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Original Article

Acidophilic and Acid Tolerant Actinobacteria as New Sources of Antimicrobial Agents Against *Helicobacter Pylori*

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Abstract

About half of the world's population is infected by *Helicobacter pylori*, which is related to various diseases. The increase in the resistance of *H. pylori* to antibiotics is alarming and requires new medication candidates. In this study, 83 acidic soil samples (pH 3.9-6.8) were collected from tea and rice farms, located in the semitropical strip in the north of Iran (Lahijan and Fooman cities, Gilan Province). After various pretreatments, including dry heating (120 ºC, 10 min), exposure to electromagnetic waves (800 Hz, 3 min), and centrifuging (2950 g, 15 min), 33 acidophilic or acid-tolerant actinobacteria were isolated and their potentials as a source of active metabolites against *H. pylori* were investigated. According to phenotypic and molecular identification tests, the actinobacterial isolates were classified into *Streptomyces* and *Kitasatospora* genera. Among 10 strains that had anti-*H. pylori* activity, the highest potentials were seen in the strains UTMC 3061 and UTMC 3318. The minimum inhibitory concentrations (MIC) of the related metabolites were 125 and 62.5 µg/ml, respectively. In the checkerboard test, the metabolites of these actinobacteria showed synergism with clarithromycin and reduced its MIC from 1 to 0.5 µg/ml. However, no synergism was seen between the metabolites and amoxicillin or metronidazole. The gas chromatography-mass spectrometry (GC-MS) analysis of the metabolites showed some antimicrobial agents, including carbamic acid, maltol, 2.4-di-tert-butylphenol, methyl dimendone, prolylleucyl, and oleamide. The strains UTMC 3061 and UTMC 3318 showed 99.41 and 100% similarity in 16S rRNA gene sequence to *Streptomyces spinoverrucosus* and *Streptomyces cirratus*, respectively. Their metabolites showed good antibiotic activity and limited toxicity and can be considered as promising sources of natural products against *H. pylori*.

Keywords: Acidophile, Actinobacteria, Antibiotic resistance, Antimicrobial activity, *Helicobacter pylori*

Actinobactéries Acidophiles et Tolérantes Aux Acides en Tant que Nouvelles Sources D'agents Antimicrobiens Contre *Helicobacter Pylori*

Résumé: Environ la moitié de la population mondiale est infectée par *Helicobacter Pylori*, qui est liée à diverses maladies. L'augmentation de la résistance de *H. pylori* aux antibiotiques est alarmante et nécessite de nouveaux médicaments candidats. Dans cette étude, 83 échantillons de sol acide (pH 3.9-6.8) ont été collectés dans des plantations de thé et de riz, situées dans la bande semi-tropicale du nord de l'Iran (villes de Lāhījān et Fooman, province de Gīlān). Après divers prétraitements, notamment chauffage à sec (120 ºC, 10 min), exposition aux ondes électromagnétiques (800 Hz, 3 min) et centrifugation (2950 g, 15 min), 33 Actinobactéries acidophiles ou tolérantes aux acides ont été isolées et leurs potentiels comme source de métabolites actifs contre *H. pylori* ont été étudiés. Selon des tests d'identification phénotypique et moléculaire, les isolats d'actinobactéries ont été

classés en genres *Streptomyces* et *Kitasatospora*. Parmi 10 souches qui avaient une activité anti-*H. pylori*, les potentiels les plus élevés ont été observés dans les souches UTMC 3061 et UTMC 3318. Les concentrations minimales inhibitrices (CMI) des métabolites apparentés étaient de 125 et 62.5 µg/ml respectivement. Dans le test en damier, les métabolites de ces actinobactéries ont montré une synergie avec la clarithromycine et ont réduit sa CMI de 1 à 0.5 µg/ml. Cependant, aucune synergie n'a été observée entre les métabolites et l'amoxicilline ou le métronidazole. L'analyse par chromatographie en phase gazeuse-spectrométrie de masse (GC-MS) des métabolites a montré certains agents antimicrobiens, notamment l'acide carbamique, le maltol, le 2.4-di-tert-butylphénol, le méthyl dimendone, le prolylleucyle et l'oléamide. Les souches UTMC 3061 et UTMC 3318 ont montré une similitude de 99,41 et 100% dans la séquence du gène de l'ARNr 16S avec *Streptomyces spinoverrucosus* et *Streptomyces cirratus*, respectivement. Leurs métabolites ont montré une bonne activité antibiotique et une toxicité limitée et peuvent être considérés comme des sources prometteuses de produits naturels contre *H. pylori*.
Mots-clés: Acidophile, Actinobactéries, Résistance aux antibiotiques, Activité antimicrobienne, *Helicobacter pylori*

1. Introduction

Helicobacter pylori is a gram-negative, microaerophilic bacterium that persistently colonizes the epithelium of human stomach cells. According to a comprehensive global survey, approximately 4.4 billion *H. pylori* infections were found around the world, with the distribution of 24.4% in Oceania to 70.1% in Africa (Hooi et al., 2017).

The number of diseases potentially contributed by *H. pylori* is increasing. They include gastric and extragastric diseases, such as dyspepsia, gastritis, gastric and colorectal cancers, laryngeal/pharyngeal cancers, lymphomas, iron-deficiency anemia, idiopathic thrombocytopenic purpura; some diseases in the skin, eyes, ears, nose, throat, liver, gallbladder, pulmonary, cardiovascular; diabetes, neuromyelitis optica, multiple sclerosis; and autoimmune, neurodegenerative, and pregnancy diseases (Testerman and Morris, 2014).

It should be mentioned that *H. pylori* can be treated by multiple antibiotics therapy; however, its resistance to commonly used antibiotics is increasing globally (Testerman and Morris, 2014). Therefore, it is needed to find new antimicrobial sources for its treatment.

