]. Streptomycetes are known to be producers of many metabolites, possessing antibacterial, anti-parasitic, antifungal, immunosuppressive and antitumor properties [3]. Also, Actinomycetes are the major source of important antibiotics that are suitable in human and animal treatment, in industry and food production. Since they live in various soil habitats, soil ecology has been studied in various countries and also different surveys have been done in Iran [4-6].

The increase in frequency of resistance to antibiotics by pathogens represents a global health problem and therefore it is necessary to develop antibiotics with novel mechanisms of action [7,8]. Among the cellular targets for antibiotic action, membrane has great potential because of low tendency of developing resistance to it [9,10].

According to previous studies [11-13], lipid/PDA artificial vesicle model was successfully used for the screening of membrane active metabolites. As indicated earlier, out of 67 isolates, only 3 (08-1-1, 08-3-17 and 08-34-6) showed potential membrane disruption activity and more researches have been performed to isolate and identify the metabolites existing in the fractions of the mentioned membrane active strain extracts.

In this research, detection and purification of antimicrobial compound in the membrane-active fraction produced by Actinomycetes isolated from soil was accomplished by TLC and PTLC. Structure determination was performed by ^{1}HNMR, COSY and LC-MS techniques.

EXPERIMENTAL

Chemicals

Organic solvents: hexane, chloroform, methanol and dimethyl sulfoxide were of analytic grade and ethanol was of HPLC grade, and obtained from Merck, Germany. Ethylacetate was obtained from Chem lab, Belgium. Silica gel TLC sheets were obtained from Merck, Darmstadt, Germany. Silica gel (200 - 400 mesh size) was obtained from Merck, Darmstadt, Germany.

Sabouraud maltose broth was obtained from DIFCO, Becton, Dickinson, USA. Brain heart infusion (BHI) broth was obtained from Merck, Germany. Phospholipid, dimyristoyl phosphatidyl choline (DMPC), 10,12-tricocosadiynoic acid (TCDA) and diacetylenic monomer were purchased from Sigma, USA.

Soil sample collection

Since 2008, soil samples have been collected from several parts of northern and central Iran. These include the mineral soils around Meshkinshahr (38˚29.545″ N and 047˚44.886″ E), and desert soils around Damghan (35˚54.509″ N and 053˚57.832″ E). The samples were taken from a depth of 15 cm from soil surface by a clean soil borer. The samples were placed in sterile bags, closed tightly and stored in a refrigerator as previously reported [11].

Extraction of antimicrobial metabolites

The three isolated *Streptomyces* sp.: 0811, 08346 and 08317 were grown in ISP4 medium plate and kept in an incubator for 5 - 7 days at 28 °C. A loopful of spores was scraped from the plate and inoculated onto 3 mL ISP2 and TSB. It was kept in rotatory shaker incubator at 150 rpm for a period of 7 days at 28°C and used as inoculum. Culture inoculum was put in 2000 mL Erlenmeyer flasks containing 500 mL of ISP2 and TSB then incubated at 25°C in a shaker at 200 rpm for 14 days; after that, the culture broth was centrifuged at 4000 rpm for 15 min to remove the biomass. The culture medium was washed with ethyl acetate (1:3 v/v) and the ethyl acetate layer was removed after 30 min. The process was repeated thrice. The pooled ethyl acetate extract was distilled and dried in a rotary evaporator to obtain the final extract.

Determination of minimum inhibitory concentration (MIC)

MIC of the crude extracts produced by the isolated actinomycetes strains, against one Gram positive bacteria, one Gram negative bacteria and one fungus was determined by following the broth microdilution method of testing based on NCCLS M27-A [14].

Escherichia coli ATCC 25922, *Candida albicans* ATCC 10231 and *Staphylococcus aureus* ATCC: 25923 were used as the test strains. The crude extracts were dissolved in dimethyl sulfoxide (DMSO) to obtain a concentration of 10 mg/mL.

Sabouraud maltose broth was used as the growth medium and *E. coli* and *S. aureus* were cultured at 37°C for 24 h, in brain heart infusion broth (BHI). AmB was used as a positive control in the antifungal test, streptomycin for antimicrobial test and DMSO as solvent control. The plate was incubated at 37°C for 24 to 48 h. The MIC values are the lowest concentration of the extract showing no growth in the well.

