**Siderophore and indolic acid production by *Paenibacillus triticisoli* BJ-18 and their plant growth-promoting and antimicrobe abilities**

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*Paenibacillus triticisoli* BJ-18, a N$_2$-fixing bacterium, is able to promote plant growth, but the secondary metabolites that may play a role in promoting plant growth have never been characterized. In this study, untargeted metabolomics profiling of *P. triticisoli* BJ-18 indicated the existence of 101 known compounds, including N$_2$-acetyl ornithine, which is the precursor of siderophores, plant growth regulators such as trehalose 6-phosphate, betaine and trigonelline, and other bioactive molecules such as oxymatrine, diosmetin, luotonin A, (-)-caryophyllene oxide and tetrahydrocurcumin. In addition, six compounds were also isolated from *P. triticisoli* BJ-18 using a combination of silica gel chromatography, sephadex LH-20, octadecyl silane (ODS), and high-performance liquid chromatography (HPLC). The compound structures were further analyzed by Nuclear Magnetic Resonance (NMR), Mass Spectrometry (MS), and Electronic Circular Dichroism (ECD). The six compounds included three classical siderophore fusarinines identified as deshydroxylferritriacetylfusigen, desferritriacetylfusigen, and triacetylfusigen, and three indolic acids identified as paenibacillic acid A, 3-indoleacetic acid (IAA), and 3-indolepropionic acid (IPA). Both deshydroxylferritriacetylfusigen and paenibacillic acid A have new structures. Fusarinines, which normally occur in fungi, were isolated from bacterium for the first time in this study. Both siderophores (compounds 1 and 2) showed antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis*, but did not show obvious inhibitory activity against yeast *Candida albicans*. Whereas triacetylfusigen (compound 3) showed no antibiosis activity against these test microorganisms. Paenibacillic acid A, IAA, and IPA were shown to promote the growth of plant shoots and roots, and paenibacillic acid A also showed antimicrobial activity against *S. aureus*. Our study demonstrates that siderophores and indolic acids may play an important role in plant growth promotion by *P. triticisoli* BJ-18.
Siderophore and indolic acid production by *Paenibacillus triticisoli* BJ-18 and their plant growth-promoting and antimicrobe abilities

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Abstract

*Paenibacillus triticisoli* BJ-18, a N$_2$-fixing bacterium, is able to promote plant growth, but the secondary metabolites that may play a role in promoting plant growth have never been characterized. In this study, untargeted metabolomics profiling of *P. triticisoli* BJ-18 indicated the existence of 101 known compounds, including N$_2$-acetyl ornithine, which is the precursor of siderophores, plant growth regulators such as trehalose 6-phosphate, betaine and trigonelline, and other bioactive molecules such as oxymatrine, diosmetin, luotonin A, (-)-caryophyllene oxide and tetrahydrocurcumin. In addition, six compounds were also isolated from *P. triticisoli* BJ-18 using a combination of silica gel chromatography, sephadex LH-20, octadecyl silane (ODS), and high-performance liquid chromatography (HPLC). The compound structures were further analyzed by Nuclear Magnetic Resonance (NMR), Mass Spectrometry (MS), and Electronic Circular Dichroism (ECD). The six compounds included three classical siderophore fusarinines identified as deshydroxylferritriacetylfusigen, desferritriacetylfusigen, and triacetylfusigen, and three indolic acids identified as paenibacillic acid A, 3-indoleacetic acid (IAA), and 3-indolepropionic acid (IPA). Both deshydroxylferritriacetylfusigen and paenibacillic acid A have new structures. Fusarinines, which normally occur in fungi, were isolated from bacterium for the first time in this study. Both siderophores (compounds 1 and 2) showed antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis*, but did not show obvious inhibitory activity against yeast *Candida albicans*. Whereas triacetylfusigen (compound 3) showed no antibiosis activity against these test microorganisms. Paenibacillic acid A, IAA, and IPA were shown to promote the growth of plant shoots and roots, and paenibacillic acid A also showed antimicrobial activity against *S. aureus*. Our study demonstrates that siderophores and indolic acids may play an important role in plant growth promotion by *P. triticisoli* BJ-18.

Introduction

Plant growth-promoting bacteria (PGPB) have great usage as agricultural inoculants, such as biofertilization and biocontrol of pathogens (Backer et al., 2018). Commercialized PGPB strains mainly include the members of *Agrobacterium, Azospirillum, Bacillus, Paenibacillus, Pseudomonas, Streptomyces* et al. (Lucy, Reed & Grick, 2004; Banerjee, Yesmin & Vessey, 2006). PGPB may promote plant growth directly usually by either facilitating resource acquisition (e.g., nitrogen fixation, production of indolic compounds and siderophores, phosphate solubilization, and 1-aminocyclopropane-1-carboxylate deaminase activity) or indirectly by decreasing the inhibitory effects of various pathogenic agents on plant growth and...
development (antibiotics and lytic enzymes), that is, by acting as biocontrol bacteria (Glick, 2012).

Nitrogen (N) fixation is catalyzed by molybdenum-dependent nitrogenase, which is a metalloenzyme composed of two protein components, referred to as MoFe protein and Fe protein. The atmospheric N\textsubscript{2} is reduced to bioavailable NH\textsubscript{4}\textsuperscript{+} by nitrogen fixation, providing a large amount of natural N into cultivated agricultural systems (Galloway et al., 2008). In addition to symbiotic N\textsubscript{2}-fixing Rhizobia associated with legumes, the non-symbiotic N\textsubscript{2}-fixing bacteria are also important contributors to the N nutrition of non-legumes (Gupta, Roper & Roget, 2006). It is estimated that the microbial N accounts for roughly 30–50% of the total N in crop fields (Liu et al., 2017).

