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Evaluation of growth inhibition activity of myxobacterial extracts against multi-drug resistant *Acinetobacter baumannii*

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ABSTRACT

The worldwide dissemination of multi drug resistant *Acinetobacter baumannii* strains has caused serious concern and high rate of mortality in recent decades that originate from limited effective antibiotics in the treatment of *A. baumannii* infections. Myxobacteria are Gram-negative bacteria that are important for their complex lifestyle and production of novel structurally secondary metabolites with diverse bioactivities. In this study, a total of 60 myxobacterial strains were purified by culturing and investigation of 130 soil samples. Secondary metabolite extracts of the selected strains were screened for antibacterial activity against multi-drug resistant (MDR) *Acinetobacter baumannii*. The most potent extracts derived from *Stigmatella* sp. UTMC 4081, *Stigmatella* sp. UTMC 4072, and *Archangium* sp. UTMC 4070 which were investigated by recording percentage of growth inhibition, MIC, MBC, and IC50 values. The results showed that the MIC value of extract No. 4072 was 2.5 µg/ml and its MBC value against *A. baumannii* recorded as 5 µg/ml. Extract No. 4081 was known to be active with MIC of 2.5 µg/ml and MBC of 10 µg/ml. In addition, MIC and MBC values of extract No. 4070 were found to be 10 and 25 µg/ml, respectively. Myxobacterial extracts showed no toxicity against *Artemia salina*. This study demonstrated the importance of myxobacterial metabolites as promising antimicrobial agents against multi drug resistant *A. baumannii.*

Keywords: *Myxobacteria*; *Stigmatella*; Antimicrobial activity; Multi-drug resistant bacteria

Introduction

Bacterial resistance to a number of antibiotics has been dramatically increased in last decade and caused critical worldwide problems for health care programs and treatment of patients. According to the World Health Organization (WHO) report, a high number of nosocomial infections are caused by multi-drug resistant bacteria including several Gram-negative pathogenic bacteria (1). Multi-drug resistant (MDR) Gram-negative bacteria are responsible for serious infections in patients worldwide, which are gradually exhibiting resistance to a broad-range of available antibiotics (2). *Acinetobacter baumannii* is an opportunistic pathogen that can cause a number of severe infections, especially in patients with prolonged

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hospitalization period and infants (3). The most strains of *A. baumannii* species are resistant to several classes of antibiotics (4, 5) through multiple mechanisms such as β-lactamases, efflux pumps, permeability defects, alteration of target sites, and aminoglycoside-modifying enzymes (6). Therefore, developing anti-biotic resistance mechanisms in *A. baumannii* and lack of sufficient antimicrobial agents may increase the mortality rate in future. Currently, the MDR *A. baumannii* is resistant to minimum three classes of antibiotics including cephemes, carbapenems, β-lactams, aminoglycosides, and penicillins. To overcome the widespread prevalence of infections caused by MDR pathogens, searching for new antibacterial agents is necessary.

As a valuable source of therapeutic agents, natural products and their semi-synthetic derivatives, have been accounted for 50% of drugs approved in a 25-year period between 1981 and 2007 (7, 8). These compounds originate from plants, animals and microorganisms. Out of them, microorganisms have shown the largest chemical diversity in their secondary metabolites. *Myxobacteria* are Gram-negative, predominantly soil dwelling, and high G+C content bacteria with characteristics of gliding motility, predation of microorganisms, and formation of the multicellular fruiting body. The order *Myxococcales* contains three suborders, 11 families, 30 genera and 59 species (9). *Myxobacteria* have been considered in the last decade as a rich source of bioactive secondary metabolites containing high structurally diverse molecules. They have recently received notable attention in drug discovery programs due to the novel structure and modes of action of the majority of their identified metabolites (10). Many myxobacterial antibacterial metabolites have been reported, including myxovirescin (11), althiomycin (12), myxovalargin (13), sorangicin (14, 15), saframycin (16), sorangiolid (17), chondrochlorens (18), and thuggacins (19).

This project was aimed at finding a myxobacterial source with the capability to produce effective metabolites against the current nosocomial infections of MDR *A. baumannii*. In this study, we collected several samples from various geographical areas of Iran to isolate myxobacterial strains. The isolates were identified based on morphological and molecular characteristics and were subjected to metabolite extraction. Myxobacterial extracts were screened against MDR *A. baumannii* to find a valuable source for anti-Acinetobacter natural compounds.

Materials and methods

Test microorganism

The MDR *A. baumannii* UTMC 126 was isolated from a patient at Mofid Children's Hospital in Tehran (Iran) in 2016. The strain exhibited a resistance spectrum to ceftazidime, cefepime, cefexime, ticarcillin, amikacin, cefotaxime and ciprofloxacin. For this reason, *A. baumannii* UTMC 126 was selected as an antibiotic resistant strain for further antibacterial tests. The strain was preserved in TSB medium containing 20% glycerol as the cryoprotectant and stored at -80ºC.