Actinobacteria are among the most microbial sources of bioactive metabolites and have been used as a very useful source of antibiotics. Actinobacteria produce 130 out of ~190 clinical antibiotics (Hamedi et al., 2017), including broad-range antibiotics (e.g. tetracycline by *Kitasatospora auerofaciens*) and narrow-range antibiotics (e.g. erythromycin by *Saccharopolyspora erythraea*). Given the decreasing rate of the discovery of novel antibiotics, extremophilic microorganisms, like thermophiles, which have been poorly investigated, seem to be good sources for novel and effective antibiotics (Giddings and Newman, 2015).

Acidophilic actinobacteria are among common residents in acidic habitats, such as acidic soils, and grow at pH values of 3.5-6.5, with an optimal pH of ~4.5. Acid-tolerant actinobacteria grow at pH values of 4.5-7.5 with optimal growth at 5.0-5.5 pH range. Acidophilic and acid-tolerant actinobacteria have been studied as promising strains in biotechnology. For example, bactericidal (butalactin), fungicidal-insecticidal (nikkomycin), and antiprotozoal (granaticin A) activities were reported in *Streptomyces corchorusii* (Ueki and Kinoshita, 2004), *Streptomyces tendae* (Ginj et al., 2005), and *Streptomyces lateritius*, respectively. However, there is no report on the activity of acidophilic and acid-tolerant actinobacterial metabolites against *H. pylori*. In this regard, this study aimed to isolate these actinobacteria from acidic soils and investigate their activities against *H. pylori*.

2. Material and Methods

2.1. Microbial Test Strains

The *H. pylori* strain TUMS 10 was obtained from Medical Bacteriology Lab (Dept. of Pathobiology, School of Public Health, Tehran University of Medical

Sciences [TUMS], Tehran, Iran). Other bacteria and fungi that were used as test strains in antimicrobial assays were obtained from the University of Tehran Microorganisms Collection (UTMC) and included *Escherichia coli* UTMC 1465, *E. coli* TolC UTMC 1462, *Pseudomonas aeruginosa* UTMC 1463, *Micrococcus luteus* UTMC 1461, *Mucor hiemalis* UTMC 5057, *Chromobacterium violaceum* UTMC 1466, *Candida albicans* UTMC 5055, *Staphylococcus aureus* UTMC 1467, *Pichia anomala* UTMC 5056, and *Bacillus subtilis* UTMC 1464.

2.2. Microbial Culture Media

2.2.1. Helicobacter Pylori Medium

Brucella agar (45 g/l) was enriched by defibrinated sheep blood (10% v/v) and fetal bovine serum (20% v/v). To reduce contamination, vancomycin (10 µg/ml), trimethoprim (5µg/ml), and amphotericin (5 µg/ml) were added to the medium.

2.2.2. Media for Isolation of Actinobacteria

It consisted of starch casein agar, compost agar, and rice bran agar (Hamedi et al., 2019). All media contained 15 g/l agar with pH 5.0±0.1 and were supplemented by amphotericin B (25 µg/ml) to inhibit the growth of fungi.

2.2.3. Medium for Purification and Short-time Maintenance of Actinobacteria

The ISP2 medium included malt (5 g/l), glucose (2 g/l), yeast extract (2 g/l), $CaCO_3$ (1 g/l), and agar (15 g/l).

2.3. Antibiotics

Amphotericin B (Cipla Ltd, India), vancomycin (Sigma Co., USA), trimethoprim (Sigma Co., USA), and amphotericin (Sigma Co., USA) were used for the prevention of contamination or as control in the antibacterial assay.

2.4. Soil Sampling and Pretreatment

The samples were collected from acidic soils obtained from tea and rice farms located in the semitropical strip in the north of Iran. The soil samples were collected at the depth of 10-25 cm and transported to the laboratory in less than 24 h. After air-drying, crushing, and sieving the soil samples, three pretreatments were performed on them, including heat treatment at 120 ˚C for 10 min in an oven, exposure to electromagnetic waves (800 Hz, three min), and centrifuging (2950 g for 15 min, and the supernatant was used for actinobacterial isolation) (Hamedi et al., 2019).

2.5. Isolation and Identification of the Acidophilic Actinobacteria

The treated samples were serially diluted and appropriate dilutions were cultured on the media for isolation of acidophilic actinobacteria. The plates were incubated at 28 °C for 14 days. Putative actinobacterial colonies were sub-cultured on ISP 2 medium. The isolates were deposited in UTMC.

2.6. Determination of pH Tolerance of the Actinobacteria

To study the pH tolerance of the isolates, they were cultured in ISP-2 broth in various pH levels (1-14) and incubated at 28 ºC for 14 days. Afterward, the turbidity of the broth was compared with that of the uninoculated ISP-2 broth and its increase was considered as growth and tolerance of the strain towards the related pH.

2.7. Preparation of Actinobacterial Metabolites

The isolates were cultured on the 100 ml screw-capped bottles containing ISP 2 agar slant (pH 5.5) as a sporulation medium. Appropriate concentrations of spores (~10^7-10^8/ml) were added to the 500 ml Erlenmeyer flask containing 50 ml seeding medium and incubated at 28 ºC and 180 rpm for 48 h.

The seeding material (10 v/v) was added to the 1000 ml Erlenmeyer flask containing 150 ml fermentation medium and incubated at 28 ºC and 180 rpm for 196 h. The fermentation material was centrifuged at 4000 rpm for 20 min to remove the biomass, and the supernatant was extracted by an equal volume of ethyl acetate. The organic phase (containing actinobacterial metabolites) was evaporated by a rotary evaporator at 38 ºC at reduced pressure.

