

Molecular, chemical and biological screening of soil actinomycete isolates in seeking bioactive peptide metabolites

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ABSTRACT

Background and Objective: Due to the evolution of multidrug-resistant strains, screening of natural resources, especially actinomycetes, for new therapeutic agents discovery has become the interests of researchers. In this study, molecular, chemical and biological screening of soil actinomycetes was carried out in order to search for peptide-producing actinomycetes.

Materials and Methods: 60 actinomycetes were isolated from soils of Iran. The isolates were subjected to molecular screening for detection NRPS (non-ribosomal peptide synthetases) gene. Phylogenic identification of NRPS containing isolates was performed. Chemical screening of the crude extracts was performed using chlorine o-dianisidine as peptide detector reagent and bioactivity of peptide producing strains was determined by antimicrobial bioassay. High pressure liquid chromatography- mass spectrometry (HPLC-MS) with UV-visible spectroscopy was performed for detection of the metabolite diversity in selected strain.

Results: Amplified NRPS adenylation gene (700 bp) was detected among 30 strains. Phylogenic identification of these isolates showed presence of rare actinomycetes genera among the isolates and 10 out of 30 strains were subjected to chemical screening. *Nocardia* sp. UTMC 751 showed antimicrobial activity against bacterial and fungal test pathogens. HPLC-MS and UV-visible spectroscopy results from the crude extract showed that this strain has probably the ability to produce new metabolites.

Conclusion: By application of a combined approach, including molecular, chemical and bioactivity analysis, a promising strain of *Nocardia* sp. UTMC 751 was obtained. This strain had significant activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Strain *Nocardia* sp. UTMC 751 produce five unknown and most probably new metabolites with molecular weights of 274.2, 390.3, 415.3, 598.4 and 772.5. This strain had showed 99% similarity to *Nocardia ignorata* DSM 44496 T.

Keywords: Actinomycetes, Molecular screening, Non-ribosomal peptide synthetases, Peptide

INTRODUCTION

In recent years multi-drug resistant strains of pathogens has emerged because of extensive use

of antimicrobials in medicine, animal husbandry and agriculture (1). Also, prevalence of cancer and emergence of new diseases provoked scientists and pharmaceutical companies to discover novel therapeutic agents (2).

Small bioactive peptides are a well-known group of therapeutic agents, including β -lactams, tyrocidin, gramicidins, cyclosporine, polymyxins, lipopeptides such as daptomycin and surfactin (1). Peptides are divided into two groups based on their biosynthesis routes: non-ribosomal and ribosomal. Ribosomal

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peptides are synthesized by all living creatures including mammals, amphibians, insects, plants, bacteria, and even viruses (3). Non-ribosomal peptides are synthesized by special and well-organized multi-enzymes, non-ribosomal peptide synthetases (NRPS) in restricted organisms. Bioactive peptides comprise a wide range of biological activities including, antiviral, antiprotozoal, hypocholesterolemic, antifungal, siderophore (4), antimicrobial (5;6), antitumor (7), immunosuppression (8), antioxidant (9), anti-hypertensive (10) and immunomodulatory activities (11;12). Actinomycetes are a major group of soil inhabitant bacteria. Up to now, more than 22 thousands microbial bioactive compounds are known, that half of them are produced by actinomycetes, (13). High frequencies of NRPS biosynthetic systems (58%) is reported among actinomycetes (14). Due to more than 80 years researches on actinomycetes, the chances of finding novel bioactive molecules through the isolation and screening of predominant actinomycetes, the members of *Streptomyces* genus has dramatically decreased (15;16). Thus, it is crucial that new groups of non-*Streptomyces* actinomycetes be pursued as sources of novel pharmaceutically active metabolites (17). It is shown that rare actinomycetes, which are difficult to isolate and culture may be a unique source for future drug discovery (18).