Screening for antimicrobial membrane activity

Artificial vesicle was applied using detection method, based on a biomimetic polymer polydiacetylene (PDA) together with phospholipid as a membrane model. To polymerize the vesicle components, synthetic phospholipid, dimyristoyl phosphatidyl choline (DMPC), and diacetylenic monomer, 10,12-tricocosadiynoic acid (TCDA), were separately dissolved in dichloromethane 1 mg/mL, mixed at 2:3 molar ratio with a total concentration of 1 mM. The lipid material was dried together in vacuum followed by addition of water and sonicated for 2 min. The solution was cooled, and kept overnight at 4°C, and then irradiated at 254 nm for one hour for polymerization. The resulting vesicle solution had intense blue color appearance due to polymerization of the diacetylene [16].

Circular dichroism (CD) spectroscopy

The CD was performed on a Jasco spectropolarimeter model 810. The spectra were measured from 190 to 260 nm at room temperature and length cell was 1 mm. Data were recorded at a bandwidth of 1.5 nm and a step size of 1 nm with a scan rate of 200 nm/min. Data were reported as the accumulation of spectra for 3 consecutive recordings with concentrations of 1X, 1:5X, 1:250X and analyzed as mentioned in the result section [17].

Qualitative analysis by thin layer chromatography (TLC)

In the current study, TLC and PTLC techniques were used to screen the ethyl acetate extracts of 3 strains for secondary metabolites. Extracts were checked by TLC on analytical silica gel plates. Ethyl acetate extract was subjected to TLC in which different mobile phases like dichloromethane, chloroform and ethyl acetate/n-hexane were tried in order to separate the bioactive compounds. After drying, the plates were visualized through UV at 254 and 366 nm in TLC viewer chamber. The compounds appeared on TLC plate in the form of bands. Each fraction can be further purified using PTLC and HPLC [18].

Purification of Sourin by preparative thin-layer chromatography (PTLC)

Slurry preparation for soft layer coating

First, silica gel powder was dispersed in water to make a slurry, then the mixture was spread as a paste on a 20 x 20 cm glass plate. The resulting plate was dried and heated in an oven at 110°C for one hour in order to activate the silica gel layer. Silica gel 60, GF254 Merck, was used for PTLC. For each 1.0 mm silica layer, about 5 mg of the fraction was loaded. Mobile phases in varying proportions are n-hexane-ethyl acetate and chloroform-ethyl acetate. The scraped bands were extracted with suitable solvent (methanol) and filtration as the final step was carried out to obtain the compound by removal of the stationary phase

HPLC analysis

HPLC analysis were carried out with Perkin Elmer instrument (Flexar) equipped with a photodiode array detector (PDA) using a C18 column (4.6 × 150 mm) Perkin Elmer. The column was equipped with a guard column C18 (3.9 × 20 mm). The PDA was used according to the following conditions: temperature: 25°C; pressure: 2500 psi; wavelength: 254 nm. The PDA provided all characteristic UV wavelengths.

For analyses of the crude fraction, a standard HPLC method was set up with water (A), acetonitrile (B), gradient (100:0 to 0:100) with a total run time of 23 min. The flow rate of 1 ml/min was applied. The injection volume was 20 µL. Analysis of the pure fraction obtained from PTLC was carried out with the same HPLC condition using water (A), methanol (B), gradient (100:0 to 0:100) as mobile phase. The peak of pure compound for each fraction was obtained and then analyzed by ^{1}HNMR, ^{13}CNMR, COSY and LC-MS.

Characterization of the compound

For characterization of the compound, ^{1}HNMR, COSY and LC-mass spectra were performed.

^{1}H NMR, ^{13}CNMR and COSY spectra were recorded on Bruker 300 MHz. The pure fractions were dissolved in solvent MeOD-d4, poured in NMR tube and observed on the applied magnetic field. All 1D and 2D spectra were obtained using the standard Bruker software.