The obtained metabolite was weighed and divided into two screw-capped vials that were preserved at -20

°C. One vial was used for well agar diffusion assay as described below, and the other vial was mixed in methanol and carefully dissolved by using an ultrasonic bath for 2-3 min. Appropriate concentrations of the methanol-dissolved metabolites were poured on the blank paper disks that were put in the bottom of porcelain immunological plates. After evaporation of the solvent, the dried disk papers containing the metabolites were preserved at -20 °C in screw-capped vials.

2.8. Culture of *Helicobacter pylori* as an Inoculation Material

The *H. pylori* TUMS 10 was inoculated in a 50 ml Falcon tube containing 40 ml *H. pylori* medium (HP medium) and incubated at 37 °C in a CO_2 incubator (10 % CO_2) for 72 h. The tube was centrifuged at 3000 rpm for 5 min. An appropriate volume of HP broth was added to the bacterial sediment to achieve an appropriate concentration of *H. pylori* (~6×10^8 colony forming unit [CFU]/ml). Finally, the ~1×10^5 CFU/ml concentration of *H. pylori* was used in agar well diffusion or broth dilution methods.

2.9. Primary Screening of the Isolates for Antimicrobial Activity against *H. pylori*

Antimicrobial activity of the isolates against *H. pylori* was evaluated by agar diffusion methods using soluble extracts. Briefly, *H. pylori* inoculation material was spread onto the bacteriological plates containing HP agar. The metabolite disks were put on the surface of the plate or various concentrations of the metabolites were applied into the agar well. The plates were incubated in a humid and microaerophilic atmosphere at 37 °C for 3-4 days. Clarithromycin, amoxicillin, and metronidazole were used as controls.

Furthermore, the activity of the actinobacterial metabolites was studied against *E. coli* UTMC 1465, *E. coli* TolC UTMC 1462, *P. aeruginosa* UTMC 1463, *M. luteus* UTMC 1461, *M. hiemalis* UTMC 5057, *C. violaceum* UTMC 1466, *C. albicans* UTMC 5055, *S. aureus* UTMC 1467, *P. anomala* UTMC 5056, and *B. subtilis* UTMC 1464 by agar dilution method (Hamedi et al., 2019).

2.10. Determination of Minimum Inhibitory Concentrations of the Metabolites

The minimum inhibitory concentrations (MIC) of the selected metabolites (0-1000 *µg*/ml) against *H. pylori* were determined by the broth microdilution method (Meletiadis et al., 2010).

2.11. Combinatorial Effects of Actinobacterial Metabolite-antibiotics

The synergism or antagonism of the combination of actinobacterial metabolites and the antibiotics was performed by the checkerboard technique (Meletiadis et al., 2010). For this purpose, stock solutions and serial twofold dilutions of the antibiotics, including clarithromycin, amoxicillin, and metronidazole were prepared according to the protocols of the National Committee for Clinical Laboratory Standards.

Moreover, serial dilutions of the antibacterial metabolites were prepared as described above. Each one of the 24 wells on the microplate was filled by the molten HP medium. When the medium was solid, each well was inoculated with the inoculation material (0.1 mL) and then incubated for 72 h at 37 °C with 10% CO_2. The interaction was assessed by determining the fractional inhibitory concentration (FIC), which is the MIC of each antibacterial in combination divided by the MIC of each antibacterial when used alone. An FIC index lower and higher than one was considered as synergy and antagonism, respectively (Meletiadis et al., 2010).

2.12. Molecular Identification of Acidophilic Actinobacteria

Molecular identification of the actinobacterial isolates was performed by PCR amplification of 16S rRNA gene using universal primers 9F (5AAGAGTTTGATCATGGCTCAG-3) and 1541R (5-AGGAGGTGATCCAACCGCA-3) and their subsequent sequencing (Hamedi et al., 2019). The actinobacteria were cultured in BHI broth medium and incubated at 28 ˚C for 48 h. The biomass was obtained by centrifugation at 4000 rpm for 10 min, and the genomic DNA was extracted using a DNA extraction kit (Pooya Gene Azma Co., Tehran, Iran) and

amplified. It should be noted that the PCR products were sequenced by Macrogen Inc. (South Korea). The sequences were blasted in National Center for Biotechnology Information (NCBI) and EzTaxon data bank, and finally, the sequences were submitted to GenBank (NCBI).

2.13. Brine Shrimp Lethality Bioassay

To find the toxicity of the metabolites, brine shrimp lethality bioassay was used by applying various concentrations of bioactive metabolites (200, 100, 50, and 25 µg/ml) to *Artemia salina* (Salimi et al., 2018).

2.14. High-performance Liquid Chromatography of Bioactive Metabolites

The metabolites obtained after extraction of the fermentation broth of the bioactive strains against *H. pylori* were analyzed by high-performance liquid chromatography (HPLC)-UV. Reversed-phase HPLC experiments were performed using XBridge C18 column 100×2.1 mm (Waters), 3.5 µm, solvent A (H_2O−acetonitrile [95/5], 5 mmol NH_4 acetate, 0.04 mL/L CH_3COOH), solvent B (H_2O−acetonitrile [5/95], 5 mmol NH_4 acetate, 0.04 mL/L CH_3COOH), and gradient system with 10% B increasing to 100% B in 30 min with a flow rate of 0.3 mL/min at 40 °C.

2.15. Chemical Constituents of the Bioactive Extract Using Gas Chromatography-mass Spectrometry Analysis

The chemical ingredients of the metabolites of selected strains were analyzed using GC-MS (Almasi et al., 2018).