Iron is the fourth most abundant element on earth but its bioavailability is extremely limited due to its poor solubility (Braun & Braun, 2002; Miethke & Marahiel, 2007; Kazamia et al., 2018). Microbes have evolved strategies to obtain sufficient amounts of iron, such as chelation, reduction, and protonation (Guinot, 1994). The use of a siderophore to transport iron by chelation is vitally important for bacteria. Siderophores are low molecular weight compounds (500–1500 Da) possessing a high affinity for Fe\textsuperscript{3+} (\(K_f > 10^{30}\)) (Haas & Defago, 2005) and they are synthesized by bacteria through non-ribosomal pathways (Hutchins et al., 1999; Khan et al., 2017). Many PGPB have been reported to produce siderophores, such as Bacillus subtilis, Paenibacillus polymyxa SK1, Mesorhizobium sp., Brevibacillus brevis GZDF3 (Franco-Sierra et al., 2020; Khan et al., 2020; Menéndez et al., 2020; Sheng et al., 2020). The chelate of siderophores and ferric iron can be directly absorbed by plants and are called mechanism III, which is thought to be used by plants to resist iron stress (Shanker et al., 1992; Yehuda et al., 1996; Chen, Dick & Streeter, 2000). When grown under iron-limiting conditions, mung bean plants by inoculation with the siderophore-producing Pseudomonas strain GRP3 showed reduced chlorotic symptoms and an enhanced chlorophyll level compared to uninoculated plants (Sharma et al., 2003). Tomato seedlings inoculation with these two Mesorhizobium strains that produce siderophores and IAA showed significantly higher plant growth traits than uninoculated seedlings (Menéndez et al., 2020). In addition, siderophores secreted by PGPB can suppress plant pathogens by competing for trace amounts of iron in the environment (Glick, 2012). It has been suggested that biocontrol PGPB produce siderophores that have a much greater affinity for iron than do fungal pathogens so that the fungal pathogens are unable to proliferate in the root rhizosphere of the host plant due to lack of iron (Schippers et al., 1987; O’Sullivan & O’Gara, 1992). For examples, siderophores have biocontrol roles against plant pathogens, such as Piricularia oryzae (Yuquan et al., 1999), Stagonospora curtisii (Shuangya, Yongxiang & Xiangqun, 2003), Fusarium oxysporum (Duijff et al., 1993), Macrophomina phaseolina (Arora, Kang & Maheshwari, 2001) and Cryphonectria parasitica (Chen et al., 2006).
Indolic acids include several compounds, of which indole-3-acetic acid (indole acetic acid, IAA) is by far the most common as well as the most studied auxin (Xie et al., 2005). They stimulate cell division and promote cell elongation (Katzy et al., 1990; Weyers & Paterson, 2001). They are abundant in higher plants and rhizospheric microorganisms and play a vital role in plant–microbe interactions (Beneduzi et al., 2008; Costacurta & Vanderleyden, 1995; Lambrecht et al., 2000; Beck, Hansen & Lauritsen, 2003; Mao et al., 2014). Previous studies have demonstrated that Paenibacillus spp. can produce indolic compounds to promote plant growth (Kumari & Thakur., 2018; Castellano et al., 2018; Lebuhn, Heulin & Hartmann, 1997). Endophytic bacteria promote growth of the medicinal legume Clitoria ternatea by IAA production, P and K-solubilization (Aeron, Maheshwari & Meena, 2020).

Paenibacillus triticisoli BJ-18 (=DSM 25425T = CGMCC 1.12045T) is a N₂-fixing bacterium isolated by our laboratory from wheat rhizosphere soil in Beijing (Wang et al., 2013). Recently, we have shown that inoculation with P. triticisoli BJ-18 significantly promotes the growth of tomato, maize and wheat (Xie et al., 2016; Shi et al., 2016; Li et al., 2019). The ¹⁵N-isotope-enrichment experiment indicated that plant seedlings inoculated with P. triticisoli BJ-18 derived 12.9–36.4% N from nitrogen fixation (Li et al., 2019). However, the secondary metabolites of P. triticisoli BJ-18 have never been isolated or characterized. In this study, six compounds, composed of three siderophores and three indolic acids, were isolated and characterized from P. triticisoli BJ-18. Notably, a new siderophore and a new indolic acid were here identified. Our results will provide insight into the mechanisms by which P. triticisoli BJ-18 promotes plant-growth, including nitrogen fixation and the secretion of siderophores and indolic acids.

Materials & Methods

Bacterial strain

The bacterial strain Paenibacillus triticisoli BJ-18 (=DSM 25425T = CGMCC 1.12045T) was used in our study.

Untargeted metabolomics by LC-MS

The equipment and raw data for untargeted metabolomics were provided by Beijing Novogene Technology Co., Ltd. P. triticisoli BJ-18 was cultured in Lysogeny Broth (LB) (10 g tryptone, 5 g yeast, 10 g NaCl, 15 g agar per 1 L H₂O) at 30°C for 3 days. A single colony was inoculated in 20 mL CH medium (30 g sucrose, 6.4 g tryptone, 7 g yeast, 0.6 g MgSO₄·7H₂O, 3.5 g NaCl, 0.1 g K₂HPO₄, 0.4 g KH₂PO₄ per 1 L H₂O) and cultured at 30 °C 200 rpm for 48 h. The seed solution was inoculated into 150 mL of CH medium at an inoculation amount of 2% and cultured at 30 °C 200 rpm for 48 hours. Cells were harvested by centrifugation at 6000 rpm at 4 °C for 10 min, ground with liquid nitrogen, and resuspended with 500 μL of 80% methanol solution containing 0.1 % formic acid. The homogenate was incubated on ice for 5 min and was
centrifuged at 15000 rpm at 4 °C for 10 min. 200 μL of supernatant was subsequently transferred to a fresh Eppendorf tube with a 0.22 μM filter and was centrifuged at 15000 rpm at 4°C for 10 min. LC-MS/MS analyses were performed using the Vanquish UHPLC system (Thermo Fisher) coupled with an Orbitrap Q Exactive series mass spectrometer (Thermo Fisher) in a Hyperil Gold column (100×2.1 mM, 1.9μM) with a 16-min linear gradient at a flow rate of 0.2 mL/min. The eluents for the positive polarity mode were eluent A (0.1% formic acid in water) and eluent B (methanol). The eluents for the negative polarity mode were eluent A (5 mM ammonium acetate, pH 9.0) and eluent B (methanol). The solvent gradient was set as follows: 2% B, 1.5 min; 2-100% B, 12.0 min; 100% B, 14.0 min; 100-2% B, 14.1 min; 2% B, 16 min. The Q exactive mass series spectrometer was operated in positive/negative polarity mode with spray voltage of 3.2 kV, sheath gas flow rate of 35 arb, aux gas flow rate of 10 arb, and a capillary temperature of 320°C. Compound discoverer 3.0 (CD 3.0, Thermo Fisher) was used for processing the data files to match results from the mzCloud (https://www.mzcloud.org/) and ChemSpider (http://www.chemspider.com/) databases. Accurate qualitative and relative quantitative results were obtained through statistical analysis.

