

# Isolation of Nannocystis species from Iran and exploring their natural products

Azam Moradi

Shahid Beheshti University

Mohammad Yaghoubi-Avini (✉ [m\\_yaghoubi@sbu.ac.ir](mailto:m_yaghoubi@sbu.ac.ir))

Shahid Beheshti University <https://orcid.org/0000-0001-5761-1062>

Joachim Wink

Helmholtz-Zentrum für Infektionsforschung GmbH

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## Research Article

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# Abstract

Several different techniques were employed for the isolation of *Nannocystis* from various sources. A polyphasic approach was used for identification. Twelve strains of *N. pusilla*, *N. exedens*, and *N. konarekensis* with distinctive distribution between climates were identified. The bioactivity was examined against a panel of eight bacteria, two yeasts, and one fungus; cytotoxicity was tested on the L929 fibroblast cell line. Eleven strains mainly inhibit Gram-positive bacteria, and only one isolate was cytotoxic. The extracts analysis by HPLC and LC-MS were compared to Myxobase, and eight different compounds were detected; a correlation was observed between compounds and producing species. 70% of strains had the potential to produce structurally diverse compounds. Nannochelins and althiomycin were the most abundant metabolites. The discovery of a new species of *Nannocystis* and the high potentiality of strains to produce secondary metabolites encourage further sampling and in-depth analysis of extracts to find new active metabolites.

## Introduction

Myxobacteria are a fascinating monophyletic group of *δ-proteobacteria*, with a large genome ranging between 8 to 13 Mb. Their complex life cycle includes two stages: the vegetative stage, in which rod-shaped cells of myxobacteria swarm on solid surfaces and prey on other bacteria or decompose cellulose. In unfavorable conditions, the cells undergo morphogenesis and exhibit an organized social behavior to develop fruiting bodies that contain heat-stable myxospores (Reichenbach and Dworkin 1992). The genus *Nannocystis*, with its three species and some genera of marine myxobacteria, constitutes the family *Nannocystaceae* in the *Nannocystineae* suborder of myxobacteria (Mohr 2018). Metagenomics studies revealed the wide distribution of myxobacteria in both terrestrial and aquatic environments. Indeed, *Myxococcales* form the 4% of OTUs in soil samples that is the fourth abundant taxa in this habitat (Zhou et al. 2014). Metagenomic studies revealed an outstanding diversity in myxobacteria with 20 suborders that only strains of *Sorangiiineae*, *Cystobacterineae*, and *Nannocystineae* were cultured and extensively explored for their secondary metabolites (Zhou et al. 2014). *Nannocystis* are considered ubiquitous myxobacteria that are mainly isolated from soil and decaying plants in diverse geographical locations. The vegetative cells of this genus have short, slender, or fat rods with small spherical sporangioles. Their colorless colonies excavate agar in a nutrient-poor medium. They live in mesophilic temperatures and degrade organic matter in aerobic conditions (Müller 2014).

Myxobacteria are famous for their high capabilities to produce diverse secondary metabolites. They are the second most proliferative producers of these compounds after *Actinobacteria*. Until now, more than 100 new core structures and over 600 derivatives are characterized in myxobacteria (Weissman and Müller 2010). However, only four structural varieties are reported from *Nannocystis* and, their potential for the production of natural products is less studied (Herrmann et al. 2017).

Previous studies showed isolation of every new species, genus, or family increases the chance of encountering new natural products, respectively (Hoffmann et al. 2018). One way to isolate such new

taxonomic groups is to search for new habitats because every ecological niche has its specific condition and microbiome that provoke certain symbiosis relationships between microbes. Thus, myxobacteria isolation efforts in under-explored environments introduce the chance of encountering new bioactive metabolites. In turn, it may help to overcome antibiotic resistance in pathogens.

The objective of this study was to thoroughly examine the less studied species of *Nannocystis* in Iran and characterize their natural products and bioactivities.

## Materials And Methods

### Sample collection

Multiple samples were collected from May 2013 to February 2015, from 45 stations located in Caspian shore, temperate forests of Mazandaran, the central desert of Iran, deserts of Sistan & Baluchistan, mountainous area of Zagreus, grasslands of northern Khorasan and Azerbaijan, and the coast and islands of the Persian Gulf. These samples include topsoil, decaying woods and barks, rabbit dungs, beach sands, and freshwater. The samples were shipped to the laboratory in capped bottles and dried at room temperature and darkness.