Because of the significant importance of non-ribosomal peptides in modern medicine, finding NRPS gene containing strains is of great value. The presence of NRPS gene in isolates can be proved by molecular screening but it alone cannot show production of non-ribosomal peptides. Therefore, expression of NRPS clusters was tracked by conducting chemical screening of the strains. Chemical screening is an easy and rapid approach that recruits specific reagents to visualize the biosynthetic capacity of strain in production of different skeleton of secondary metabolites, including sugars, lipids, flavonoids, phenols, arylamines, steroids, terpenes, chloroamines, carbamates, antibiotics, surfactants, peptides, sulfonamides (19;20).

With the aim of attaining sources of bioactive peptides and due to the high diversity of non-ribosomal peptides in actinomycetes, recovered soil actinomycetes were subjected to molecular screening in order to find NRPS gene containing strains. Strains encompassing NRPS clusters were chemically screened and evaluation of their bioactivity was determined by antibacterial bioassay. HPLC (high

pressure liquid chromatography) measurements in combination with mass spectrometry, providing an impression of the metabolite diversity produced by selected strain (21) which was used in metabolite analysis of the selected peptide producing strain.

MATERIALS AND METHODS

Soil sampling and isolation of actinomycetes.

The soil samples were collected aseptically from 20 cm depth of the various habitats of Iran. The samples were immediately taken to the laboratory and stored at 4 °C. The soil samples were dried at room temperature for 1 week, then they crushed and sieved using 2 mm sieve (22). Then, the soil samples were pretreated at 50 °C for 24h before isolation of actinomycetes. One gram of each soil sample was suspended in 10 ml sterile distilled water and vortexed for 45 seconds. The mixtures were allowed to settle, and various concentrations of the spore suspensions were inoculated and spread on the surface of ISP2 agar (malt extract 10 g, yeast extract 4 g, glucose 4 g, agar 15 g in one liter of tap water) and incubated at 28 °C for 7-21 days. The actinomycete putative colonies were selected and transferred to fresh ISP2 agar. The actinomycete isolates were purified by repeated sub-culturing on ISP2 medium and the pure cultures were maintained at -20 °C for further use.

Molecular screening. The isolates were revived on ISP2 agar at 28 °C for 7 days. For genomic DNA isolation, one single colony was cultured in LB medium and was incubated at 28 °C at 220 rpm. After 48 h, the biomass were harvested and stored in -20 °C. DNA extraction was performed according to standard protocols (23).

PCR amplification of NRPS gene and DNA sequencing. PCR amplification of NRPS gene in the isolated strains was done using A3F, A7R degenerate primers (A3F, GCS TAC SYS ATS TAC ACS TCS GG; A7R, SASGTCVCCSGTSCGGTAS) (24).

Each PCR reaction contained 2 µl of genomic DNA, 0.5 µM of primers, 1.5 mM MgCl₂, 0.15 µM dNTPs, 5% DMSO, 1X standard *Taq* reaction buffer, 2 units of *Taq* DNA polymerase (Genet Bio) and dH₂O up to 50 µl. PCR was performed according to the following condition: 96 °C for 3 min, 35 cycles of (96°C for 30s, 59-54 °C for 40s, 68°C for 140-190 s), 72 °C for 10 min. PCR products were observed

through 1% agarose gel electrophoresis and ethidium bromide staining. The PCR products were sequenced by Macrogen (South Korea) and compared with that of other validated species in EzTaxon database.

Molecular characterization of selected isolates.

The isolates containing NRPS gene were subjected to PCR amplification of 16S rRNA gene with two universal primers, 9F (AAG AGT TTG ATC ATG GCT CAG) and 1541R (AGG AGG TGA TCC AAC CGC A) (25). PCR condition was same as past PCR (for NRPS gene) except annealing temperature which was 54°C. After detection of PCR products via 1% agarose gel electrophoresis, the product was sequenced by Macrogen (South Korea) (26;27).

Fermentation and extraction of metabolites.