Reagents and instruments for separation and purification of compounds 1-6

HPLC data were acquired using a Waters 2695 instrument. HRESIMS and HPLC-ESI-MS data were obtained using an Accurate-Mass-Q-TOF LC/MS 6520 instrument (Agilent, USA) in positive ion mode. ¹H and ¹³C NMR data were obtained by Bruker Avance-500 spectrometers (Rheinstetten, Germany) with solvent signals (DMSO-d₆, δH 2.500/δC 39.520) as references, and HSQC and HMBC experiments were optimized for 145.0 and 8.0 Hz, respectively. The optical rotations measurement was conducted on an Anton Paar MCP200 polarimeter (Anton Paar, Austria). Optical rotations were conducted using a Perkin-Elmer 241 polarimeter, and UV data were detected by a Shimadzu Biospec-1601 spectrophotometer. The absorbance of 96-well clear plate was measured on a microplate reader (Molecular Devices, SpectraMax® Paradigm®). Analytically pure solvents including methanol, dichloromethane, and ethyl acetate were used for extraction and chromatographic separation. TLC was carried out on silica gel HSGF₂₅₄ plates and spots were visualized by UV at 254 nm or sprayed with 10% H₂SO₄ and heated. Silica gel (150–250 μM, Qingdao Haiyang Chemical Co., Ltd.), octadecylsilyl (ODS, 50 μM, YMC CO., LTD), and Sephadex LH-20 (Amersham Biosciences) were used for column chromatography (CC). HPLC separation was performed on an Agilent 1200 HPLC system with a Diode Array Detector (DAD) detector using an ODS column (C₁₈, 250 × 9.4 mM, YMC Pak, 5 μM) at a flow rate of 2.0 mL/min.
Extraction and isolation of the secondary metabolites

*P. triticisoli* BJ-18 was grown in a 500 L fermenter (Biotech, Shanghai Baoxing Biological Equipment Engineering Co., Ltd.) filled with 200 L of CH medium at pH 5.0 with 40% to 60% dissolved oxygen and shaking at 100 rpm and at 30 °C for 48 h. For doing this, this bacterium was inoculated into 20 mL of CH medium and then scaled-up to 20 L. The seed culture was inoculated into 200 L of CH medium with an inoculation amount of 1% in a 500 L fermenter.

The fermented culture was passed through a 200 mesh macroporous adsorption resin following 3 periods of 30-minute ultrasonic cell-breaking and was eluted with analytically pure methanol. The organic solvent was collected and evaporated using rotary evaporators to obtain the crude extract (7 g). The aqueous phase was discarded after being extracted and separated three times between the ethyl acetate and the aqueous phases, and the organic phase was retained and evaporated using rotary evaporators. The final product was 4 g of EtOAc (ethyl acetate) extract.

The EtOAc extract was separated into 20 subfractions (Pt-1 to Pt-20) after being subjected to ODS column chromatography with a gradient of methanol-water (5-100%). The fraction Pt-10 was further partitioned by Sephadex LH-20 CC separated by 50% methanol in water to create 26 subfractions (Pt-10-1 to Pt-10-26). Compound 4 (4.2 mg, *t* <sub>R</sub> 31.2 min) was obtained from fraction Pt-10-24 (21.3 mg) by RP-HPLC using 55% methanol in acidic water. Fraction Pt-13 was divided into 16 fractions, Pt-13-1 to Pt-13-16, after being subjected to ODS column chromatography with a gradient of MeOH-H_{2}O (40–80%). Compounds 1-3 (2 mg, 3.1 mg and 8 mg, *t* <sub>R</sub> 62.5 min, 58.4 min and 37.4 min, respectively) were obtained from fractions Pt-13-6 (42.5 mg) by RP-HPLC using 20% acetonitrile in acidic water. Compounds 1-3 were isolated from fraction Pt-13-6 (928 mg) by RP-HPLC (C8) using 80% methanol in acidic water.

Deshydroxylferritriacetylfusigen (compound 1): yellow powder; [α]<sup>25</sup> <sub>D</sub> = −14.0 (c 0.10, MeOH); UV (MeOH) *λ*<sub>max</sub> (log ε) 214 (2.44) nm; NMR data (500 MHz, DMSO-<sub>d6</sub>) is shown in Table 1; positive HRESIMS *m/z* 837.4242 [M + H]<sup>+</sup> (calculated for C<sub>39</sub>H<sub>61</sub>N<sub>6</sub>O<sub>14</sub>, 837.4246, Δ=0.0004).

Desferritriacetylfusigen (compound 2): yellow powder; [α]<sup>25</sup> <sub>D</sub> = −8.0 (c 0.05, MeOH); UV (MeOH) *λ*<sub>max</sub> (log ε) = 214 (2.43) nm; positive HRESIMS *m/z* 853.4203 [M + H]<sup>+</sup>, (calculated for C<sub>39</sub>H<sub>60</sub>N<sub>6</sub>O<sub>15</sub>, 853.4195 Δ=0.0008).
Triacetylfusigen (compound 3): brown powder; $[\alpha]_{D}^{25} = -216.0 \ (c \ 0.025, \text{MeOH})$; UV (MeOH) $\lambda_{\text{max}}$ (log $\varepsilon$) 211 (2.35) nm; positive HRESIMS $m/z$ 906.3316 [M + H]$^+$ (calculated for C$_{39}$H$_{58}$N$_6$O$_{15}$Fe, 906.3309 $\Delta = 0.0007$).

Paenibacillic acid A (compound 4): white powder; $[\alpha]_{D}^{25} = -39.99 \ (c \ 0.10, \text{MeOH})$; UV (MeOH) $\lambda_{\text{max}}$ (log $\varepsilon$) 211 (2.33) nm; NMR data (500 MHz, DMSO-$d_6$) is shown in Table 2; positive HRESIMS $m/z$ 303.1704 [M + H]$^+$ (calculated for C$_{17}$H$_{23}$N$_2$O$_3$, 303.1708 $\Delta = 0.0004$).

3-Indoleacetic acid (IAA) (compound 5): white amorphous powder; UV (MeOH) $\lambda_{\text{max}}$ (log $\varepsilon$) 234 (4.22), 272 (3.77), 280 (3.80), 290 (3.72) nm; positive HRESIMS $m/z$ 176.0709 [M + H]$^+$ (calculated for C$_{10}$H$_{10}$NO$_2$, 176.0701 $\Delta = 0.0008$).

3-Indolepropionic acid (IPA) (compound 6): white amorphous powder; UV (MeOH) $\lambda_{\text{max}}$ (log $\varepsilon$) 235(4.25), 270 (3.77), 280 (3.80), 290 (3.72) nm; positive HRESIMS $m/z$ 190.0871 [M + H]$^+$ (calculated for C$_{11}$H$_{12}$NO$_2$, 190.0868 $\Delta = 0.0003$).