### Isolation of strains

Three methods were employed to isolate *Nannocystis* species:

I. WCX media (0.15%  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.15%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 50mM HEPES, 100 mg/ml cycloheximide, 1.8% agar, pH 7.2) supplemented with 1ml/L vitamin solution (0.2% biotin, 2% nicotinic acid, 1% thiamine, 1% 4-aminobenzoic acid, 0.5% pantothenate, 5% pyridoxamine, 2% cyanocobalamin, filter sterilized) cross streaked with live *E. coli* as bait and inoculated with pea-sized samples in the tips of lines. Inoculated media incubated at 30°C for up to 8 weeks. The plates were investigated twice a week for characteristics of the swarm and fruiting bodies of *Nannocystis* by the stereomicroscope (Olympus SZX-10, Japan).

II. Four pieces of filter paper (2×2 cm) were placed on ST21 agar (Shimkets et al. 2006) containing 100 µg/ml cycloheximide and a pea-sized sample transferred to the center of the filter papers. The plates were incubated at 30°C for eight weeks and searched for fruiting bodies and swarms twice a week by the stereomicroscope.

III. Freshwater samples were filtered through a sterile nitrocellulose membrane filter (0.45 µm, Sartorius, USA). The filters were transferred to the rabbit dung agar (Shimkets et al. 2006) and incubated at 34°C. After 3–4 days, plates were studied under the stereomicroscope.

### Purification of cultures

Because of the massive contamination of isolation plates, application of the following protocols was necessary to purify the *Nannocystis* isolates:

1. The tip of young fruiting bodies or the edge of swarms was picked by a sterile needle under a stereomicroscope and transferred to the center of the CY or VY/2 agar plate (Reichenbach and Dworkin 1992). Repeating this procedure several times results in pure cultures.
2. An antibiotic solution (Table 1) was prepared and distributed on the surface of the CY, VY/2, or WAT agars by a Drigalsky bar. After the solution adsorption, the vegetative cells or fruiting bodies of *Nannocystis* strains were inoculated to the center of these media and incubated at 30°C.
3. The same method also was used to reveal antibiotic resistance patterns of purified strains.

Table 1  
Antibiotic solutions for purification of *Nannocystis* isolates

Antibiotic stock solutions	Final concentration
Ampicillin 10 mg/ml	100 µg/ml
Bacitracin 10 mg/ml	50 µg/ml
Cephalosporin 10 mg/ml	50 µg/ml
Chloramphenicol 9 mg/ml <sup>a</sup>	30 µg/ml
Fusidic acid 10 mg/ml	50 µg/ml
Gentamicin 10 mg/ml	50 µg/ml
Hygromycin 50 mg/ml	150 µg/ml
Kanamycin 10 mg/ml	50 µg/ml
Oxytetracycline 10 mg/ml	10 µg/ml
Polymycin 10 mg/ml	50 µg/ml
Spectinomycin 10 mg/ml	50 µg/ml
Trimethoprim 5mg/ml <sup>a</sup>	50 µg/ml
Thiosterpton 10 mg/ml <sup>b,c</sup>	50 µg/ml
<sup>a</sup> in methanol	
<sup>b</sup> in DMSO	
<sup>c</sup> stored at -20°C	

For long-term storage, a piece of WAT agar medium with fully developed fruiting bodies was cut and placed on a sterile filter paper on a plate and dried under the vacuum between 2 to 3 days. The paper cuts were recovered in a cryovial under the sterilized condition and stored at room temperature.

## Identification and phylogenetic analysis

The images were taken from the fruiting bodies, fringes of swarms, and the vegetative cells grown on CY or VY/2 agar by the stereomicroscope and under 40X magnification. The taxonomy was presumed by comparing morphological features with Bergey's Manual of Systematic Bacteriology (Reichenbach 2005) and The Prokaryotes (Rolf Muller 2014).