Production of bioactive compound of the strains containing NRPS gene was done by fermentation in flasks. Actinomycete isolates were inoculated in 50 ml of ISP2 medium as seeding medium into 250 ml-Erlenmeyer flasks under aseptic conditions and incubated at 28°C for 48h at 220 rpm. Appropriate seeding material (5% v/v) was inoculated into 250-ml of ISP2 medium as fermentation broth medium into 1000ml-Erlenmeyer flasks and was incubated in a rotary shaker at 220 rpm, 28°C, for 7 days (28). After fermentation, the media were centrifuged at 4000 rpm for 20 min to remove the cell and debris. The supernatant was extracted by equal volume of ethyl acetate. The organic solvent was evaporated at low temperature and pressure to obtain metabolite extract (29;30). The extract of uninoculated fermentation medium was obtained as described above and used as negative control during the experiments.

Chemical screening of crude extracts. The crude extracts were dissolved in dichloromethane-methanol (CH₂Cl₂/MeOH) 92:8 before analysis. TLC screening performed on a silica gel 60 F₂₅₄ plate (Mecherey-Nagel). After drying, the silica plates were sprayed with chlorine o-dianisidine as peptide detection reagent. Green colored zones produced on a plate background were the indication of positive results. Ninhydrin reacting with free amines was used in order to detect the linear or cyclic nature of the peptides. Yellow colored zone formation was the indication of positive results.

Retention factor (R_f) of the compound was also determined after running thin layer chromatography.

Virginamycin was used as a positive control.

Bioassay of antimicrobial activity. Antimicrobial bioassay was carried out by agar well diffusion method against *Bacillus subtilis* UTMC 1416, *Pseudomonas aeruginosa* UTMC 1404, MRSA (methicillin resistant *staphylococcus aureus*) UTMC 1401, *Aspergillus niger* UTMC 1431 and *Candida albicans* UTMC 1430. After fermentation, the media were centrifuged at 4000 rpm for 20 min to remove cell and debris. The wells were aseptically bored in plates seeded with pathogenic strains and filled with 100-250 µl of culture filtrates and incubated for 72 hours at 28°C and 24 hours at 35°C for fungi and bacteria, respectively. The diameter (mm) of the inhibition zone around each well was measured (31-33). The bioassays were done in triplicates.

Chromatographic conditions for dereplication using HPLC-MS analysis. Crude extract of the selected isolate was subjected to mass analysis and HRESIMS data were recorded on a Maxis ESI TOF mass spectrometer (Bruker Daltonics), and molecular formulas were calculated including the isotopic pattern (Smart Formula algorithm).

Analytical RP HPLC was carried out with an Agilent 1260 HPLC system equipped with a diode-array UV detector (DAD) at 200-600 nm and a Corona Ultra detector (Dionex) or a Maxis ESI TOF mass spectrometer. HPLC conditions: Waters Acquity UPLC BEH C18 column 50 × 2 mm, 1.7 µm, solvent A: H₂O, 0.1% HCOOH; solvent B: acetonitrile, 0.1% HCOOH; gradient system: 5% B for 1 min, increasing to 95% B in 20 min; flow rate 0.6 mL/min; 40 °C.

RESULTS

Isolation of actinomycete strains. From 15 soil samples of different habitats, 60 putative actinomycetes strains were isolated based on Gram staining and colony morphology. The isolates were observed to have the colonies with white, brown, yellow, cream, orange, surfaces. The isolates strains were deposited in University of Tehran Microorganisms Collection (UTMC).

Screening of actinomycetes with NRPS genes. All 60 strains were subjected to DNA extraction and PCR using degenerate specific NRPS primers. A band with about 700 bp length was detected in 30 isolates

by PCR screening of the A domain (Fig 1). Analysis of the NRPS sequences with registered genes in the NCBI database confirmed the 60-80% similarity of 10 among 30 investigated isolates. Afterwards phylogenetic identification of the strains containing NRPS gene were done using 16SrRNA gene amplification. Sequences were compared with that of other validated species in EzTaxon to database. All of the 30 bacteria show high similarity (>98%) to the relatives in GenBank (34). Phylogenetic identification of these 30 isolates shows presence of NRPS gene in rare actinomycetes such as *Nocardia*, *Saccharothrix*, *Micromonospora* in addition to *Streptomyces* genus. The sequenced genes were submitted to the GenBank and their accession numbers are listed in Table 1. Finally 10 out of 30 investigated isolates were selected for chemical screening.