Conversion of compound 3 into desferritriacetylfusigen

The removal of the ferric ion was conducted as reported by Kodani et al. (2015) with additional changes. Compound 3 (4 mg) was dissolved in 1.5 mL of water and then 1.5 mL of 1M 8-quinolinol was added. The solution was stirred for 25 min and mixed with 3 mL of CH$_2$Cl$_2$, then left to stand until bilayer separation occurred. The water layer was evaporated using a freeze-dryer vacuum. This was repeated 3 times to eliminate ferri-8-quinolinol and obtain the total dry material. The sample was then dissolved in 100 $\mu$L of methanol and HPLC purification was performed with the C18 column (7.8 $\times$ 300 mM, Waters, 7 $\mu$M; detector: UV), eluted with 20 % acetonitrile in water at a flow rate of 1 mL/min, and monitored at OD$_{210}$ to create 2.0 mg of desferri-compound 3. Positive HRESIMS and NMR of desferri-compound 3 was the same as compound 2.

Computations

Systematic conformational searches of structure 4a were carried out using MMFF94 force field and CONFLEX (Liu et al., 2018).

Determination of plant growth promoting capacity of indolic compounds

The seeds of Arabidopsis thaliana var. Columbia were sterilized using 75% ethanol for 45 seconds, 5% NaClO for 15 minutes, and were washed in sterile water 3 times. The seeds were then placed horizontally on solid 1/2 Murashige and Skoog (MS) (Murashige & Skoog, 1962) medium supplemented with 1.5% sucrose, 0.35% (w/v) agar, and different concentrations and combinations of 1 mg/L paenibacillic acid A, IAA, and IPA for direct regeneration. At least ten Arabidopsis strains were cultured in each group of treatments. The cultures were incubated at 25 $\pm$ 2 $^\circ$C under cool fluorescent light (2000 lux 16 h/day photoperiod) for 14 days. The ability of
the three indolic acids to promote plant growth was evaluated by measuring the dry weight of the stems and roots of *Arabidopsis thaliana*.

**Detection of indolic compounds**

The Salkowski Reagent (PC technique) was used to determine concentrations of indolic compounds (Glickmann & Dessaux, 1995). IAA standard solutions with concentrations of 0, 5.0, 10.0, 15.0, 20.0, and 25.0 μg/mL were used to react with an equal volume of PC reagent in the dark for half an hour and the OD$_{530}$ value was measured to draw the Salkowski calibration curve. The R$^2$ value should be greater than 0.99.

*P. triticisoli* BJ-18 was grown overnight in 50 mL of LB medium at 200 rpm 30°C. The cells were collected by centrifugation, washed three times with deionized water, and resuspended in 1 mL of deionized water. 100 μL of the bacterial suspension was added to 20 mL nitrogen-deficient medium (prepared with deionized water and supplemented with 100 mM NH$_4$Cl as a nitrogen source) with 36 mg/L iron citrate or no iron citrate. After three days of incubation at 200 rpm and 30°C, all samples were treated with the same method to measure the corresponding OD$_{530}$ value. The content of indolic compounds of each sample was calculated using the Salkowski calibration curve. The nitrogen-deficient medium with deionized water was used as a blank control. Each sample was treated three times.

**Antimicrobial assay**

The antimicrobial assay was performed according to the recommended guidelines of the National Center for Clinical Laboratory Standards (NCCLS) (Li et al., 2008). The bacterial cells of *Escherichia coli* (ATCC1.0090), *Staphylococcus aureus* (ATCC6538), and *Bacillus subtilis* (ATCC6663) were grown in Mueller-Hinton Broth (MHB) medium (5.0 g beef powder, 1.5 g starch, 17.5 g acid hydrolyzed casein, and 1 L H$_2$O) at 37 °C for 24 h. The fungus (yeast), *Candida albicans* (CGMCC2.2086), was grown in Saurer’s Dextrose Broth (SDB) medium (5 g casein trypsin digest, 5 g gastric enzyme digest, 20 g dextrose and 1 L ddH$_2$O) at 28°C for 48 h.

The cells of bacteria and yeast cultivated in MHB and SDB media as described above were seeded onto each well of a 96-well clear plate, then the gradient concentrations of test compounds 1-6 were added to each well and mixed with the targeted microbes to reach a final volume of 100 μL. The inoculation concentrations of bacteria and yeast were 0.5–2.5 × 10$^5$ cfu/mL and 0.5–2.5 × 10$^3$ cfu/mL, respectively. The suspension was cultured at 37°C for 1 day and 28°C for 2 days, respectively. Gentamicin, ampicillin, sulphate streptomycin, and amphotericin B were used as positive controls. A microplate reader was employed to perform at the OD$_{595}$. 
The IC₅₀ of compounds 1-6 were plotted, calculated, and obtained. Each antimicrobial assay was tested in triplicate.

**Siderophores detection by blue agar CAS assay**

Siderophores were detected using a blue agar CAS medium as described by Schwyn and Neiland (1987). The solid CAS medium was composed of 1 mL 20% sucrose solution, 3 mL sterilized 10% casamino acid, 100 μL 1 mmol/L CaCl₂, 5 mL CAS dye solution (a mixture of 0.012 g of chrome azurol S in 10 mL of ddH₂O, 2 mL 1 mmol/L FeCl₃, and 0.015 g hexadecyltrimethylammonium bromide in 8 mL of deionized H₂O), PIPES buffer (pH 6.8-7.0), and 2 g agar powder per 100 mL H₂O.

*P. triticisoli* BJ-18 was inoculated in 20 mL of liquid LB medium with shaking at 200 rpm at 30 °C for 48 h. 1mL of the bacterial solution was centrifuged, washed with sterile deionized water, and then suspended with 200 μL of sterile deionized H₂O. 10 μL of the suspension was inoculated on a CAS plate. The color changed from blue to light orange, indicating the presence of iron carriers.

**Results**

**Untargeted metabolomics profiling of P. triticisoli BJ-18**

The untargeted metabolomics profiling of *P. triticisoli* BJ-18 were analyzed by using LC-MS and the data were comparatively analyzed with the databases of mzCloud, ChemSpider and mzVault. A total of 101 compounds were measured, the majority of which were common compounds involved in the basic metabolism (Table S1), including carbohydrates, amino acids, peptides, alcohols, aldehydes, ketones, fatty acids, lipids, nucleic acids, vitamins, alkaloids, cyclics and their respective derivatives (Fig 1A). Of the 101 compounds, 46 have the molecular weights of 100-200 Da, 34 have the molecular weights of 200-300 Da, 18 have the molecular weights of more than 300 Da and 3 have the molecular weights of less than 100 Da (Fig. 1B).