The homogenates of strains cultivated in liquid media were used to determine biochemical profiles by API ZYM® (bioMérieux, France) rapid tests. VY/2 agar cultures incubated at 22, 30, and 44°C for 1 to 3 weeks used for determination of optimum growth temperatures and strains cultured at 30°C for 1–3 weeks on the same medium prepared in pH 5, 6, 7, 8, and 9 indicate the best growth condition.

The genomic DNA was extracted with the Gram-negative protocol of the spin plant mini kit (Invitex, Germany). Amplification of 16S rDNA was done by PCR using F27 and R1525 universal primers and the following conditions: initial denaturation 5 min at 95°C, 34 cycles of denaturation at 95°C for 0.5 min, annealing at 52°C for 0.5 min, and extension at 72°C for 0.5 min, and a final extension at 72°C for 10 min. The PCR products were separated by electrophoresis on 0.8 % agarose gel to determine amplicon size and purified using NucleoSpin Extract II (Macherey-Nagel, Germany). 16S rDNA sequenced by Sanger dideoxy method and contigs assembled by Seqman II software (Ver 6.00) using default settings. The isolates were identified by comparing their contigs with type strain sequences in the EzBioCloud database (Yoon et al. 2017). Multiple alignment and curation were performed by MUSCLE and Gblocks, respectively, using phylogeny.fr web tools (Dereeper et al. 2008). The phylogenetic analysis was conducted by MEGA-X software (Kumar et al. 2018), applying the Maximum-Likelihood method and Hasegawa-Kishino-Yano model (Hasegawa et al. 1985) with a bootstrap analysis of 100 replications. The sequences were submitted to the Genbank database of NCBI, and the accession numbers are obtainable from the phylogenetic tree (Fig. 3).

## Secondary metabolite production and extraction

The agar pieces containing fruiting bodies or swarms from a pure culture were excised and transferred to a 20 ml of myxovirescin medium (1.0% casein peptone, 0.005% CaCl<sub>2</sub>, 0.025% MgSO<sub>4</sub>, 1 mg/l CoCl<sub>2</sub>, 100mM HEPES, pH 7.0) amended with 10% A medium (0.8% starch, 0.4% soy flour, 0.2% yeast, 0.1% CaCl<sub>2</sub>, 0.1% MgSO<sub>4</sub>, 100 mM HEPES, 1 ml/l Fe-EDTA, 4 ml/l glycerin, pH 7.4) and vitamin B12 (final concentration, 500 µg/l), the fermentation experienced for 10–14 days at 30°C. After this period, *Nannocystis* has grown to the spherical masses of aggregated cells. The whole medium was used to inoculate 100 ml of the same medium containing 2% XAD-4 resin (Sigma-Aldrich, USA) and incubated under the same conditions. At the end of fermentation, the resin and biomass were harvested and washed with deionized water, then extracted with acetone for at least 3 hours in darkness. The acetonetic extract was dried in a rotary evaporator (Heidolph, Germany) and resolved in 1 ml methanol, and stored at -20°C for further experiments.

## Antimicrobial activity and cytotoxicity

The spectrum of antibacterial activity of each crude extract was determined by micro broth dilution method against Gram-positive bacteria *Bacillus subtilis* (DSM 10), *Micrococcus luteus* (DSM 1790) and *Staphylococcus aureus* (Newman), Gram-negative *E. coli* (DSM 1116), *E. coli* TolC mutant (DSM 104619), *Pseudomonas aeruginosa* (PA14) and *Chromobacterium violaceum* (DSM 30191), *Mycobacterium smegmatis* (ATCC 700084) and yeasts—*Candida albicans* (DSM 1665), *Pichia anomala* (DSM 6766), and filamentous fungus *Mucor hiemalis* (DSM 2656). The bacteria were cultured in Muller-Hinton broth and diluted by the same medium to the 0.05 turbidity at 600 nm. Yeasts and fungus were cultured in Mycosel broth (Cazin et al., 1989) and were diluted to 0.01 absorbance at 600 nm. One hundred and fifty microliters of the cell suspensions were pipetted to each well of the microtiter plates (Brand, Germany), and 20 µl of each crude extract was added to the first row and serially diluted with 150 µl of the same suspension. Upon visual investigation on plates, those extracts that inhibit indicator microorganism growth further than row C were considered bioactive and were chosen for LC-MS studies.