Primary detection of peptide compounds by chemical screening. Results of primary chemical screening using absorption measurement at 254 nm and 366 and reaction with chlorine o-dianisidine were revealed that strains UTMC 751, 827, 736, 509 and 555 were recognized as peptide producing strains. None of the spots react with ninhydrin reagent.

Antimicrobial bioassay of selected strains. According to Table 2, the antimicrobial assays against five microbial pathogens showed that strain *Nocardia* sp. UTMC 751 has a broad-spectrum antimicrobial activity against tested bacterial and fungal pathogens.

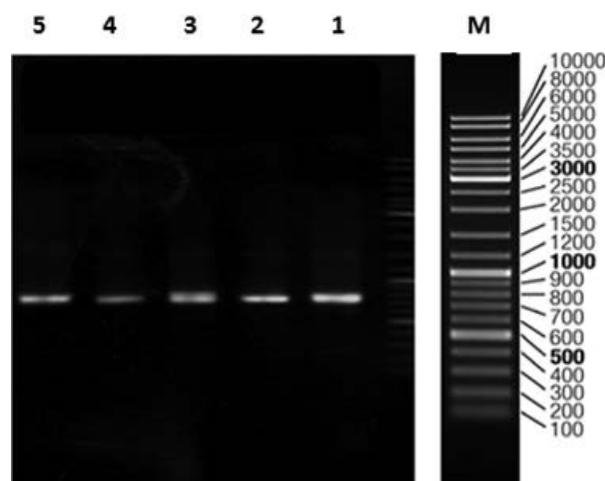


Fig. 1. PCR amplification of NRPS gene sequences using primers A3F/A7R on agarose gel 0.8%. Lines, M: Marker, 1: *Micromonospora* sp. UTMC 509, 2: *Micromonospora* sp. UTMC 555, 3: *Micromonospora* sp. UTMC 736, 4: *Nocardia* sp. UTMC 827 and 5: *Nocardiasp.* UTMC 751.

Detection of *Nocardia* sp. UTMC 751 secondary metabolites. Analysis of produced secondary metabolites by *Nocardia* sp UTMC 751 in ISP2 medium revealed a number of compounds which are produced in enough amounts which are retrievable from the crude extract after separation process. Some compounds were attributed to insostamycin or monensin (656.4), mycolacton (714.5) and miamycin (615.3) based on their molecular weight values and UV absorption spectra.

Also, *Nocardia* sp. UTMC 751 showed the ability

Table 1. Phylogenetic features of 10 selected actinomycete strains and similarity with registered NRPS gene in GenBank.

| UTMC Code | Length (bp) | Accession number | Closest relative and its accession number | % Identity |
|-----------|-------------|------------------|---|------------|
| UTMC 751 | 936 | KF770971 | <i>Nocardia ignorata</i> DSM 44496(T) | 99% |
| UTMC 1046 | 900 | KF770976 | <i>Streptomyces griseorubiginosus</i> NBRC 13047(T) | 100% |
| UTMC 428 | 933 | KF770978 | <i>Streptomyces rectiviolaceus</i> NRRL B-16374(T) | 99.8% |
| UTMC 728 | 939 | KF356151.1 | <i>Streptomyce skasugaensis</i> M338-M1(T) | 99.6% |
| UTMC 736 | 934 | KF356152.1 | <i>Micromonospora endolithica</i> DSM 44398(T) | 99.9% |
| UTMC 537 | 886 | KF770972 | <i>Saccharothrix yanglingensis</i> Hhs.015(T) | 99.3% |
| UTMC 555 | 811 | KF770973 | <i>Micromonospora aurantiaca</i> ATCC 27029(T) | 100% |
| UTMC 509 | 929 | KF770974 | <i>Micromonospora sedimicola</i> SH2-13(T) | 99.3% |
| UTMC 827 | 941 | KF770975 | <i>Nocardia ignorata</i> DSM 44496(T) | 99% |
| UTMC 875 | 941 | KF770977 | <i>Micromonospora tulbaghia</i> TVU1(T) | 100% |