Notably, N²-acetyl ornithine, a precursor to fusarinines, is included among these compounds. Also, plant growth regulators such as trehalose 6-phosphate (T6P, resistant to drought and salt stress. Wingler et al., 2012; Prasad et al., 2014; Kretzschmar et al., 2015), betaine (a non-toxic osmotic regulator. Cho et al., 2003; Tramontano & Jouve, 1997) and trigonelline (resistant to salt stress. Minorsky, 2002; Tramontano & Jouve, 1997), and other active molecules such as oxymatrine (a drug used to treat hepatitis B and tumors. Lu et al., 2003; Song et al., 2006),
diosmetin (in food or medicine with anti-oxidant properties, anti-infective, and anti-shock functions. Pallab et al., 2019), luotonin A (anti-tumor drug. González-Ruiz et al., 2010), α-humulene (hippone, sesquiterpene, with anti-inflammatory effect. Rogerio et al., 2009), (-)-caryophyllene oxide (anti-tumor and antifungal drug. Yang et al., 1999; Park et al., 2011), tetrahydrocurcumin (hepatotoxicity prevention drug and natural whitening ingredients. Pari & Murugan, 2004; Trivedi et al., 2017) were also included among these compounds.

Structural determination of secondary metabolites from *P. triticisoli* BJ-18

The cells of *P. triticisoli* BJ-18 were fermented and concentrated, and 4 g of EtOAc extracts was obtained. The extracts were separated using a combination of silica gel chromatography, sephadex LH-20, ODS column chromatography, and HPLC. Six compounds (compounds 1-6) were ultimately obtained and were further analyzed using NMR and MS to establish their structures. Further the structure and characters of the six compounds (compounds 1-6) were compared with the corresponding compounds in the literature. Among the six compounds, compounds 1-3 were identified as fusarinines that were classical siderophores, while compounds 4-6 were identified as indolic acids (Fig. 2). Both compound 1 and 4 have new structures.

Compound 2 was a cyclic triopolymer which has three same monomers (m/z 284.1366) identified as desferritriacetylfusigen (Anke, 1977). It was characterized by $^1$H NMR (500 MHz, DMSO-$d_6$), $\delta_H$ 9.70 (s), 8.21 (d, $J = 7.5$ Hz), 6.32 (s), 4.30 – 4.03 (m, 3H), 3.33 (s), 2.81 (m), 1.88 (s), 1.85 (s), 1.75 – 1.39 (m, 4H) (Fig. S1) and by $^{13}$C NMR (125 MHz, DMSO-$d_6$ $\delta_C$ 22.7, 23.5, 25.8, 28.5, 32.4, 46.8, 52.3, 63.5, 117.9, 150.1, 167.4, 170.0, 172.6 (Fig. S2). Compound 5 was identified as 3-indoleacetic acid (IAA) (Gathungu et al., 2014). As shown in Fig. S3, it was characterized by $^1$H NMR (500 MHz, DMSO-$d_6$), $\delta_H$ 12.14 (s), 10.90 (s), 7.49 (d, $J = 7.9$ Hz), 7.34 (d, $J = 8.1$ Hz), 7.22 (d, $J = 2.3$ Hz), 7.07 (t, $J = 7.6$ Hz), 6.98 (t, $J = 7.4$ Hz), 3.63 (s, 2H).

Compound 6 was identified as 3-indolepropionic acid (IPA) (Rustamova et al., 2019). As shown in Fig. S4, it was characterized by $^1$H NMR (500 MHz, acetone-$d_6$), $\delta_H$ 9.97 (s), 7.16 (s), 7.09 (t, $J = 7.5$ Hz), 7.02 (t, $J = 7.5$ Hz), 7.01 (t, $J = 7.5$ Hz), 3.06 (t, $J = 7.7$ Hz), 2.70 (t, $J = 7.7$ Hz).

Compound 1 was a new member of fusarinines (siderophores) and compound 4 was a new member of the indolic acids. Their structures were further determined by extensive spectroscopic experiments. The structure of compound 3 was determined by NMR after iron was removed and it was identified as triacetylfusigen. Overall, the six compounds include three fusarinines (siderophores) and three indolic acids. (Fig. 2).

Compounds 1, 3, and 4, are described in greater detail as follows:

Deshydroxylferritriacetylfusigen (compound 1) was a yellow powder with a molecular formula of $C_{39}H_{60}N_6O_{14}$ (thirteen degrees of unsaturation) by HRESIMS $m/z$ 837.4242 [M + H]$^+$
Manuscript to be reviewed

(Manuscript to be reviewed) indicating that the double bond was in a
spectrum (Fig. S12) showed that CH
confirmed by combining
that there were only two hydroxyls in compound
Although the NMR of compound
unique monomer is 16 less than that of others, which is the molecular weight of an oxygen atom.
Thirteen carbon signals in the
NMR of compound 1 was similar to compound 2, the integration of N-OH protons
in 1H NMR of compound 1 was less than compound 2. The molecular formula also suggested
that there were only two hydroxyls in compound 1 rather than three. The planar structure was
confirmed by combining 1H-1H COSY and HMBC (Figs. S10 & S11 Fig. 3). The ROESY
spectrum (Fig. S12) showed that CH3-11 had the NOE correlation with the olefinic proton (δH
6.31) indicating that the double bond was in a Z-configuration. Compound 2 was deduced to
come from ornithine (Schrettl et al., 2007), and the absolute configuration of compound 1 was
determined to be 5S. Our data suggested that compound 1 has a new structure and we proposed
the name deshydroxylferritriacetylfusigen for the new number of fusarines (Table 1).

Compound 3 was a brown powder with a molecular formula of C39H57N6O15Fe by HRESIMS
([M + H]^+ at m/z 906.3316, calculated for C39H58N6O15Fe, 906.3309). Compound 3 had no signal
of NMR (Fig. S13) due to its metal-shielding characteristics. A strong complexing agent (Kodani
et al., 2015) desferri-compound 3 was a yellow powder with a molecular formula of C39H60N6O15
(thirteen degrees of unsaturation) by HRESIMS ([M + H]^+ at m/z 853.4203, calculated for
C39H60N6O15, 853.4195) obtained following the precipitation and separation of the metal ions by
8-quinolinol. Analysis of its HRESIMS and 1H NMR (Fig. S14) data revealed that desferri-
compound 3 had the same structure as compound 2. Compound 3 was determined to be a ferri-
complex compound, identified as triacetylfusigen.