LC-MS was performed using Agilent Technologies 6410 Triple Quadrupole LC-MS, LC 1200 series. LC condition was set as follows: mobile phases A: water, B: acetonitrile. The flow was equal to 1 mL/min. Analysis program: The first 8 min was run with 20% B, for the next 3 min with 50% B; for the following 5 min with 100% B, and for the 7 last min with 20% B. The total run time was 23 min. Column was C18, (250 × 4.6 × 5 mm), and the detector was photodiode array

(DAD) 254 nm, and injection volume was 20 µL. Mass condition was ESI positive mode.

Statistical analysis

Statistical analysis was performed by averaging the antimicrobial assay. All results were repeated in duplicates and the average was obtained, and this analysis gave high reproducibility of MIC assay which is adequate at this level [11-13].

RESULTS

Isolated actinomycete strains and antimicrobial activity

As previously mentioned, a total of 60 Actinomycete strains were isolated from soil samples [12-14]. Among them, 16 strains showed antimicrobial activity and were effective against *E. coli, C. albicans* and *S. aureus*. The number of strains with membrane-active ethyl acetate extract that also showed good activity against the test microorganisms was three.

The isolates are shown in Table 1. The results show that the selected strains produced metabolites present in ethyl acetate extract that inhibited the growth of bacteria and yeast in the assay.

Optimized culture conditions

According to previous report, the best duration for incubation is 14 days [12]. The suitable medium for the generation of bioactive compounds, ISP2 and TSB culture media, were used. According to the TLC and bioactivity results, strain 0811 (TSB) and 08346 (ISP2, TSB) were the best for generation of bioactive metabolites. TSB medium was the most appropriate one for the growth of all strains.

PDA vesicles

Membrane activity was measured by PDA vesicles. The color changes were generated by interaction of vesicle solution with membrane active metabolites present in the extract. The transition of blue to red was observed in the extract of strains 08346, 08317, 0811 in both TSB and ISP2 media.

Membrane activity

Graphs produced by artificial membrane on CD spectroscopy show undesirable results with 1X, 1:5X and 1:250X concentrations, hence, the tested concentration was changed to 1:2500X. Although, there was no significant difference among the CD graphs, in the absorbance section of the CD, the difference between the control group (vesicle only) and the other groups is evident.

Metabolite profile

HPLC generates a sensitive and rapid estimation of the profile for secondary metabolites present in ethyl acetate extracts. In Figure 1, the chromatographic profile of ethyl acetate for strain 08346 (ISP2) performed by HPLC at 254 nm, is shown.

Extensive LC-MS fragmentation experiments on the strain extract resulted in the molecular mass, to help in identification of metabolite and find the corresponding structure by NMR (Figure 2).

Table 1: Antimicrobial activity of ethyl acetate fraction of actinomycete cultures against *E. coli, S. aureus and C. albicans,* expressed as µg/mL, using broth dilution method

Strain code/medium	*C. albicans* ATCC 10231		*E. coli* ATCC 29922		*S. aureus* ATCC 25923
	24 h	***48 h***	***24 h***	***48 h***	***24 h***
08-1-1/ ISP2	500	1000	>1000	>1000	>1000
08-1-1/ TSB	500	1000	500	500	250
08-3-17/ISP2	250	250	>1000	>1000	46.8
08-3-17/ TSB	500	1000	1000	>1000	62.5
08-34-6/ ISP2	125	1000	>1000	>1000	>1000
08-34-6/ TSB	250	1000	1000	1000	>1000
Ketoconazol	1.5	1.5	-	-	-
AmB	<0.78	0.78	-	-	-
Streptomycin	-	-	6.25	6.25	50
Kanamycin	-	-	-	-	25
DMSO (v/v)	5%	10%	>10%	>10%	5%

(-) Not tested

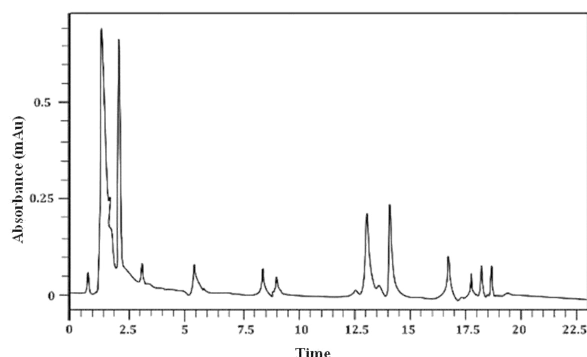

Figure 1: Analysis of HPLC-PAD at 254 nm of the ethyl acetate extract from strain code 08346 cultured on ISP2