3. Results

In total, 33 isolates were obtained from 83 acidic soil samples (pH 3.9-6.8). It should be noted that 20 and 13 of the isolates were obtained from the samples with pH levels of 3.9-5.0 and 5.4-6.8, respectively. Moreover, 25 and 8 isolates were collected from tea and rice farms, respectively. Table 1 summarizes the results of the molecular identification, sources of the isolates, and their related accession No. in Genbank. The majority of the isolates (91%) belonged to the *Streptomyces* genus while three of them (7%) belonged to the *Kitasatospora* genus. Some of the isolates were non-actinobacteria, including *Methylobacterium* and *Varivorax*; they were removed from the research. It is noteworthy that both of these isolates belonged to the *Proteobacteria* phylum.

3.1. pH Profile of the Isolates

Table 2 tabulates the results of the growth of the isolates in ISP-2 broth. It must be noted that 12% of the isolates, including *Streptomyces* sp. UTMC 3061, *Streptomyces* sp. UTMC 3218, *Kitasatospora* sp. UTMC 3221, and *Streptomyces cirratus* UTMC 3318, showed maximum growth at a pH level of less than 7 and were considered acidophiles.

Moreover, four strains (12%), including *Streptomyces* sp. UTMC 3019, *Streptomyces* sp. UTMC 3028, *Streptomyces* sp. UTMC 3055, and *Streptomyces pratensis* UTMC 3217 could grow at a higher pH than eight and were considered as alkaliphiles. The minimum pH for the growth of the strains was related to the *Streptomyces* sp. UTMC 3320.

3.2. Antimicrobial Susceptibility Profile of *Helicobacter pylori*

The *H. pylori* TUMS 10 used in the research was susceptible to clarithromycin, amoxicillin, and metronidazole in broth microdilution assay. Its minimum inhibitory concentrations among the aforementioned antibiotics were 0.5, 1, and 8 µg/ml, respectively.

3.3. *Anti-Helicobacter pylori* Activity of Actinobacterial Isolates

When the agar dilution method was used, no activity against *H. pylori* was observed in metabolite disks of actinobacterial isolates. However, in the broth microdilution method, 10 out of 33 isolated strains showed anti-*H. pylori* activity, including, *Streptomyces* sp. UTMC 3019, *Streptomyces* sp. UTMC 3026, *Streptomyces coelicoflavus* UTMC 3052, *Streptomyces* sp. UTMC 3061, *Streptomyces* sp. UTMC 3066, *Streptomyces* sp. UTMC 3211, *Streptomyces* sp. UTMC 3312, *Streptomyces mirabilis* UTMC 3317, *S. cirratus* UTMC 3318, and *Streptomyces* sp. UTMC 3320. Among them, two strains, namely *Streptomyces* sp.

UTMC 3061 and *S. cirratus* UTMC 3318, were selected for secondary screening due to higher antibacterial activity and the results of their MICs against *H. pylori* were 125 and 62.5 µg /ml, respectively.

3.4. Checkerboard Analysis

Table 3 summarizes the results of the combinatorial effects of the selected actinobacterial metabolites and clarithromycin, amoxicillin, and metronidazole. As can be seen, the metabolites of *Streptomyces* sp. UTMC 3061 and *S. cirratus* UTMC 3318 at concentrations of 32 and 62.5 µg/ml, respectively, reduced the MIC of *H. pylori* to clarithromycin from 1 ug/ml to 0.5 µg/ml. However, no synergism was observed between the metabolites of the studied actinobacterial strains and amoxicillin and metronidazole since the actinobacterial metabolites did not reduce the MIC of these antibiotics from 1 and 16 µg/ml, respectively.

3.5. Other Biological Activities of the Isolates

3.5.1. Toxicity of the Metabolites

Toxicity of *Streptomyces* sp. UTMC 3061 and *S. cirratus* UTMC 3318 metabolites against brine shrimp indicated that the metabolites of both strains had no toxicity at concentrations of less than 32 µg/ml. Metabolites of *Streptomyces* sp. UTMC 3061 and *S. cirratus* UTMC 3318 at 125 and 62.5 µg/ml had 30% and 15% toxicity against *A. salina,* respectively.

3.5.2. Other Antimicrobial Activities of the Isolates

Among 33 actinobacterial isolates, 9 strains (27.3%) had other antimicrobial activities. More specifically, 6, 1, 1 and 3 isolates had antimicrobial activities against *M. luteus* UTMC 1461, *B. subtilis* UTMC 1464, *P. anomola,* and *C. albicans,* respectively (Table 4). No antimicrobial activity was observed against *E. coli* UTMC 1465, *E. coli* TolC UTMC 1462, *P. aeruginosa* UTMC 1463, *M. hiemalis* UTMC 5057, *C. violaceum* UTMC 1466, and *S. aureus* UTMC 1467 by agar dilution method.

3.5.3. Thin-Layer Chromatography–bioautography of Selected Metabolites against *H. pylori*

No inhibition zone was observed around the thin-layer chromatography plates of the metabolites obtained from the strains, *Streptomyces* sp. UTMC 3061 and *S. cirratus* UTMC 3318.

3.5.4. Chemical Analysis of the Metabolites

According to the results of HPLC analysis, there were four and six UV-active compounds in the fermentation broth extracts of the strains *Streptomyces* sp. 3061 UTMC (Figure 1A) and *S. cirratus* UTMC 3318 (Figure 1B) at the wavelengths of 254 and 366 nm, respectively. Figure 2 shows the results of the GC-MS analysis of the metabolites of the selected strains that were obtained after ethylacetate extraction.

Analysis of the results of the metabolites of *Streptomyces* sp. 3061 UTMC and *S. cirratus* UTMC 3318 led to five and four compounds, respectively. Metabolites of *Streptomyces* sp. 3061 UTMC consisted of carbamic acid (retention time [RT]: 5.788 min), maltol (RT: 8.182 min), 2.4-di-tert-butylphenol (RT: 13.549 min), methyl dimendone (RT: 18.094 min), and oleamide (RT: 22.233 min). Moreover, the metabolites of *S. cirratus* UTMC 3318 contained maltol (RT: 8.191 min), 2.4-di-tert-butylphenol (RT: 13.548 min), prolylleucyl (RT: 18.268 min), and oleamide (RT: 22.171 min).

