



Antibacterial and Antifungal Activity of Novel Freshwater Bacterium *Tabrizicola aquatica* as a Prominent Natural Antibiotic Available in Qurugol Lake

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Abstract

Background: Recently, resistant pathogenic microorganisms have become increasingly wide spread. The search for new natural antibiotics is a viable solution to this problem. For this aim we investigated the antimicrobial ability of *Tabrizicola aquatica*, the novel bacterium isolated from Qurugol Lake located nearby Tabriz city, Iran.

Methods: The antimicrobial properties of *Tabrizicola aquatica* was investigated using well diffusion test. *Tabrizicola aquatica* was incubated at 40°C in shaking incubator at 150 rpm for 14 days. The culture was centrifuged to obtain cell free supernatant, which was sterilized using 0.2 µm filter paper and lyophilized. Microorganisms were lawn and then wells were prepared over the agar plates. About 100 ml of the diluted lyophilized supernatant was added to the wells. The plates then were incubated at 37°C. After 48 hours, antimicrobial activity was defined by measuring the inhibition zone diameter.

Results: The bacterial filtrates had considerable antagonistic effect against *Escherichia coli*, *Rhizobium radiobacter*, *Pseudomonas syringae*, *Erwinia amylovora*, *Botrytis cinerea*, *Neurospora crassa* and *Fusarium oxysporum*. However, the filtrates did not show any inhibitory action on the *Aspergillus flavus* and *Klebsiella pneumoniae*. The supernatant decreased the growth zone on *Streptococcus aureus*, *Pseudomonas aeruginosa*, *Shigella flexneri*, *Xanthomonas camoestrus* and *Bacillus cereus*. The result of MIC against pathogens was found for *Neurospora crassa* in the 50 µg/mL.

Conclusion: The results, suggested that *Tabrizicola aquatica* and similar bacteria can be helpful to control freshwater natural water sources from pathogenic microorganism. Moreover, microbial natural products are still the most promising source of new antibiotics. Our results point out a scope for characterization of the metabolites and could be a candidate in the identification of novel antibiotics.

Introduction

Nowadays, rapid emergence of resistant bacteria is occurring worldwide and multi- drug resistant microorganisms are a major health problem in pharmaceutical and medicine. The World Health Organization (WHO) has announced that antibiotic resistance is one of the three most important public health threats of the 21st century. Therefore, exploring the new sources of compounds with antimicrobial effect is important to find novel agents against resistant pathogen microorganisms.^{1,2} Tracking the novel natural antibiotics that defeat antibiotic resistance of

pathogenic microorganisms is a vital solution for the obstacle.³⁻⁶ Nowadays, microbial natural products could be the source of the majority of the antibiotics which are commonly used in different fields. Unfortunately, the novel antibiotics which are under development in the pharmaceutical industry, now, are scarce. However, microbial natural products are the most hopeful source of new antibiotics, although novel methods are needed to progress the yield of the discovery procedures.⁷ The first report of aquatic bacterium which produces a new antibiotic was in 1966.⁸ Different micro-biome, including

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archaea, bacteria and fungi also show a role in production of several antimicrobial compounds.⁹ Hence, maybe antimicrobial compounds isolated from new bacteria represent an alternative source of novel antibiotics which can be used to accumulate the starved pharmaceutical business. Many researches have been conducted in several part of ocean regions like Atlantic, Pacific Ocean and the Mascarene Islands and represent an extremely fertile reservoir of metabolic novelties.^{10,11} Until now, no study has been conducted about the antibiotic properties of marine isolated from Qurugol Lake neighboring Tabriz city, Iran. *Tabrizicola aquatica* *gen. nov. sp. nov.*, is a new alphaproteobacterium which is isolated from Qurugol lake neighboring Tabriz city,¹² Iran. The bacterium is a gram-negative, non-motile and rod-shaped and can grow chemo-organoheterotrophically and chemo-lithoautotrophically.¹³ The present study evaluates the antimicrobial activity of *Tabrizicola aquatica* RCRI19^T which was isolated from Qurugol Lake neighboring Tabriz city, Iran.

Materials and Methods

Sample growth conditions

The frozen sample of *Tabrizicola aquatica* Strain RCRI19^T in -70°C was thawed and cultured into complete sterile marine broth medium (Lab made) without NaCl.¹⁴ The pH of medium was adjusted to 7.6 ± 0.2 before autoclaving. The bacterial culture was incubated at 30°C for 72h.