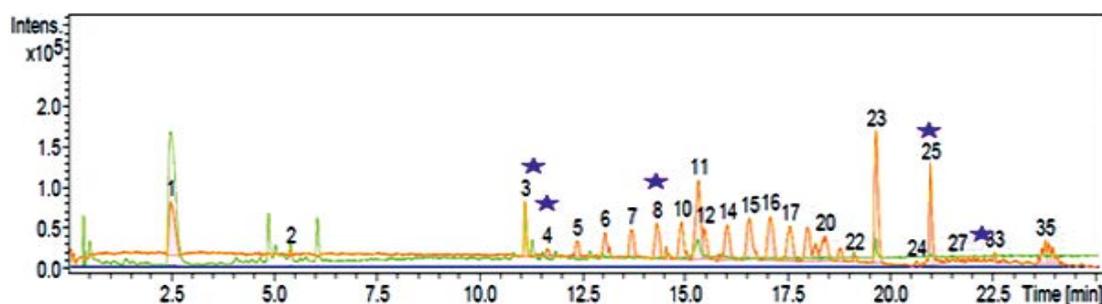


Fig. 2. Total ion chromatogram of major secondary metabolites of *Nocardia* sp. UTMC 751 crude extract. The compounds are assigned based on retention time and intensity of compounds in crude extract (Y: Intensity and X: Retention time). Metabolites with probable new structure features are indicated by star: Cmpd 3 11.27 min: 274.2 D, Cmpd 4, 11.74 min: 598.4D, Cmpd 8, 14.50 min: 415.3 D, Cmpd 25 21.31 min: 390.3 D, Cmpd 30, 22.07 min: 772.5D

to produce five new metabolites with molecular weights of 274.2, 390.3, 415.3, 598.4 and 772.5 probably, based on their molecular weight values and UV absorption spectra which did not matched with the natural compounds recorded in natural product database (Figs. 2 and 3).

DISCUSSION

Most of the known peptide compounds in actinomycetes, including penicillins, cephalosporins, glycopeptides, vancomycin and actinomycins are non-ribosomal peptides which are synthesized by NRPS enzymes. The high detection levels (50%) of NRPS biosynthetic systems observed in our isolates confirmed the wide distribution of these sequences in this bacterial group.

In addition, the chance of finding novel peptides through screening of actinomycetes especially the genus *Streptomyces* has decreased. Therefore, the probability of discovering a novel peptide can be increased via screening of rare actinomycetes containing NRPS genes. In the present study phylogenetic identification of NRPS genes containing isolates shows presence of NRPS genes in rare

actinomycetes such as *Nocardia*, *Saccharothrix*, *Micromonospora* in addition to *Streptomyces* genus. Since molecular screening by itself cannot prove production of non-ribosomal peptides, chlorine o-dianisidine that reacts with peptides was used to find peptide-producing the strains. Selection of the strains for chemical screening was performed based on phylogenetic analysis and percent identity of NRPS gene to their closest neighbors in GenBank. Due to the existence of $-NH-CO-$ groups in peptides, chlorine o-dianisidine has greater ability to detect peptides. Positive reaction of TLC spots with chlorine o-dianisidine reagent suggests they are peptides and the absence of coloration of the TLC spots with ninhydrin suggests they are cyclic (35).