Myxobacteria isolation and purification

One hundred thirty soil samples were collected from different geographical areas of Iran, air dried and kept at 4ºC before culturing. The isolation media included WCX ($CaCl_2.2H_2O$, 0.1%; agar, 1.5%; and pH, 7.2) and chitin agar (chitin, 0.5%; $MgSO_4.7H_2O$, 0.1%; $CaCl_2.2H_2O$, 0.1%; KNO_3, 0.1%; $(NH_4)_2SO_4$, 0.02%; K_2HPO_4, 0.02%; agar, 1.5%; and pH, 7.4) supplemented by cycloheximide (30 μg/ml). A small amount of each soil sample was placed onto the isolation agar media and incubated at 30ºC up to 4 weeks, depending on the strains. The myxobacterial fruiting bodies were recognized and transferred to the VY2 medium (Barkers yeast, 0.5%; $CaCl_2.2H_2O$, 0.1%; agar, 1.5%; and pH, 7.2) as the purification medium. The purification process was performed several times until complete pure swarms were obtained (20). The purified strains were cultured in liquid CY broth medium and maintained at -70ºC without cryoprotectant.

Morphological identification

All isolates were cultivated on VY2 and CY (casiton, 0.3%; yeast extract, 0.1%; $CaCl_2.2H_2O$, 0.1%; agar, 1.5%; and pH, 7.2) media for stereomicroscopic assessment (Hund, Germany) of the swarming pattern and morphology of the fruiting bodies. The MD1

medium (casitone, 0.3%; $CaCl_2.2H_2O$, 0.1%; $MgSO_4.7H_2O$, 0.2%; vitamin B12, 0.5mg/l) (20) was used in morphology determination of vegetative cell, myxospore and pigmentation property of the strains in the liquid culture. Morphological properties and taxonomic position were identified using Bergey's Manual of Systematic Bacteriology (21).

Molecular identification and phylogenetic analysis

Extraction of DNA was carried out using the chloroform-phenol method, and the DNA samples were kept at -20ºC (22). Bacterial 16S rRNA gene was amplified (SensoQuest Thermocycler, Denmark) using universal 16S rRNA gene primers (9F, 1541R). PCR products were purified using the Expin combo GP (Geneall, Korea) kit and were run using 0.8% agarose gel. After staining with ethidium bromide, they were visualized using an Image Analyzer GelDoc (UVP, England).

Myxobacterial metabolite extracts

The representative strains from different genera were selected based on phylogenetic characteristics and were cultured in H broth medium (soy meal, 0.2%; glucose, 0.2%; starch, 0.8%; yeast extract, 0.2%; $MgSO_4.7H_2O$, 0.1%; $CaCl_2.2H_2O$, 0.1%; Fe-EDTA, 0.0008%, pH, 7.2) as a seeding medium. The fermentation medium, H medium supplemented with 1% Amberlite XAD-16 resin (Sigma-Aldrich, USA), was inoculated with 10% of 48h grown seeding culture. After 5 days incubation at 30ºC (180 rpm), biomass and XAD resin were harvested and extracted several times using methanol. The extracts were concentrated under reduced pressure at 40ºC using a rotary evaporator (Heidolph, Germany) and maintained at -20ºC.

Cytotoxicity assay

The eggs of *Artemia salina* were hatched in aerated artificial sea water at 25ºC within 48h of incubation to obtain nauplii. Dilutions of extracts were made using DMSO in a 48-well microplate. A suspension containing 15-20 nauplii was added to each microplate well, containing extracts dilutions and incubated at 25ºC for 24 h. DMSO and dichromate potassium were used as negative and positive controls, respectively. The number of dead cells was counted and toxicity percentage of extracts were calculated (23).

Antibacterial activity assay

Primary screening of antibacterial activity of myxobacterial extracts against *A. baumannii* was performed by disc diffusion method with two concentration levels of the extracts (50 and 100 µg). Imipenem disc (10 µg) was used as a positive control against *A. baumannii*, and the inhibition zones were reported in mm.

Chemical screening and fractionation of extracts

Thin layer chromatography was performed on TLC aluminum sheets, silica-gel 60 F254 (Merck 5554, Germany). The crude extracts, dissolved in methanol, were located on TLC plate and run using dichloromethane/methanol (9:1; v/v) under solvent vapor saturation condition in a tank. Visualization was carried out using UV irradiation at 254 and 366 nm, and subsequent staining with anisaldehyde reagent.