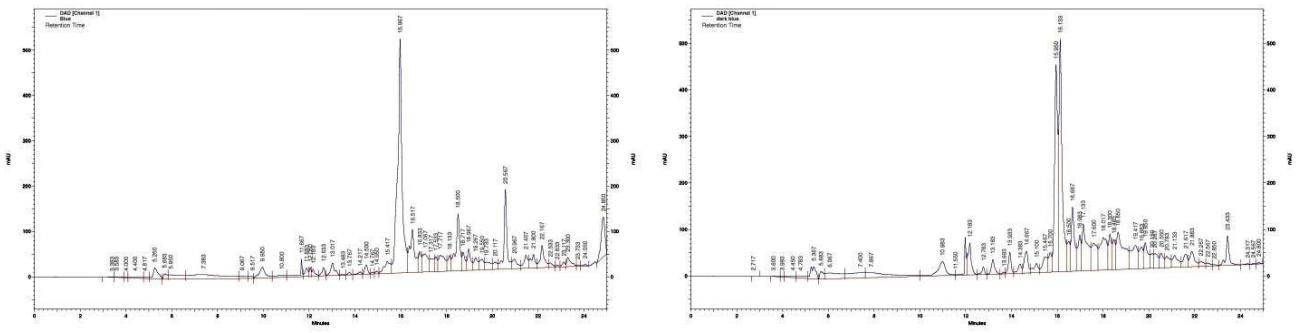

Figure. 1 Fractionation of the metabolites of *Streptomyces* sp. UTMC 3061 (**A**) and *Streptomyces cirratus* UTMC 3318 (**B**) by HPLC at wavelengths 254 and 366 nm

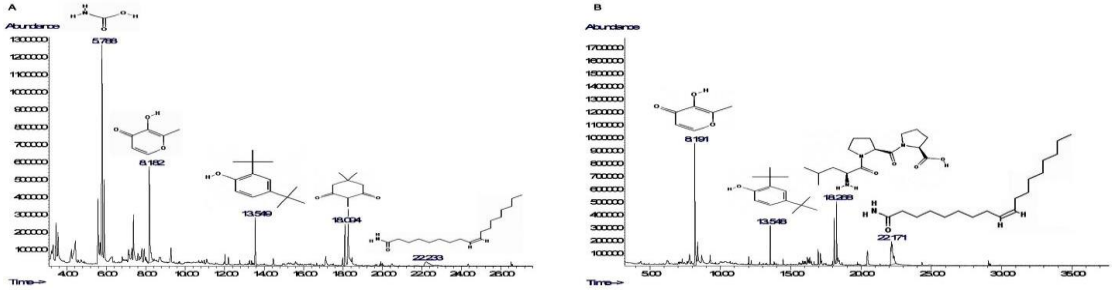

Figure. 2 The chemical constituents of the metabolites of *Streptomyces* sp. UTMC 3061 (**A**) and *Streptomyces cirratus* UTMC 3318 (**B**) found by gas chromatography and mass spectrometry analysis

Table 1. This table shows the diversity of actinobacteria isolated from acidic soils obtained from tea (*) and rice (**) farms as well as the nearest species according to similarity (%) in the 16S rDNA sequence

No.	Source	Soil pH	UTMC code	NCBI code	Nearest neighborhood
1	Fooman*	4.92	3019	SUB6393744	*Streptomyces lateritius* (99.7%)
2	Fooman**	5.78	3022	SUB6393839	*Streptomyces coelicoflavus* (100%)
3	Fooman**	5.62	3026	SUB6393849	*Streptomyces albogriseolus* (99.87%)
4	Fooman**	5.62	3028	SUB6393858	*S. albogriseolus* (99.88%)
5	Fooman**	5.62	3038	SUB6393866	*S. lateritius* (99.65%)
6	Fooman**	5.78	3039	SUB6393896	*Streptomyces lienomycini* (100%)
7	Fooman**	5.78	3052	SUB6393907	*S. coelicoflavus* (100%)
8	Fooman**	4.17	3055	SUB6393949	*S. albogriseolus* (99.87%)
9	Fooman*	4.17	3056	SUB6393967	*S. lateritius* (99.74%)
10	Fooman*	6.8	3061	SUB6394180	*Streptomyces spinoverrucosus* (99.41%)
11	Fooman*	6.7	3066	SUB6394182	*Streptomyces xantholiticus* (99.87%)
12	Lahijan, Gerd-Korf*	4.87	3211	SUB6395102	*Streptomyces yokosukanensis* (99.8%)
13	Lahijan, Choushal*	4.75	3212	SUB6395184	*Streptomyces aureus* (99.87%)
14	Lahijan, Darreh-Jir*	5.66	3214	SUB6396310	*Streptomyces corchorusii* (99.61%)
15	Lahijan, Baz-kia-gourab*	3.90	3215	SUB6396312	*Streptomyces griseolus* (100%)
16	Lahijan, Dehsar*	4.26	3216	SUB6396331	*Streptomyces yanglinensis* (99.07%)
17	Lahijan, Sardar-Jangal*	4.53	3217	SUB6396334	*Streptomyces pratensis* (100%)
18	Lahijan, Baz-kia-gourab*	6.72	3218	SUB6396339	*Streptomyces yaanensis* (99.27%)
19	Lahijan, Sukhteh-Kouh*	4.89	3220	SUB6396342	*S. pratensis* (100%)
20	Lahijan, Choushel*	4.49	3221	SUB6396347	*Kitasatospora aureofaciens* (99.6%)
21	Lahijan, Baz-kia-gourab*	6.72	3312	SUB6396359	*Streptomyces kebangsaanensis* (96.35%)
22	Lahijan, Roujour-Azberm*	4.45	3313	SUB6396364	*Streptomyces neopeptinius* (99.83%)
23	Lahijan, behind Justice*	4.33	3314	SUB6396368	*Streptomyces katrae* (99.46%)
24	Lahijan, Kouh-Bijar*	4.89	3315	SUB6396374	*S. griseolus* (100%)
25	Lahijan, Behind Justice*	4.32	3316	SUB6401885	*Kitasatospora psammotica* (100%)
26	Lahijan, Choushel*	5.0	3317	SUB6401894	*Streptomyces mirabilis* (100%)
27	Lahijan, Mian-Mahaeh*	5.0	3318	SUB6401902	*Streptomyces cirratus* (100%)
28	Lahijan, Bande-Bion-Paein*	4.98	3320	SUB6401909	*Streptomyces bungoensis* (99.61%)
29	Lahijan, Ahandan*	4.52	3322	SUB6401914	*Streptomyces shaanxiensis* (99.03%)
30	Lahijan, Baz-Kia-Gourab*	6.72	3323	SUB6401923	*Streptomyces rhizosphaerihabitans* (99.09%)
31	Lahijan, Gerd-Korf*	5.0	3329	SUB4848576	*Streptomyces tendae* (100%)
32	Lahijan, Gerd-Korf*	4.27	3333	SUB6401929	*Kitasatospora niigatensis* (100%)
33	Fooman, Foosheh-Roudkhan**	5.62	3477	SUB6402077	*S. xantholiticus* (99.88%)