Easy to perform, speed, and low cost of TLC has made it a widely used technique. Using chemical screening, we were able to determine to some extent the peptide-producing strains and diversity of metabolites of the strains before dereplication (rapid identification of known compounds) and large-scale production. Peptides have a wide range of biological activity (36). When a peptide component shows a kind of biological activity, the probability of finding more activities can be increased. Since short peptides

Table 2. Antimicrobial activities of selected isolates.

| Strain | Microbial test strains | | | | |
|------------------------------------|------------------------|------------------|-----------------|----------------------|--------------------|
| | <i>C. albicans</i> | <i>S. aureus</i> | <i>A. niger</i> | <i>P. aeruginosa</i> | <i>B. subtilis</i> |
| <i>Micromonospora</i> sp. UTMC509 | - | - | - | - | - |
| <i>Micromonospora</i> sp. UTMC 736 | - | - | - | 11 | - |
| <i>Micromonospora</i> sp. UTMC 555 | - | 12 | - | - | - |
| <i>Nocardia</i> sp. UTMC 751 | - | 19 | 14 | 18 | - |
| <i>Nocardia</i> sp. UTMC 827 | - | 12 | - | 12 | - |

(-) no activity, weak activity (5–10 mm), mediocre activity (10–15 mm) and high activity (15–20 mm).