Minimum inhibitory concentration (MIC)

The MIC values were determined by microdilution method according to the standard of CLSI-2015 (24). Myxobacterial extracts were diluted in DMSO and 20 µl of the extract dilution plus 80 µl of Mueller-Hinton broth (Merck, Germany) and 100 µl of a 10^7 CFU/mL of *A. baumannii* solutions were added to each well of a sterile 96-well plate and the final volume in each well was 200 µl. Each concentration was tested with five replications. Imipenem powder was prepared in Mueller-Hinton broth and used as a positive control. The microplate was shaken for 20 sec; thereafter, incubated at 37ºC and turbidity was measured at 600 nm after 24 h. The MIC value was considered as the lowest concentration of the extract, in which no bacteria growth was observed. To obtain the minimum bactericidal concentration (MBC) 10 µl of clear wells suspension was removed and plated on Mueller-Hinton agar medium. The percentage of the growth inhibition was calculated according to the formula $((Ac–At)/Ac) \times 100$, where *Ac* was the average of absorbance of negative controls and *At* was the average of absorbance

of samples at wavelength 600 nm.

Results

Myxobacterial strains

A total of 60 out of 100 myxobacterial strains were purified from 130 soil samples collected from different habitats of Iran. Most of the isolates were observed on WCX medium with *E. coli* streaks as the baiting. According to the morphological traits (shapes of vegetative cells, myxospores, and fruiting bodies) and molecular characterization, the isolates were classified into seven genera, including *Myxococcus*, *Corallococcus*, *Archangium*, *Stigmatella*, *Chondromyces*, *Cystobcater*, and *Nanocystis*.

Cytotoxicity assay

According to the results of brine shrimp lethality assay, the extracts showed no toxicity against *Artemia salina* at the concentration of 200 µg/ml. In addition, DMSO at concentration of 1.2 mM was almost non-toxic for cells as it rendered 2.5% mortality of cells.

Antibacterial activity of extracts

The extracts of *Stigmatella* sp. UTMC 4072, *Stigmatella* sp. UTMC 4081 and *Archangium* sp. UTMC 4070 exhibited antibacterial inhibitory effect against *A. baumannii*. The inhibition zone diameters of extracts and imipenem as the reference antibiotic are presented in Table 1. Among the active extracts, *Stigmatella* sp. UTMC 4081 and *Archangium* sp. UTMC 4070 presented the most and least activities against *A. baumannii* with 18 mm and 11 mm inhibition zones diameter at the concentration of 100 µg/disc, respectively.

Table 1. Antibacterial activity of myxobacterial extracts against *Acinetobacter baumannii* in disc diffusion method. The diameter of inhibition zone of imipenem (10 µg) as a positive control was 15 mm.

	Diameter of inhibition zone (mm)	
	A. baumannii	
Strain	**50 µg**	**100 µg**
Stigmatella sp. UTMC 4081	16	18
Corallococcus sp. UTMC 4066	-	-
Corallococcus sp. UTMC 4061	-	-
Chondromyces sp. UTMC 4096	-	-
Archangium sp. UTMC 4059	-	-
Stigmatella sp. UTMC 4072	10	16
Archangium sp. UTMC 4070	-	11

Minimum inhibitory concentration

Antibacterial effect of *Stigmatella* sp. UTMC 4081, *Stigmatella* sp. UTMC 4072, and *Archangium* sp. UTMC 4070 extracts were evaluated on multi-drug resistant *A. baumannii* by microdilution method. Antimicrobial activities of extracts were reported as MIC, MBC and IC50 values (Table 2). Interestingly, the extracts from two *Stigmatella* sp., including UTMC 4081, and UTMC 4072 exhibited identical values for MIC (2.5 µg/ml) and IC50 (1.48 µg/ml) values on *A. baumannii*. However, the former strain showed two-time higher value for MIC, compared with that of the latter one. Antibacterial activity of *Archangium* sp. UTMC 4070 extract was reported with MIC 10 µg/ml, MBC 25 µg/ml and IC50 7.13 µg/ml against the same test microorganism.

The percentages of the bacterial growth inhibition are illustrated in Fig. 1 which shows quite a low IC50 (< 1 µg/ml) for extracts of strains UTMC 4081 (a) and UTMC 4072 (b).

Table 2. Antibacterial activity index of myxobacterial extracts against *Acetinobacter baumannii* UTMC 126. MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; IC50, the half maximal inhibitory concentration.