Preparation of pathogenic microorganisms

The gram positive bacteria consist of: *Streptococcus aureus* strain ATCC35668^T and *Bacillus cereus* strain ATCC11778^T and the gram negative bacteria including *Escherichia coli* strain O157^T, *Shigella flexneri* PTCC1234^T, *Klebsiella pneumonia* PTCC10031^T and *Pseudomonas aeruginosa* ATCC10231^T were prepared as the human pathogenic microorganisms. In addition, *Aspergillus flavus* strain IBRC-M30029^T, *Fusarium oxysporum* IBRC-M30067^T, *Neurospora crassa* IBRC-M30138^T, *Erwinia amylovora* strain IBRC-M10748^T, *Botrytis cinerea* strain IBRC-M30162^T, *Pseudomonas syringae* *pv. Syringae* strain IBRC-M10702^T, *Rhizobium radiobacter* strain IBRC-M10701^T and *Xanthomonas campestris* strain IBRC-M10644^T were prepared as the plant pathogenic microorganisms. All strains were obtained from Iranian Biological Research Center. The pathogenic microorganisms were cultured in nutrient broth at 37°C for 24 h and in a rotary shaker. The cultures were centrifuged at 10,000 rpm for 5 min and the pellets of bacteria were dissolved in ddH₂O in order to obtain the optical density at 600 nm (OD₆₀₀). The fungal inoculums were prepared from old culture grown on MEA medium in 10 day. The petri dishes were rinsed with 10 ml of distilled water and the conidia were scraped using sterile spatula. The spore density for each fungus was adjusted in OD:595 nm in order to obtain the final concentration of about 10⁵ spores/ml.

Solvent extraction

Tabrizicola aquatica RCRI19^T was incubated in marine broth medium without NaCl (lab made) at 30 °C for 48h. The cells were provided in 10 mL of marine broth to obtain the condensation to 0.5 McFarland. The bacterial solution was dissolved in the 1 L of marine broth and incubated for two weeks at 30 °C on an orbital shaker 150 rpm. Bacterial culture was centrifuged at 5000 rpm for 10 min and the extracts, biomass and secondary metabolites were obtained from the broth culture by passing through a 0.22 µm (Millipore Corporation USA) pore filter membrane based on agar well diffusion method according to described by Ennahar *et al.*, (2000). The supernatant was frozen and freeze-dried and then maintained in sterile bottles at -20 °C for next experiment.¹⁵

Anti-bacterial testing

The antibacterial activity of *Tabrizicola aquatica* Strain RCRI19^T was measured using agar dilution technique. All pathogenic bacterial strains were cultured in Mueller Hinton medium (MHB, Sigma) for 24 h at 37°C. 0.5 McFarland standard (10⁸ cfu/ml) of bacterial suspensions were prepared by dilution in Mueller Hinton broth and pureed on Mueller Hinton agar plates. Subsequently, the plates were gently shaken for spreading the microorganisms to the media to obtain suitable mix. The 6mm in diameter wells were prepared by using a sterile cylinder on the surface of plates. Each well was occupied with 0.1 mL of the *Tabrizicola aquatica* strain RCRI19^T extracts. All of the plates were incubated at 37°C for 24 h. The microbial inhibition of dishes was considered after incubation. Positive control was streptomycin sulphate (10 µg mlG) and negative control was methanol solvent (100 µg mlG). The diameters of the inhibition zones were measured in mm.¹⁵ Each test was carried out triplicate and the mean of the inhibition zones was calculated for every strain.

Antifungal activity

The pathogenic fungal were cultured on MEA medium for 10 days. The antifungal activity by *Tabrizicola aquatica* RCRI19^T was carried out by disc diffusion method as described above. Nystatin (10 µg disc) and solvent methanol were used as positive and negative controls, respectively. The antifungal activity of *Tabrizicola aquatica* aquatic strain RCRI^T was investigated after 72 h of incubation at 30°C. The diameters of the inhibition zones were measured in mm. Each test was carried out triplicate and the mean of inhibition zones was calculated for each fungus.

Minimum inhibitory concentration and Minimum bactericidal concentration

Micro broth dilution method was carried out for evaluation of the Minimum inhibitory concentration (MIC) according to Clinical Laboratory Standards method described by the National Committee (NCCLS). The MIC value is detected as the lowest concentration for completely inhibition of the bacterial growth after 48 h. In addition, for detection of

the MIC rate against experimented pathogens, the various concentrations including (10^{-1} g / ML to 10^{-8} g / ml) were carried out according to described method by Elyasifar *et al.* (2019).¹⁶ Moreover, 5 μ l of liquid from every well which exhibited no growth were taken and incubated for 24 h in the same condition for determination of MBC. The lowest concentration after sub-culturing that prevented no obvious bacterial growth was taken as MBC.¹⁶

Minimum inhibitory concentration and minimum fungal concentration

In this stage of study, the rate of MIC from bacterial extract was carried out against fungal pathogens according to macro broth dilution method. Several dilutions of bacterial extract including 10^{-1} g/ml to 10^{-8} g/ml were provided and 1 mL of extracts was added to 50 mL suitable medium in tube 1 then contents were complexed and 5 mL was dissolved to tube 2. This periodical dilution was re-done through to tube 10. Nine hundred microliter of inoculum was transferred to tubes 1 – 10. The negative control is involved an antimicrobial extract and culture media and the positive controls including the mix of culture medium with 0.5 McFarland of the pathogen. Moreover, the MFC was determined as the lowest concentration with three or fewer colonies incubated at 30 °C for 72 h.¹⁶

Statistical analysis

The analysis of data was performed using SPSS software version 16 to calculate the average of inhibition zones on experimented microorganisms.