UTMC: University of Tehran Microorganisms Collection, NCBI: National Center for Biotechnology Information

Table 2. Results of pH profiles of the actinobacterial isolates from acidic soils. None of the strains had any growth on the media with 4>pH>12. higher growth of the strain is shown with more +.

UTMC code	4	5	6	7	8	9	10	11	12
3019	-	-	+	++	+++	+	+	-	-
3022	-	+	++	+++	++	+	+	+	+
3026	-	-	+	+	++	+	+	-	-
3028	-	-	+	++	+++	+++	++	+	+
3038	-	-	-	+	++	-	-	-	-
3039	-	-	+	++	+	+	-	-	-
3052	-	-	+	+	+	+	+	+	+
3055	-	-	+	++	+++	++	+	+	+
3056	-	-	+	+	++	+	+	+	-
3061	-	++	+++	++	+	+	+	+	+
3066	-	-	+	+++	++	+	+	+	-
3211	-	+	+	++	++	+	+	+	+
3212	-	-	+	+	++	+	+	-	-
3214	-	+	+	+++	++	+	+	-	-
3215	-	+	+	+	++	+	+	-	-
3216	-	+	+	++	+	+	+	-	-
3217	-	-	-	++	+++	++	+	-	-
3218	-	++	+++	++	++	+	+	-	-
3220	-	+	+	+++	++	+	+	+	+
3221	-	+++	++	++	++	-	-	-	-
3312	-	-	+	++	+	+	+	+	+
3313	-	-	+	++	+	-	-	-	-
3314	-	-	+	++	+	-	-	-	-
3315	-	-	+	++	+	-	-	-	-
3316	-	-	++	+++	++	+	+	-	-
3317		++	++	+++	++	+	+	-	-
3318	-	++	+++	++	++	-	-	-	-
3320	+	+	++	+++	++	+	+	-	-
3322	-	-	+	++	+	+	+	-	-
3323	-	-	+	++	+	+	+	-	-
3329	-	+	+	++	++	+	+	+	+
3333	-	+	++	+++	++	++	+	+	+
3477	-	-	+	++	+	-	-	-	-

UTMC: University of Tehran Microorganisms Collection

Table 3. Study of synergism of the metabolites of *Streptomyces* sp. UTMC 3061 and *Streptomyces cirratus* UTMC 3318 with clarithromycin, amoxicillin, and metronidazole by the checkerboard method

		Clarithromycin				Amoxicillin				Metronidazole			
Metabolites	µg/ml	0	0.25	0.5	1	0	0.25	0.5	1	0	4	8	16
3061	8	G	G	NG	NG	G	G	G	NG	G	G	G	NG
3318		G	G	NG	NG	G	G	G	NG	G	G	G	NG
3016	16	G	G	NG	NG	G	G	G	NG	G	G	G	NG
3318		G	G	NG	NG	G	G	G	NG	G	G	G	NG
3061	32	G	G	NG	NG	G	G	G	NG	G	G	G	NG
3318		G	NG	NG	NG	G	G	G	NG	G	G	G	NG
3061	62.5	G	NG	NG	NG	G	G	G	NG	G	G	G	NG
3318		NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG
3061	125	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG
3318		NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG

Table 4. The actinobacterial isolated from acidic soil with antimicrobial activity. Zone inhibition (mm) is shown in the table.

UTMC code	***Micrococcus luteus***	***Bacillus subtilis***	***Candida albicans***	***Pichia anomola***
3019	-	-	-	14
3026	18	-	-	-
3039	25	-	-	-
3212	21	-	-	-
3217		-	-	25
3220	24	-	-	-
3315		17	-	-
3317	18	-	-	-
3477	14	-	17	30

UTMC: University of Tehran Microorganisms Collection

4. Discussion

Previous studies have noted the importance of acidophilic microorganisms in biotechnology. This study aimed to assess the importance of acidophilic actinobacteria in antimicrobial production against *H. pylori*. All actinobacteria isolated in this study were obtained from tea and rice fields with pH levels of 3.9-6.8 and should be considered acid tolerant. Moreover, 12% of the strains had maximum growth at acidic conditions and were acidophiles.