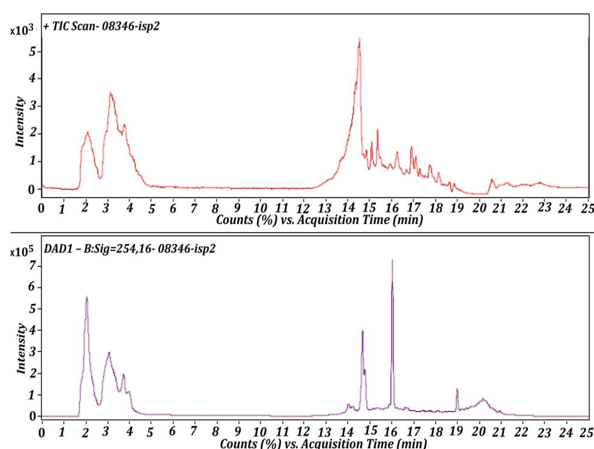

Figure 2: LC-MS chromatogram of crude ethyl acetate fraction from strain 08346/ ISP2. Top: MS detector (total ion count, TIC), bottom: diode array detector (DAD)

Identified components of strains

The best mobile phase for purification of strains was ethyl acetate/hexane 50:50. In this solvent system, TLC showed many metabolite spots that are produced by strain 08-34-6 according to their Rf values (0.22, 0.35 and 0.42). TLC in this solvent system showed clear spots with the mentioned Rf value for extracted 08346 strain.

Ethyl acetate/hexane 50:50 mobile phase system was used for PTLC chromatography for the purification of compounds. One compound, PAF (named by our team as Sourin) was obtained from the bioactive fraction of strain 08-34-6.

Purity of compounds

Purity of the bioactive compounds was analyzed by HPLC and the chromatogram is shown in Figure 3. A sharp single peak was obtained at the retention time of 13.60 min with purity of about 90% for PAF compound isolated.

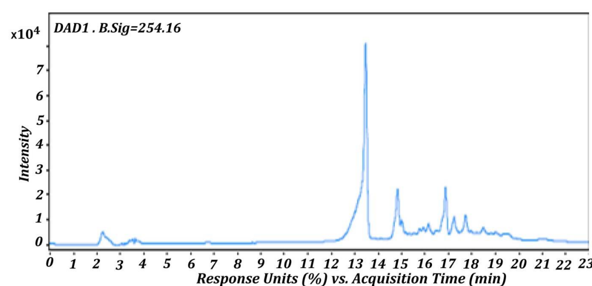

Figure 3: Chromatogram of PAF compound

Mass spectra

The mass spectrum of the purified compound is shown in Figure 4. The purified PAF compound at positive ion mode and the ESI spectrum showed major (M + H) peak at 279 m/z. The calculated molecular mass of the purified compound was found to be 278 for PAF.

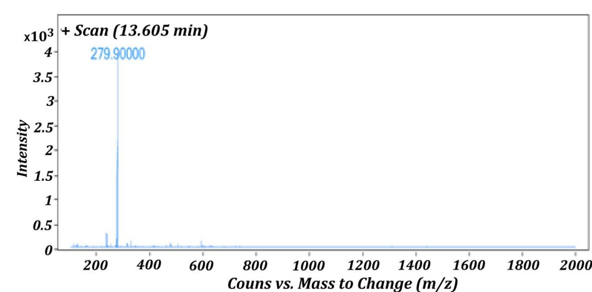

Figure 4: LC-MS spectrum of purified PAF

NMR spectra

^{1}H NMR of the purified compound was obtained with 300 MHz instrument. From the ^{1}H NMR spectra, the chemical shifts were observed and calculated with 4029 NS (number of scans).