Results

Antimicrobial and Antifungal activity of *Tabrizicola aquatica* RCRI19^T

The antimicrobial activity of new marine bacterium genus (*Tabrizicola aquatica* RCRI19^T) has been investigated against six bacterial species of pathogens (Figure 1) including *Bacillus cereus* strain ATCC11778^T, *Streptococcus aureus* strain ATCC35668^T, *Escherichia coli* strain O157 PTCC1276^T, *Shigella flexneri* strain PTCC1234^T, *Klebsiella pneumoniae* strain PTCC10031^T and *Pseudomonas aeruginosa* strain ATCC10231^T. Additionally, antifungal activities of stain RCRI19^T was screened against plant pathogens including *Aspergillus flavus* strain IBRC-M30029^T, *Fusarium oxysparum* IBRC-M30067^T,

Neurospora crassa IBRC-M30138^T, *Erwinia amylovora* strain IBRC-M10748^T, *Botrytis cinerea* strain IBRC-M30162^T, *Pseudomonas syringae* pv. *Syringae* strain IBRC-M10702^T, *Rhizobium radiobacter* strain IBRC-M10701^T and *Xanthomonas campestris* strain IBRC-M10644^T were prepared as the plant pathogenic microorganisms. Our results indicated that, *Tabrizicola aquatica* RCRI19^T has ability of production of inhibition zones against human pathogen, *Bacillus cereus*, *Shigella flexneri*, *Escherichia coli* O157 and *Streptococcus aureus*. However, it was not active *Klebsiella pneumoniae*. The highest inhibition zone was observed against *Streptococcus aureus* (6 mm) (Table 1).

Tabrizicola aquatica RCRI19^T depicted antimicrobial effect against plant pathogen *Fusarium oxysparum*, *Neurospora crassa*, *Botrytis cinerea*, *Pseudomonas syringae*, *Xanthomonas campestris*, *Erwinia amylovora*, *Rhizobium radiobacter*. However, *Tabrizicola aquatica* was not active against *Aspergillus flavus*. This isolate showed the highest inhibition zone against *Neurospora crassa* (8 mm)(Table 2). The MIC and MBC values showed that *Tabrizicola aquatica*

Table 1. The zones of inhibition (diameter in mm) against various human bacterial pathogens.

Human pathogens	<i>Tabrizicola aquatic</i> RCRI19 ^T
<i>Bacillus cereos</i>	4±0.3
<i>Escherichia coli</i> O157	2.5±0.4
<i>Klebsiella pneumoniae</i>	NI
<i>Shigella flexneri</i>	3±0.5
<i>Streptococcus aureus</i>	6±0.1

Data are expressed as the average of three determinations ± standard deviations. NI: Not Inhibited

Table 2. The zones of inhibition (diameter in mm) against various plant bacterial pathogens.

Plant Pathogen	<i>Tabrizicola aquatic</i> RCRI19 ^T
<i>Pseudomonas syringae</i> pv. <i>Syringae</i>	1.1±0.5
<i>Xanthomonas campestris</i>	3±0.2
<i>Erwinia amylovora</i>	1.4±0.2
<i>Rhizobium radiobacter</i>	7.5±0.4
<i>Fusarium oxysparum</i>	7.5±0.2
<i>Aspergillus flavus</i>	NI
<i>Neurospora crassa</i>	8±0.3
<i>Botrytis cinerea</i>	6.5±0.2

Data are expressed as the average of three determinations ± standard deviations. NI: Not Inhibited