Another important finding was that 76% of the strains were isolated from the tea field, and the average pH of the tea fields was 0.5 units less than that of the rice fields. All three *Kitasatopsora* isolates were obtained from the tea field with an average pH of 4.36. It can be concluded that tea-field soils are better sources for isolation of acidophilic actinobacteria than rice-field soils. However, the actinobacteria isolated from tea fields did not show any antibacterial activity against *H. pylori*. All of the 10 active actinobacterial isolates belonged to the *Streptomyces* genus, including *Streptomyces* sp. 3061 UTMC, and *S. cirratus* UTMC 3318.

Streptomyces sp. 3061 had 99.41% similarity to *Streptomyces spinoverrucosus* obtained from National Brain Research Centre with the code NBRC 14228 (T). Until now, two strains of *S. spinoverrucosus* have been reported, namely *S. spinoverrucosus* Diab 163MA^T (NBRC 14228=NCIB 11666) (Diab and Al-Gounaim, 1982) and *S. spinoverrucosus* SNB-032.

The first strain was the type strain isolated from air in

Kuwait, while the second strain was isolated from a marine sediment sample collected from Trinity Bay, Texas, USA and had 99% similarity in 16S rRNA gene sequence to type strain (Hu et al., 2012). From the second strain, three anthraquinones were discovered, including galvaquinones A-C (Hu et al., 2012); however, no antibacterial activity was reported in galvaquinones.

The *S. cirratus* UTMC 3318 had a 100% similarity to *S. cirratus* obtained from Northern Regional Research Lab with the code B-3250^{T}. *S. cirratus* 248-Sq2 that was isolated from the soil in Norikura highland, Japan, and produced cirratiomycin A, cirramycin A, and cirramycin B that were active against *Lactobacillus casei* and some strains of *Streptococcus* and *Mycobacterium* (Shiroza et al., 1982). The *S. cirratus* F2-2 that was isolated from a banana plantation in Zhangzhou, China had good antibacterial activity against some gram-negative bacteria, including *Pseudomonas putida*, *P. fluorescens*, *Burkholderia sepacia,* and *Escherichia coli* (Shirokikh et al., 2018).

The most obvious findings that emerged from the chemical analysis of the metabolites of *Streptomyces* sp. UTMC 3061 and *S. cirratus* UTMC 3318 was that both of them produced maltol, 2.4-di-tert-butylphenol, and oleamide. Maltol is a naturally occurring organic compound in plants and is used primarily as a flavor enhancer in bread and cake (Han et al., 2015). This is in line with the results of previous studies which have indicated that maltol is also produced by various members of actinobacteria (Kornsakulkarn et al., 2014).

Some antibiotic activities were reported from maltol derivatives (Salsbury et al., 2015). It should be noted that 2,4-di-tert-butylphenol is an alkylbenzene and a member of phenols that are produced by actinobacteria with antibacterial and antifungal activities (Belghit et al., 2016). Oleamide is an amide of oleic acid and occurs naturally in plants and animals and has antimicrobial activity (Shao et al., 2016).

Streptomyces sp. 3061 UTMC also produced carbamic acid and methyl dimendone. Carbamic acid had various biological activities, including antibacterial activity against *Mycobacterium tubercolosis* (Zanatta et al., 2006) and methicillin-resistant *S. aureus* (Han et al., 2004) and inhibition of histidine acetylase (Rayudu, 1990). *H. pylori* produced carbamic acid by affecting its urease on urea. It should be mentioned that urease can speed up carbamic acid production (by hydrolysis of urea) or consumption (by the synthesis of urea) (Zimmerli and Schlatter, 1991).

Given the vital importance of urease as the main shield of *H. pylori* from gastric, an increase in the carbamic acid may decrease the activity of urease and help the removal of *H. pylori* from the stomach. It should also be noted that 2-Methyldimedone or 1,3 cyclohexanedione was found in essential oils of some leptospermone plants and has weak antibacterial activity against *Clostridium difficile* and *Clostridium perfringens* (Jeong et al., 2009).

Prolylleucyl is another metabolite that was detected in the extract of *S. cirratus* UTMC 3318. This is also consistent with our earlier observations which indicated the antimicrobial activity of this dipeptide that had been isolated as a metabolite of actinobacteria (El Euch et al., 2018).

Based on the findings, it can be said that acidophilic actinobacteria can be promising sources of active metabolites against *H. pylori*. The metabolites of *S. cirratus* UTMC 3318 and *Streptomyces* sp. UTMC 3061 showed limited toxicity against Eukaryotes. They also showed good synergism with clarithromycin, a current medication used for *H. pylori* treatment. This finding suggests that these acidophilic actinobacteria can be good sources of active metabolites. Therefore, future studies on the current topic are recommended to define the details of the chemical composition of the metabolites and compare their effects with those of pure compounds obtained from plant or animal sources or chemical synthesis.

Authors' Contribution

Study concept and design: J. H.
Acquisition of data: L. E.

Analysis and interpretation of data: J. H. and R. B.
Drafting of the manuscript: J. H.
Critical revision of the manuscript for important intellectual content: J. H.
Statistical analysis: L. E.
Administrative, technical, and material support: J. H., Gh. Z. and R. B.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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References

Almasi, F., Mohammadipanah, F., Adhami, H.R., Hamedi, J., 2018. Introduction of marine-derived Streptomyces sp. UTMC 1334 as a source of pyrrole derivatives with anti-acetylcholinesterase activity. J Appl Microbiol 125, 1370-1382.

Belghit, S., Driche, E.H., Bijani, C., Zitouni, A., Sabaou, N., Badji, B., *et al.*, 2016. Activity of 2,4-Di-tert-butylphenol produced by a strain of Streptomyces mutabilis isolated from a Saharan soil against Candida albicans and other pathogenic fungi. J Mycol Med 26, 160-169.