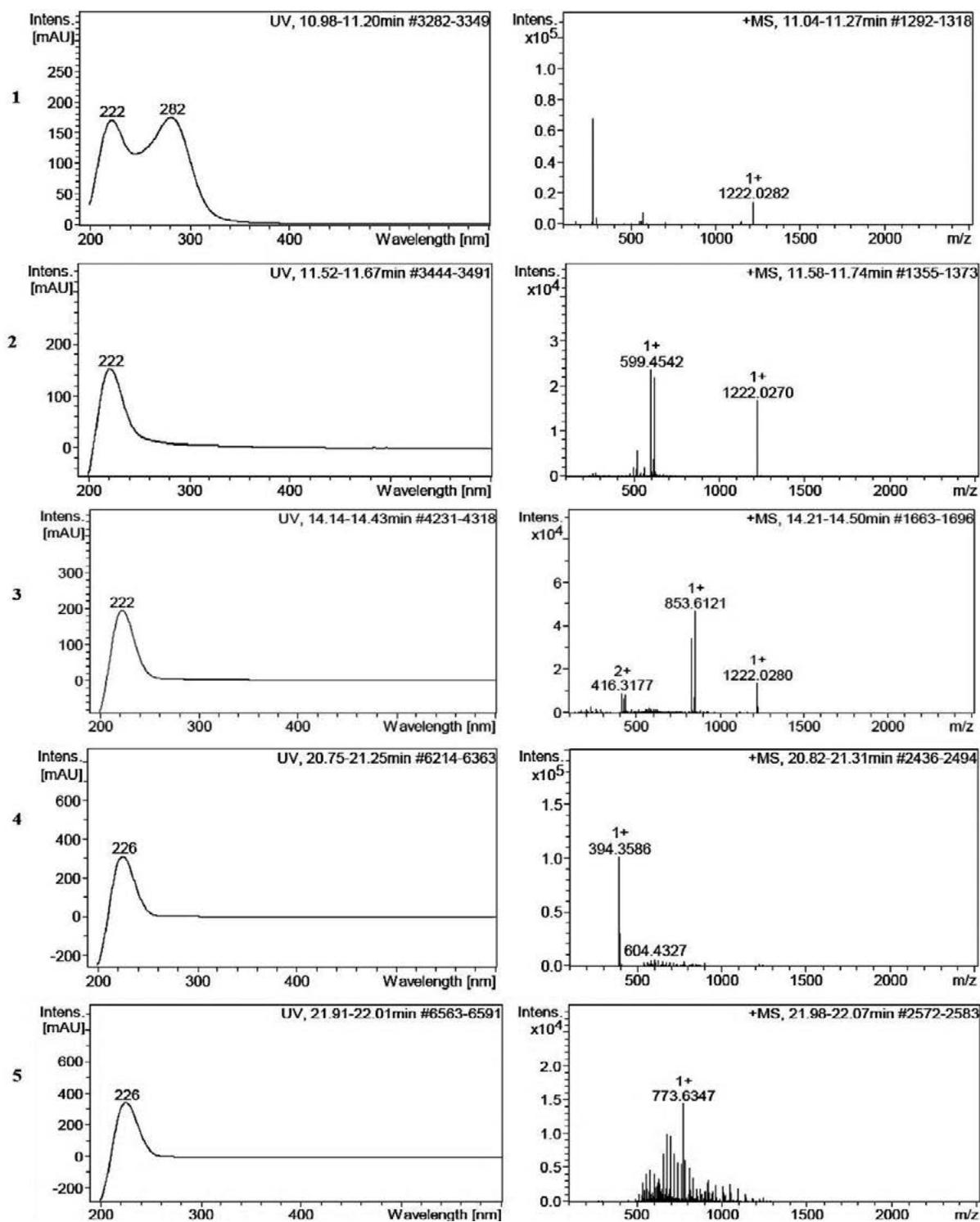


Fig. 3. Molecular mass and UV-Vis absorption spectra of probable new compounds. 1: Cmpd 3 11.27 min: 274.2 D, 2: Cmpd 4, 11.74 min: 598.4D, 3: Cmpd 8 14.50 min: 415.3 D, 4: Cmpd 25 21.31 min: 390.3 D, 5: Cmpd 30, 22.07 min: 772.5D.

(1–50 amino acids) with cationic and hydrophobic properties, react with anionic components of the cell membrane through electrostatic interactions and providing activity against a wide variety of pathogenic microorganisms such as Gram-negative and Gram-positive bacteria, fungi, viruses, and parasites (37). In this study, in order to decrease the risk of selection peptide-producing strain, antibacterial bioassay against microbial pathogens was carried out to find strain with broad antimicrobial spectra and strain *Nocardia* sp. UTMC 751 was obtained based on molecular screening and chemical screening.

Strain *Nocardia* sp. UTMC 751 showed activity against *P. aeruginosa* and *S. aureus* which are quickly increasing as antibiotic resistant strains and the efficacy of many antibiotics against these pathogens has become limited. *P. aeruginosa* as a Gram-negative bacteria are often isolated from patients with MRSA infections (38). Hence monotherapy with active drugs against Gram-positive bacteria is not effective against polymicrobial infections by *S. aureus* and *P. aeruginosa* (39).

Different antimicrobial spectra exhibited the potential chemical diversity of its bioactive metabolites of *Nocardia* sp. UTMC 751 and represent the production of diverse peptides by this strain. Since many species of the genus *Nocardia* can cause serious infections in humans and animals, they are considered to be biologically more active than non-pathogenic species and so they can produce a variety of pharmaceutical agents.

HPLC-MS analysis of the crude extract produced by this strain gave a complete picture of its metabolic diversity. The molecular masses resulting from HPLC-MS measurements were searched in natural product database. The results indicate that five compounds with molecular weights of 274.2, 390.3, 415.3, 598.4 and 772.5 D have probably not been isolated from microorganisms before.

CONCLUSION

In summary, concerning actinomycetes importance in producing numerous natural compounds, we screened NRPS gene among actinomycetes isolated from soils of Iran. Consequently, 10 strains of actinomycetes containing NRPS genes were phylogenetically characterized which showed that that most of them were rare actinomycetes and were subjected for chemical and biological assay. In fact,

the combined strategy of molecular and chemical analysis in parallel to bioactivity, was found as an effective approach in screening of bioactive peptide producing strains. Accordingly, strain *Nocardia* sp. UTMC 751 is introduced as valuable candidates of strain harboring the capacity of producing bioactive peptides. The bioactive peptide produced by this strain probably is among these five unknown compounds which will be subjected to structural elucidation after large scale fermentation and separation process that will be accomplished in further work.

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