The extracts cytotoxicity was assayed against mouse fibroblast L929 cell line by adding 3 µl of crud extracts in 87 µl of the DMEM medium with 10% FBS in a microtiter plate. The L929 cells were cultured under a 5% CO<sub>2</sub> atmosphere for seven days at 37°C. After this period, the cells were washed twice by PBS buffer, harvested by the addition of 0.05% trypsin, and counted using a Neubauer chamber. Subsequently, the cells were diluted to 50000 cells/ml by adding the same medium and 120 µl (6 × 10<sup>3</sup> cells) inoculated to each well. The plates were incubated at 37°C for five days and studied under the inverted microscope for morphological changes in the cells.

## Fractionation and LC-MS analysis of extracts

The extracts were fractionated with an HPLC (Agilent 1100) equipped with an X-Bridge C18 column; 3.5 µm, 2.1 × 100 mm (Waters, Milford, USA), and DAD detector (200–400 nm). From each bioactive extract, 5 µl were injected into the column and eluted with a gradient of 100% A: 950 ml of 0.05 mM ammonium acetate buffer, pH: 5 and 50 ml ACN to 100% B: 0.05 mM ammonium acetate in ACN at 0.3 ml/min flow rate. Fractions were collected every 30 s in wells of a microplate and dried at 40°C in nitrogen gas flow by a MiniVap (Porvair Sciences, UK). Subsequently, 150 µl of each indicator microorganism that earlier was inhibited by the same extract was added to the wells and incubated at the appropriate conditions. Wells with growth inhibition were compared with the HPLC chromatogram to filtering out crude extracts that their activities attributed to the fatty acids. The others were analyzed further by an LC-MS system made of an Agilent 1200 HPLC with DAD detector (200–600 nm) and a maXis UHR-TOF (Bruker Daltonics, USA) mass spectrometer. The analysis of samples was done by an ACQUITY UPLC BEH C18 (Waters, Milford, USA) column 2.1 × 5 mm, 1.7 µm in 0.1% formic acid as solvent A, 0.1% formic acid in ACN as solvent B by the gradient of 0.5 min 95% A; reach to 95% B in 19.5 min and hold on 95% B for 10 min at the flow rate of 0.6 ml/min; oven temperature 40°C. The compounds were identified by comparing mass data, UV spectrums, elution times, and bioactivities with the “Myxobase” database held in Helmholtz Center for Infection Research, Braunschweig, Germany. Correlation between each species and secondary metabolite production was investigated by clustering data with the ClustVis web tool and visualized in a heatmap graph (Metsalu and Vilo 2015).

## Results

### Strains isolation, purification, and identification

After 2 to 3 weeks of incubation, tiny spherical or elliptical, colorless to dark orange sporangioles of *Nannocystis* species appeared on or into the agar. Microscopic studies have shown wave-like colorless to yellow swarms that etch agar with blunt-ended short rod cells and round or oval myxospores in sporangioles (Fig. 1). Isolation and purification efforts succeeded in pure cultures of 12 isolates. Phenotypic and phylogenetic markers identified eight strains of *N. pusilla* isolated from 7 stations in temperate forests and Zagros mountains and three strains of *N. exedens* from stations of the central desert (Fig. 2). The type strain of *N. konarekensis* DSM 104509<sup>T</sup> was isolated from the deserts of southeastern Iran. Table 2 presents sample type, purification method, physiology, and phenotype of strains, except for the *N. konarekensis* type strain that was described in detail elsewhere (Mohr 2018).

Table 2

Summary of isolation and purification techniques of *Nannocystis* species and phenotypic and physiological characteristics.