Compound 4 was isolated as white amorphous powder with the molecular formula of
C17H22N2O3 (eight degrees of unsaturation) determined by HRESIMS m/z 303.1704 [M + H]^+
(calculated for C17H23N2O3 303.1708). The 1H and 13C NMR combining with HSQC (Figs. S15,
S16 & S17) revealed one methyl group [δCH 13.6/0.78 (t, J = 7.4Hz)], six methylenes, including
two O-methylene [δCH 67.1/3.30 (m) and 72.3/5.46 (s)], one ethane [δC 56.1/3.63 (dd, J = 10.3,
5.0)], eight aromatic/olefinic carbons (δC 108.2, 109.8, 118.1, 119.7,121.9, 126.2, 129.4 and
137.2) and one carboxylic carbons (δC 170.0). These data suggested that compound 4 was similar
to lycoperdine-1 (Yahara et al., 2004). The $^1$H–$^1$H COSY data (Fig. S18) revealed four isolated proton spin-systems of C-1–N–C-3–C-4, C-5–C-6, C-7–C-8, and C-3’–C-4’–C-5’–C-6’, respectively. The HMBC spectrum (Fig. S19) showed correlations from H-2’ to C-3’, C-8a and C-9a, from H-1 to C-3 and C-9a, from H-3 and H-4 to C-1, from H-4 to C-9a and C-4a, from H-5 to C-5a and C-4a, and from H-7 and H-8 to C-8a. The planar structure of compound 4 was established by linking these fragments (Fig. 3 & Table 2). The ECD calculation method (Ma et al., 2014) was used to determine the absolute configurations. The structure of compound 4 was simplified into two stereoisomers 4a (3S) and 4b (3R). The calculated ECD curve of compound 4a correlated with the experimental CD spectrum of 4 (Fig. 4). The absolute configuration of compound 4 was established as 3S using the time-dependent density functional theory (TDDFT) at the B3LYP/6-311G (d,p) level (Fig. S20). Compound 4 was the derivative of IPA (here named paenibacillic acid A).

**Effects of indolic acids compounds on plant growth promotion**

The effects of indolic acids compounds on growth of *Arabidopsis thaliana* var. Columbia were investigated. Paenibacillic acid A, IAA, and IPA were shown to promote the growth of plant shoots and roots (Fig. 5) as well as the dry weight of the plants (Fig. 6). The growth-promoting ability increased as the concentration of paenibacillic acid A, IAA, and IPA increased until their highest growth-promoting ability was attained, and then their plant growth-promoting ability decreased with increasing concentration. The significant efficiencies of plant-growth promotion were obtained when the concentrations of paenibacillic acid A, IAA and IPA were at 100 nM, 250 nM and 50 nM, respectively, suggesting that IPA has the strongest ability of plant growth promotion among the three indolic acids compounds.

We further determined the content of indolic compounds produced by *P. triticisoli* BJ-18 when it was measured in the nitrogen-deficient medium with and without iron. The results showed that *P. triticisoli* BJ-18 produced 37.03±1.21 μg/mL of the indolic compounds in a medium with an iron ion concentration of 4.409 $\times$ 10$^{-4}$ mM and 13.457±0.78 μg/mL of the indolic compounds in a medium without iron, suggesting that the content of indolic compounds in *P. triticisoli* BJ-18 is positively related to the iron in the environment.

**Antimicrobial activity**

The antimicrobial properties of siderophores and indolic acids were assayed against the indicator strains (bacteria *E. coli*, *S. aureus* and *B. subtilis*, and yeast *C. albicans*) (Table 3). Both compounds 1 and 2 that are siderophores showed antimicrobial activity against *E. coli*, *S. aureus* and *B. subtilis*, but did not show obvious inhibitory activity against yeast *C. albicans*. 
Gentamicin, ampicillin, streptomycin sulfate and amphotericin B were used as positive controls against *E. coli*, *S. aureus*, *B. subtilis* and *C. albicans*, respectively. The compounds were tested at concentrations of 5 μM, 10 μM and 20 μM. The IC$_{50}$ was calculated using the Spearman-Karber’s method.

**Siderophore detection by blue agar CAS assay**

Siderophore production by *P. triticisoli* BJ-18 was determined by blue agar CAS assay as described in materials and methods. A yellow ring appeared around each colony after seven days of *P. triticisoli* BJ-18 growth on a blue agar plate, indicating that *P. triticisoli* BJ-18 had the ability to secrete siderophores and transfer iron from the environment to the bacterial cells (Fig. S21). The data are consistent with the above results that compounds 1-3 are siderophores.

**Discussion**

*P. triticisoli* BJ-18, a N$_2$-fixing bacterium, significantly promotes plant growth, but the secondary metabolites produced by this bacterium have never been characterized. In this study, 101 known compounds of *P. triticisoli* BJ-18 were measured by untargeted metabolomics profiling. These compounds include N$_2$-acetyl ornithine, which is the precursor of fusarinines (siderophores). There are 7 types of siderophores: fusarinines, rhodotorulic acids, ferrichromes, ferrioxamines, aerobactins, enterobactins, and mycobactins (Hossain et al., 1980). Fusarinines are synthesized by aminoacyl bonds, making them different from other types of siderophores that are polymerized using peptide linkages. Fusigen, a cyclic trimester of fusaricine, was identified by Diekmann (Diekmann & Zähner, 1967) and was considered to be the iron ionophore for *Fusarium roseum* (Sayer & Emery, 1968). Studies have shown triacetylfusigen, which has three acetyl group in place of the H atoms of the amino, was isolated from *Aspergillus fumigates* (Diekmann & Krezdorn, 1975) and *Aspergillus nidulans* (Charlang et al., 1981), while desferritriacetylfusigen was found in *Aspergillus deflectus* (Anke, 1977) and *Emericella* sp. (Cruz et al., 2012). These siderophores were isolated from fungi but bacterial fusarinines have never been identified before our study. Here, three fusarinines were isolated from *P. triticisoli* BJ-18 and identified as dehydroxylferritriacetylfusigen, desferritriacetylfusigen, and triacetylfusigen, of which dehydroxylferritriacetylfusigen was a new structure of fusaricine. Here is the first study to report the bacterial fusaricine. Ornithine is the only amino acid of fusaricine (Charlang et al., 1982). The Kyoto Encyclopedia of Genes and Genomes (KEGG, https://www.kegg.jp/) shows that fusaricine can be synthesized by some specific ornithylesterases including fusaricine-C ornithineesterase, ornithine esterase and 5-N-acetyl-L-ornithine-ester hydrolase. Studies on fusaricine in fungi have shown that the specific
ornithylesterases promote cellular iron-exchange by hydrolysis of the ester bonds of the ferric ionophores (Emery, 1976).