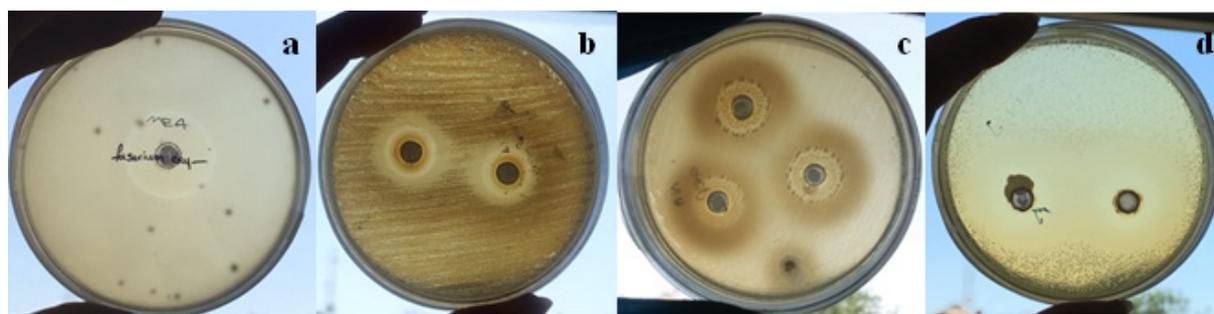


Figure 1. The antimicrobial activity of novel marine bacterium genus *Tabrizicola aquatica* RCRI19^T on concentrated pathogenic cell free culture medium on the growth a) *F. oxysporum* b) *R. radiobacter* c) *S. aureus* d) *E. amylovora* aureus d) *E. amylovora*

RCRI19^T display antimicrobial activity just against *Neurospora crassa*. The activity of *Tabrizicola aquatica* RCRI19^T against *Neurospora crassa* was considerable in the highest values (MIC=50 µg/ml). However, the extraction of *Tabrizicola aquatica* biomass didn't show any activity against human and the others pathogens.

Discussion

During the last decades, the incidence of antimicrobial resistance has increased dramatically due to overuse of antibiotics worldwide. Today's, antimicrobial resistance is one of the great health problems which threatens to human health worldwide.^{5,6,17} Therefore, the finding of new antibiotics is important issue for dissolving the problem.¹⁸ Marine bacteria are capable to product novel bioactive compounds with antimicrobial and antitumor effects.¹⁹

Here, the antimicrobial production ability of *Tabrizicola aquatic* RCRI19^T, a new bacterium isolated from Qurugol Lake located neighboring Tabriz city, Iran, is reported for the first time.¹³ The whole genome of *Tabrizicola aquatica* was sequenced. It is available in the National Center for Biotechnology Information (NCBI) with accession number of NZ_PJON000000001.1. *Tabrizicola aquatica* has 3848 genes that 3753 of them are encoding genes. Several studies investigated the abilities of this novel genus such as polycyclic aromatic hydrocarbons (PAHs) bio-remediation, Heavy metal bio-absorption and ability of photo-litho/chemo trophy.²⁰ In this study, the antimicrobial activity was verified against bacterial and fungal pathogens by agar well diffusion. Previous studies using plant secondary metabolites reported that gram-positive bacteria were more resistant to the antimicrobial agents than gram-negative bacteria,^{21,22} however, no obvious difference of susceptibility of the tested antimicrobial agents was found between gram-positive and gram-negative bacteria in this study. The supernatant of the bacterium failed to exhibit antimicrobial activity against *Aspergillus flavus* and *Klebsiella pneumonia*.

In the past decade, researches have been conducted to find new bio-control agents active against phytopathogenic bacteria in order to reduce the use of chemicals.²³ The control of fruits and vegetables diseases using biological methods such as microorganisms proposes another program that has shown significant potential. Therefore, focusing on various environment to find new and capable antimicrobial compounds against these phytopathogenic microorganisms is important.²⁴ In the present study, the inhibitory effect of the *Tabrizicola aquatica* against several phytopathogens was verified. The results showed that the supernatant of the bacterium was active against *Rhizobium radiobacter*, *Erwinia amylovora*, *Pseudomonas syringea* and *Xanthomonas campestris*.

Recently, fear on chemical fungicide which remains in the environment, food and feeds have led to a restriction of some of the agents generally used to control the pathogens of plant and also post-harvest diseases. Several researchers have proposed the biological control²⁵⁻²⁷ or usage of microbial fungicides²⁸ can be potential strategy to chemical fun-

gicides. Here, we showed that *Tabrizicola aquatica* produces extracellular antifungal compounds which inhibited the growth of *Botrytis cinerea*, *Neurospora crassa* and *Fusarium oxysparum*.

Conclusion

Our results confirmed that *Tabrizicola aquatica* RCRI19^T was a relatively abundant source of benefit secondary metabolites. These secondary metabolites displayed antifungal and antibacterial effects and empowered the bacterium to live in its natural environment. These results point to the potential industrial significance of the bacterium. It seemed that *Tabrizicola aquatica* RCRI19^T and the other similar freshwater bacteria had a potential to be used in remediation of water from pathogenic microorganisms. However, more investigations including purification and characterization of the antimicrobial compounds and also their valuation for toxicity and disintegration in the environment are needed.

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Conflict of interest

We declare that there is no conflict of interest. There are neither ethical nor financial conflicts of interest involved in the manuscript.

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