^{1}HNMR (MeOD-d6, 300MHz) for PAF: 8.55 (1H,s,H-OH), 7.80 (2H,d,H-3,3´), 7.49 (1H,d,H-6), 7.17 (1H,d, H-5,7), 6.82 (1H, d, H-2,2'), 5.64 (1H, s, NH-8), 4.67 (2H,t,H-9), 1.85 (3H,s,H-4), 1.43 (2H,t,H-10), 1.30 (2H,s,H-11) and 1.16 (3H,d,H-12).^{1}H NMR (MeOD-d6, 300MHz).

2D COSY results

2D COSY experiments were performed in MeOD-d6 with 300 MHz NMR instrument. Inter molecular cross peaks between H-11 and H-12, H-9 and H-10, H-6 and H-5,7, and H-2,2' and H-3,3' are shown in the contour plots of the COSY spectra of PAF. The ^{1}H-^{1}H COSY spectrum correlation amongst signals in the area of 7 ppm indicates the aromatic ring system for PAF.

Inter-molecular cross peaks appear in the contour plots of COSY spectra and show cross

peaks between protons that are adjacent and interacting.

These chemical assignments obtained from the UV, LC-ESI-MS and ^{1}HNMR spectroscopy and COSY helped in the structure elucidation of compound PAF and hence the proposed structure is shown in Figure 5. The empirical formula of the purified compound was suggested to be $C_{19}H_{21}NO$ for PAF (Sourin).

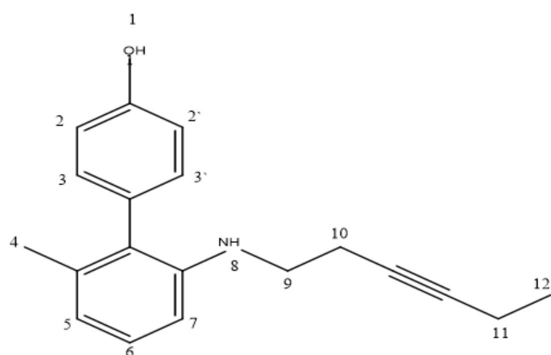

Figure 5: Proposed structure of the compound isolated from strain 08-34-6 (PAF-Sourin).

Based on the molecular weight obtained by the mass spectrum and search in databases like Resurrecting, Chemical Entities of Biological Interest, Super Natural database of natural products, massbank, BML-NMR and processing NMR spectra, it was confirmed that PAF is a new natural product. This report is the first to present data for this compound.

DISCUSSION

Actinomycetes are usually dispersed in the environment and have the ability to produce several biologically active constituents like antiviral, antifungal, antibacterial and antitumor agents. Rahman et al. [19] examined twenty isolates out of 150 Actinomycetes that had antimicrobial activity. Arifuzzaman *et al* [20] investigated twenty isolates that showed activity against bacteria. Also, Dehnad *et al* studied the bioactivity of Streptomyces isolates from Iran [21]. The current study was done to investigate the antimicrobial activity of Streptomyces isolated from soil and purify metabolite from it. The metabolite that was purified from the extract of soil *Streptomyces* was named Sourin and is one of the alkaloid-alkyne type compounds. The functional group characteristics and molecular mass of this molecule were confirmed by NMR and LC-MS techniques. This compound was isolated and purified from the membrane active ethyl acetate fraction. Similar compounds that showed multiple biological activities were reported by Minto and Blacklock [22]. Also, in another research, novel molecules with diverse antibacterial and antifungal activities were reported [23]. None of the reported compounds were associated with membrane disrupting activity.

In other studies, successful application of PTLC and HPLC techniques were reported for the isolation and characterization of biologically active secondary metabolites from *Streptomyces* sample. TLC is a fast and easy procedure that gives a quick glimpse as to how many components could exist in a mixture [24]. Accordingly, CD had been used to monitor interaction of natural products with membrane ingredients [25].

CONCLUSION

In the present study, there was effort to identify membrane active fraction and find Actinomycetes strains that exhibit activity against several opportunistic and microbial pathogens. The results indicate that it is possible to identify different soil living microorganisms that can produce membrane acting substances. Plasma membrane, as a new drug target, is important since most microorganisms can generate resistance to marketed antibiotics and membrane can act as a cellular target, which may not cause resistance to it. Structure determination of compounds in the active fractions is crucial to scale up the production of extracts containing such substances.

DECLARATIONS

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Conflict of interest

The authors declare that no conflict of interest is associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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