El Euch, I.Z., Frese, M., Sewald, N., Smaoui, S., Shaaban, M., Mellouli, L., 2018. Bioactive secondary metabolites from new terrestrial Streptomyces sp. TN82 strain: Isolation, structure elucidation and biological activity. Med Chem Res 27, 1085-1092.

Giddings, L.-A., Newman, D.J., 2015. Bioactive Compounds from Terrestrial Extremophiles. In: Giddings, L.-A., Newman, D.J. (Eds.), Bioactive Compounds from Terrestrial Extremophiles, Springer International Publishing, Cham, pp. 1-75.

Ginj, C., Ruegger, H., Amrhein, N., Macheroux, P., 2005. 3'-Enolpyruvyl-UMP, a novel and unexpected metabolite in nikkomycin biosynthesis. Chembiochem 6, 1974-1976.

Hamedi, J., Kafshnouchi, M., Ranjbaran, M., 2019. A Study on actinobacterial diversity of Hampoeil cave and screening of their biological activities. Saudi J Biol Sci 26, 1587-1595.

Hamedi, J., Poorinmohammad, N., Wink, J., 2017. The Role of Actinobacteria in Biotechnology. In: Wink, J., Mohammadipanah, F., Hamedi, J. (Eds.), Biology and Biotechnology of Actinobacteria, Springer International Publishing, Cham, pp. 269-328.

Han, C., Shen, R., Su, S., Porco, J.A., Jr., 2004. Copper-mediated synthesis of N-acyl vinylogous carbamic acids and derivatives: synthesis of the antibiotic CJ-15,801. Org Lett 6, 27-30.

Han, Y., Xu, Q., Hu, J.N., Han, X.Y., Li, W., Zhao, L.C., 2015. Maltol, a food flavoring agent, attenuates acute alcohol-induced oxidative damage in mice. Nutrients 7, 682-696.

Hooi, J.K.Y., Lai, W.Y., Ng, W.K., Suen, M.M.Y., Underwood, F.E., Tanyingoh, D., *et al.*, 2017. Global Prevalence of Helicobacter pylori Infection: Systematic Review and Meta-Analysis. Gastroenterology 153, 420-429.

Hu, Y., Martinez, E.D., MacMillan, J.B., 2012. Anthraquinones from a marine-derived Streptomyces spinoverrucosus. J Nat Prod 75, 1759-1764.

Jeong, E.-Y., Jeon, J.-H., Kim, H.-W., Kim, M.-G., Lee, H.-S., 2009. Antimicrobial activity of leptospermone and its derivatives against human intestinal bacteria. Food Chem 115, 1401-1404.

Kornsakulkarn, J., Saepua, S., Supothina, S., Chanthaket, R., Thongpanchang, C., 2014. Sporaridin and sporazepin from actinomycete Streptosporangium sp. BCC 24625. Phytochem Lett 10, 149-151.

Meletiadis, J., Pournaras, S., Roilides, E., Walsh, T.J., 2010. Defining fractional inhibitory concentration index cutoffs for additive interactions based on self-drug additive combinations, Monte Carlo simulation analysis, and in vitro-in vivo correlation data for antifungal drug combinations against Aspergillus fumigatus. Antimicrob

Agents Chemother 54, 602-609.

Rayudu, S.R., 1990. Ester of carbamic acid useful as a microbicide and a preservative. Google Patents.

Salimi, F., Hamedi, J., Motevaseli, E., Mohammadipanah, F., 2018. Isolation and screening of rare Actinobacteria, a new insight for finding natural products with antivascular calcification activity. J Appl Microbiol 124, 254-266.

Salsbury, L.E., Robertson, K.N., Flewelling, A.J., Li, H., Geier, S.J., Vogels, C.M., et al., 2015. Anti-mycobacterial activities of copper (II) complexes. Part II. Lipophilic hydroxypyridinones derived from maltol. Can J Chem 93, 334-340.

Shao, J., He, Y., Li, F., Zhang, H., Chen, A., Luo, S., *et al.*, 2016. Growth inhibition and possible mechanism of oleamide against the toxin-producing cyanobacterium Microcystis aeruginosa NIES-843. Ecotoxicology 25, 225-233.

Shirokikh, I.G., Shirokikh, A.A., Ashikhmina, T.Y., 2018. Assessing the Antagonistic Potential and Antibiotic Resistance of Actinomycetes Isolated from Two Zheltozems of Southeastern China. Eurasian Soil Sci 51, 857-864.

Shiroza, T., Ebisawa, N., Furihata, K., Endō, T., Seto, H., Ōtake, N., 1982. Isolation and Structures of New Peptide Antibiotics, Cirratiomycin A and B. Agric Biol Chem 46, 865-867.

Testerman, T.L., Morris, J., 2014. Beyond the stomach: an updated view of Helicobacter pylori pathogenesis, diagnosis, and treatment. World J Gastroenterol 20, 12781-12808.

Ueki, T., Kinoshita, T., 2004. Stereoselective synthesis and structure of butalactin and lactone II isolated from Streptomyces species. Org Biomol Chem 2, 2777-2785.

Zanatta, N., Borchhardt, D.M., Alves, S.H., Coelho, H.S., Squizani, A.M., Marchi, T.M., *et al.*, 2006. Synthesis and antimicrobial activity of new (4,4,4-trihalo-3-oxo-but-1-enyl)-carbamic acid ethyl esters, (4,4,4-trihalo-3-hydroxy-butyl)-carbamic acid ethyl esters, and 2-oxo-6-trihalomethyl-[1,3]oxazinane-3-carboxylic acid ethyl esters. Bioorg Med Chem 14, 3174-3184.

Zimmerli, B., Schlatter, J., 1991. Ethyl carbamate: analytical methodology, occurrence, formation, biological activity and risk assessment. Mutat Res Genet Toxicol 259, 325-350.