MDR test bacteriUM	4081 (µg/ml)			4070 (µg/ml)			4072 (µg/ml)		
	MIC	MBC	IC_{50}	MIC	MBC	IC_{50}	MIC	MBC	IC_{50}
A. baumannii UTMC 126	2.5	10	1.48	10	25	7.13	2.5	5	1.48

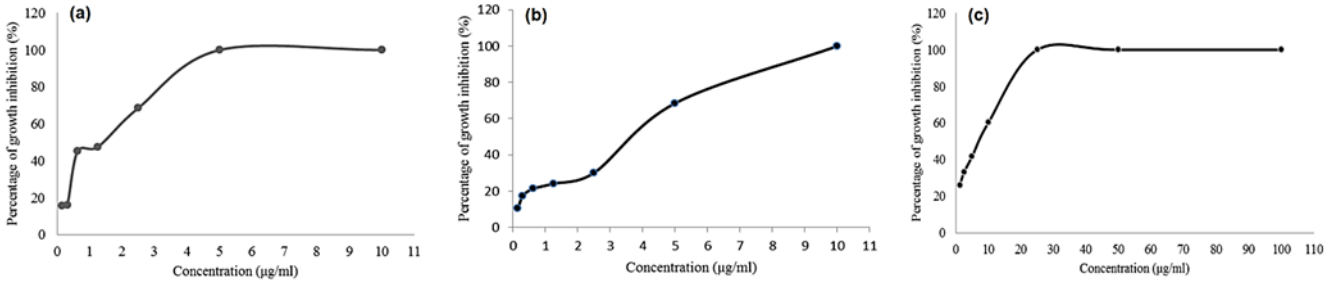

Figure 1. Percentage of growth inhibition of *A. baumannii* by myxobacterial extracts of strains UTMC 4081(a), UTMC 4072 (b) and UTMC 4070 (c).

Discussion

The emergence of antimicrobial resistance has threatened patients' lives worldwide and imposed an economic burden on the health care systems and patients. In 2013, the Centers for Disease Control and Prevention (CDC) stated that the situation is similar to pre-antibiotic era (25). The most severe Gram-negative infections are commonly caused by members of *Enterobacteriaceae* such as *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*. Some *Ainetobacter baumannii* strains are resistant to almost all current antibiotics especially carbapenems which are usually the last drugs for treatment. In this situation, developing new drugs by pharmaceutical industry and renewing research in this area is vital for surviving millions of patients.

Myxobacteria have recently received a remarkable attention because of their significant potential for the production of bioactive compounds. In the present study, among the seven active extracts, three selected strains, *Stigmatella* sp. UTMC 4081, *Stigmatella* sp. UTMC 4072, and *Archangium* sp. UTMC 4070 showed the most antibacterial activity against *A. baumannii*. It is worth noting that the extracts derived from strains UTMC 4081 and UTMC 4072 displayed efficient antibacterial activities at low concentrations as they could totally inhibit the growth of *A. baumannii* at 10 µg/ml and 5 µg/ml, respectively.

The genera of *Stigmatella* and *Archangium* have been shown to produce various bioactive natural products including stigmatellins (26), aurachines (27), myxalamids (28), myxochelin (29), argyrin (30), archazolid (31), aurafuron (32), and myxochromide (33), that present anti-bacterial, anti-fungal and cytotoxic activities. Antibacterial activity of plant extracts has been reported against *A. baumannii* in some studies. For example, antibacterial effect of the extract obtained from *Mentha arvensis* was invest-igated against MDR *A. baumannii* with MIC 23.5 and MBC 72.1 µg/ml (34). Essential oils extracted from plants have also been known as antibacterial compounds against MDR bacteria. *Eucalyptus camaldulensis* oils were assessed, for example, against *A. baumanni*. Accordingly, two main essential oils derived from *E. camaldulensis* inhibited the growth of *A. baumannii* with MIC 0.5 to 2 µl/ml (35). In addition, Epigallocatechin-3-Gallate, the antibacterial active compound in green tea, was examined against clinically isolated *A. baumannii* strains: the MIC of epigallocatechin-3-Gallate extract ranged from 64 to 512 µg/ml (36). Antimicrobial activity of fermentation broth of *Streptomyces* isolates has also been investigated against *A. baumannii*. The findings showed the MIC and MBC values of 125 and 1000 µg/ml for *Streptomyces* metabolites, respectively (37).

The current work was the first attempt on antibiotic

discovery against *A. baumannii* from myxobacteria which revealed the effectiveness of their secondary metabolites on such MDR pathogen. The results obtained in this study confirmed the antibacterial activity of some myxobacterial extracts even against MDR bacteria such as *A. baumannii*. The antibacterial activity of the genera *Stigmatella* and *Archangium* against drug resistant *A. baumannii* that are found in this project can offer a valuable source for finding effective compounds against the multi-drug resistant *A. baumannii*. However, further studies on identification of the active compound in the extract and its mechanism of action are in progress.

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