Indolic compounds are natural auxins in plants and rhizosphere microorganisms and can promote the formation of shoot tips, buds, and roots. The auxins commonly used in agriculture are IAA, indolebutyric acid, 2,4-dichlorophenoxyacetic acid and naphthylacetic acid. Three indolic acids identified as paenibacillic acid A, IAA, and IPA were isolated from P. triticisoli BJ-18, of which paenibacillic acid A was a new structure. The results of the plant growth promoting capacity assay showed that the three compounds can promote the growth of the shoots and roots of A. thaliana and can increase the dry weight of the plant. The effect of paenibacillic acid A on A. thaliana is similar to that of IAA. IPA was the best promoter of plant growth among the three indolic compounds. Indolic compounds such as indole, skatole, and indirubin were concurrently identified by untargeted metabolomics profiling. Our results showed that P. triticisoli BJ-18 can promote plant growth by synthesizing plant growth hormones, including indolic acids.

Indolic compounds were usually synthesized in two pathways, either by iron (III) complexed by the ligand of indolic compounds or by the reduction of the soluble iron (II) complex. These parallel reactions cannot proceed without iron (Gazaryan et al., 1996; Kovács et al., 2008; Xie et al., 2016). Indolic acid assays showed that the content of indolic compounds of P. triticisoli BJ-18 was related to the presence or absence of iron ions. The content of indolic compounds of P. triticisoli BJ-18 can reach 37.03 ± 1.21 μg/mL in the medium with an iron ion concentration of 4.409 × 10^{-4} mM, while the content is 13.457 ± 0.78 μg/mL in the iron-free environment, indicating the importance of iron for the synthesis of indolic compounds.

Phytopathogens must sequester iron to develop and sustain infections (Ratledge & Dover, 2000). Competing for iron is a mechanism taken by PGPR to inhibit the growth of phytopathogens in the soil (Chet et al., 1990). The six compounds were evaluated for their activities against a panel of microbes. Results showed that desferritriacetylfusigen and deshydroxylferritriacetylfusigen were antimicrobial against E. coli, S. aureus, and B. subtilis. Triacetylfusigen showed no antibiosis activity against any targeted microorganism due to its complexion with iron.

Plant growth regulators such as betaine and trigonelline, and other active molecules such as luotonin A, aphidicolin, oxymatrine, diosmetin, pilocarpine and tetrahydrocurcumin were also identified by untargeted metabolomics profiling. Besides, deshydroxyferritriacetylfusigen had weak cytotoxic activity against AsPC-1 with IC_{50} value of 81.2±3.9 μM (supplemental file 1), indicating that siderophores may have potential as a new therapy for human cancers (Kalinowski & Richardson, 2005; Ji, Juarez-Hernandez & Miller, 2012). The chemical composition and application of P. triticisoli BJ-18 is far more complicated and promising.
P. triticisoli BJ-18 has been shown to promote plant growth using several mechanisms (Fig. 7). P. triticisoli BJ-18 provides a nitrogen source for plant growth by N$_2$-fixation, produces indolic acids to promote plant growth, generates siderophores to capture iron atoms to synthesize nitrogenase and indolic acids, and synthesizes plant growth regulators such as T6P, betaine and trigonelline, and secretes fusarinines and paenibacillic acid A to resist phytopathogens.

Our results provide chemical evidence for the use of P. triticisoli BJ-18 as a PGPR biofertilizer in agriculture. The discovery of the many compounds identified in our study shows the agricultural and medical value of P. triticisoli BJ-18. The metabolic regulation of fusarinines and other bioactive compounds requires further study.

Conclusions

In this study, six compounds were isolated and characterized from P. triticisoli BJ-18, a N$_2$-fixer. The six compounds included three classical siderophore fusarinines identified as deshydroxylferritriacetylfusigen, desferritriacetylfusigen, and triacetylfusigen, and three indolic acids identified as paenibacillic acid A, 3-indoleacetic acid (IAA), and 3-indolepropionic acid (IPA). Both deshydroxylferritriacetylfusigen and paenibacillic acid A have new structures. Fusarinines, which normally occur in fungi, were isolated from bacteria for the first time in this study. Both siderophores (compounds 1 and 2) showed antimicrobial activity against E. coli, S. aureus and B. subtilis, but did not show obvious inhibitory activity against yeast Candida albicans. Whereas triacetylfusigen (compound 3) showed no antibiosis activity against these test microorganisms. Paenibacillic acid A, IAA, and IPA were shown to promote the growth of plant shoots and roots, and paenibacillic acid A also showed antimicrobial activity against S. aureus. Our study demonstrated that siderophores and indolic acids may play an important role in plant growth promotion by P. triticisoli BJ-18.

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Figure 1

untargeted metabolomics profiling of *P. triticisoli* BJ-18

A: Number of compounds of different types; B: Number of compounds in different molecular weight.

A: 
- 26 carbohydrates
- 7 amino acids and peptides
- 8 alcohols, aldehydes, ketones, fatty acids and lipids
- 4 nucleic acids
- 8 vitamins
- 6 alkaloids
- 4 cyclics

B: 
- <100: 3
- 100-200: 46
- 200-300: 34
- 300-400: 11
- 400-500: 4
- >500: 3

molecular weight
Compounds isolated from *P. triticisoli* BJ-18

Six compounds were isolated from *P. triticisoli* BJ-18, including three classical siderophore fusarines identified as (1) deshydroxyferritriacetylfusigen, (2) desferritriacetylfusigen and (3) triacetylfusigen, and three indolic acids identified as (4) paenibacillic acid A, (5) 3-indoleacetic acid (IAA), and (6) 3-indolepropionic acid (IPA).
Figure 3

Key \(^1\)H-\(^1\)H COSY and HMBC correlations of compounds 1 and 4

\(^1\)H-\(^1\)H COSY spectra (DMSO-\(d_6\), 8 MHz); HMBC spectra (DMSO-\(d_6\), 8 MHz)
Figure 4

Experimental ECD spectra of 4 and its calculated ECD spectra of related simplified possible stereoisomers 4a and 4b.

Experimental ECD spectra of 4 was recorded in methanol. Systematic conformation analysis of 4a was conducted with CONFLEX using the MMFF94 molecular mechanics force field. Optimization with DFT calculation at the B3LYP/6-31G(d) level in MeOH by the Gaussian09 program afforded the MMFF minima. At the B3LYP/6-31G(d) level, the exciting states were calculated using time-dependent density-functional theory (TDDFT) methodology for 4a. The overall ECD spectra were then produced based on Boltzmann weighting of each conformer.
Figure 5

Plant growth-promoting capacity of indolic compounds on *Arabidopsis thaliana*

Seeds of *Arabidopsis thaliana* var. Columbia were used as plant material, and were incubated at 25 ± 2 °C under cool fluorescent light (2000 lux 16 h/day photoperiod) for 14 days.

| concentration (nM) | 0   | 50  | 100 | 250 | 500 | 1000 | 2500 | 5000 |
|-------------------|-----|-----|-----|-----|-----|------|------|------|
| *Paenibacillic acid A* | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) | ![Image](image4.png) | ![Image](image5.png) | ![Image](image6.png) | ![Image](image7.png) | ![Image](image8.png) |
| *3-Indoleacetic acid* | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) | ![Image](image4.png) | ![Image](image5.png) | ![Image](image6.png) | ![Image](image7.png) | ![Image](image8.png) |
| *3-Indolepropionic acid* | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) | ![Image](image4.png) | ![Image](image5.png) | ![Image](image6.png) | ![Image](image7.png) | ![Image](image8.png) |

— 1 cm
Figure 6

Comparison of plant growth-promoting ability of indolic compounds

At least ten Arabidopsis strains were cultured in each group of treatments. The cultures were incubated at 25 ± 2 °C under cool fluorescent light (2000 lux 16 h/day photoperiod) for 14 days.
Manuscript to be reviewed
Figure 7

*P. triticisoli* BJ-18 promotes plant growth by several mechanisms

The orange squares represent plant growth regulators such as T6P, betaine and trigonelline. The purple ring represents fusarines and the red dot represents iron atoms. The blue triangle indicates indole acids. Red radial circles represent plant pathogens. Six-component structure representing nitrogenase.
Table 1 (on next page)

NMR data of compound 1 in DMSO-d$_6$

$^1$ Recorded at 125 MHz; $^2$ Recorded at 500 MHz.
| Pos. | $\delta_C$ | $\delta_H$, mult ($J$ in Hz) |
|------|-----------|-----------------------------|
| 1    | 166.0     |                             |
| 2    | 117.5     | 6.31, s                     |
| 3    | 150.0     |                             |
| 4    | 31.9      | 2.80, br. s                 |
| 5    | 63.1      | 4.17, m                     |
|      |           | 4.12, m                     |
| 6    | 172.1     |                             |
| 7    | 51.8      | 4.19, m                     |
| 8    | 28.0      | 1.65, m                     |
| 9    | 23.0      | 1.53, m                     |
| 10   | 46.3      | 3.51 br. s                  |
| 11   | 25.3      | 1.87, s                     |
| 12   | 169.5     |                             |
| 13   | 22.3      | 1.84, s                     |
|      | N-OH      | 9.70, br. s                 |
|      | N-H       | 8.20, d (7.5)               |

1 Recorded at 125 MHz; 2 Recorded at 500 MHz
Table 2 (on next page)

NMR data of compound 4 in DMSO-$d_6$

1 Recorded at 125 MHz; 2 Recorded at 500 MHz
Table 2. NMR data of compound 4 in DMSO-$d_6$

| Pos. | $\delta_C$ | $\delta_H$, mult ($J$ in Hz) |
|------|------------|-----------------------------|
| 1    | 39.5       | 4.35, d (15.7)               |
|      |            | 4.21, d (15.7)               |
| 3    | 56.1       | 3.63, dd (10.3, 5.0)         |
| 4    | 22.9       | 3.13, dd (15.3, 5.0)         |
|      |            | 2.84, dd (15.3, 10.4)        |
| 4a   | 108.2      |                             |
| 4b   | 126.2      |                             |
| 5    | 118.1      | 7.47, d (7.5)                |
| 6    | 119.7      | 7.07, t (7.5)                |
| 7    | 121.9      | 7.17, t (7.5)                |
| 8    | 109.8      | 7.55, d (7.5)                |
| 8a   | 137.2      |                             |
| 9a   | 129.4      |                             |
| 1'   | 170.0      |                             |
| 2'   | 72.3       | 5.46, s                      |
| 3'   | 67.1       | 3.30, m                      |
| 4'   | 31.0       | 1.40, m                      |
| 5'   | 18.8       | 1.22, m                      |
| 6'   | 13.6       | 0.78, t (7.4)                |

1 Recorded at 125 MHz; 2 Recorded at 500 MHz
### Table 3 (on next page)

Antimicrobial activity of compound 1-6

Gentamicin, ampicillin, streptomycin sulfate and amphotericin B were used as positive controls against *E. coli*, *S. aureus*, *B. subtilis* and *C. albicans*, respectively. The compounds were tested at concentrations of 5 µM, 10 µM and 20 µM. The IC$_{50}$ was calculated using the Spearman-Karber's method. The horizontal line “- -” indicated that the compound had no antibacterial activity against the indicator strain.
### Table 3. Antimicrobial activity of compounds 1-6

| IC<sub>50</sub> (µM) | *E. coli* | *S. aureus* | *B. subtilis* | *C. albicans* |
|----------------------|-----------|-------------|---------------|---------------|
| compound 1           | 6.8       | 5.2         | 8.8           | --            |
| compound 2           | 4.7       | 4.3         | 6.4           | --            |
| compound 3           | --        | --          | --            | --            |
| compound 4           | --        | 7.4         | --            | --            |
| compound 5           | --        | --          | --            | --            |
| compound 6           | --        | --          | --            | --            |
| Pos.                 | 1.9       | 2.1         | 2.3           | 2.2           |

Gentamicin, ampicillin, streptomycin sulfate and amphotericin B were used as positive controls against *E. coli, S. aureus, B. subtilis* and *C. albicans*, respectively. The compounds were tested at concentrations of 5 µM, 10 µM and 20 µM. The IC<sub>50</sub> was calculated using the Spearman-Karber’s method. The horizontal line “--” indicated that the compound had no antibacterial activity against the indicator strain.