Species		<i>N. pusilla</i>	<i>N. exedens</i>
Isolation biotope	Temperate forest	5 (3,7,25,166,212) <sup>a</sup>	-
	Arid desert	-	3 (51,211,215)
	Mountainous	3 (34,35,49)	-
Isolation sample	Decaying wood	2	3
	topsoil	6	-
Isolation media	WCX	2	-
	ST21	6	3
Purification method	Subculture	1	3
	Antibiotic treatment	7	-
Fruiting bodies on CY agar	Tiny, evenly distributed, colorless to yellow	Single or clustered sporangioles, Yellow to dark orange	
Optimum temperature (°C)	30	30	
Optimum pH	7–8	8	
Antibiotic resistance <sup>b</sup> (µg/ml)			
Bacitracin (50 mg/ml)	-	-	
Chloramphenicol (30 mg/ml)	-	v	
Fusidic acid (50 mg/ml)	+	+	
Hygromycin (150 mg/ml)	-	-	
Oxytetracycline (10 mg/ml)	-	-	
Polymycin (50 mg/ml)	v	-	
Spectinomycin (50 mg/ml)	-	-	
Trimethoprim (50 mg/ml)	v	+	
Thiostrepton (50 mg/ml)	-	-	
Enzyme activity (API ZYM)			
Alkaline phosphatase	5	5	

Species	<i>N. pusilla</i>	<i>N. exedens</i>
Esterase (C4)	1	1
Esterase lipase (C8)	2	1
Leucine arylamidase	2	3
Valine arylamidase	2	2
$\alpha$ -chymotrypsin	2	1
Acid phosphatase	4	5
Naphtyl-AS-BI-phosphohydrolase	4	5
$\beta$ -glucosidase	0	1
<p><sup>a</sup> The identifier numbers of strains are represented in parenthesis.</p> <p><sup>b</sup> Only widespread antibiotic resistances among strains of each species are shown. +, resistant; -, susceptible; v, variable among strains.</p> <p><sup>c</sup> Enzyme activity measured by the manufacturer guidance from 0 for without to 5 for the highest activity.</p>		

The antibiotic resistance pattern and enzymatic activity of the isolates were almost the same in each species. While all strains of *N. pusilla* were susceptible to chloramphenicol, *N. exedens* strains showed variable susceptibility to this antibiotic. Likewise, all of *N. exedens* were susceptible to polymycin and, this susceptibility was variable among *N. pusilla* strains. Resistance to trimethoprim was widespread in *N. exedens* strains but, some of *N. pusilla* strains were also susceptible. In the API ZYM test, the leucine arylamidase, acid phosphatase, and phosphorylase activity in *N. exedens* isolates were slightly higher than in *N. pusilla* strains. The  $\beta$ -glucosidase activity was weakly present in *N. exedens*. C8 esterase activity in *N. pusilla* was higher than *N. exedens* (Table 2).

The Maximum-Likelihood tree has shown that all strains are located within the *Nannocystis* genus (Fig. 3). Except for *N. konarekensis* that always form a well-separated distinct branch in any tree topology, all phylogenetic inference models applied to the dataset had low bootstrap values that caused uncertainty in the exact position of the tree branches. Interestingly, the strains that were isolated from the same biotope were often strongly related together.

## Bioactivity of isolates

Crude extracts of *N. exedens* and *N. pusilla* fermentation broths were assayed against a panel of 11 strains from different classes of microorganisms. One isolate of *N. exedens* and three of *N. pusilla* did not show any bioactivity against indicator microorganisms (Table 3). Other examined extracts were mainly active against Gram-positive bacteria and not active against Gram-negative bacteria, yeasts, and

fungus. However, the hypersensitive TolC mutant of *E. coli* was inhibited strongly by the isolate 49 of *N. pusilla* and weakly by isolates 51 and 34. In this regard, only one isolate (212) moderately arrested *M. smegmatis* growth. The granulation and vacuolation of cytoplasm or disruption of monolayer relate to the bioactivity of extracts. Among 12 bioassays were conducted by crude extracts of *Nannocystis* strains, only the strain 215 extract showed activity with degeneration and vacuolation of the cells (Fig. 4).

Table 3  
Bioactivity of pure culture extracts against a panel of sensitive microorganisms.

Test microorganisms <sup>a,b</sup>											
Strains	<i>B.s</i>	<i>S.a</i>	<i>M.l</i>	<i>M.s</i>	<i>Ps.a</i>	<i>E.c</i>	<i>E.c T</i>	<i>C.v</i>	<i>C.a</i>	<i>Pa</i>	<i>M. h</i>
<i>N. exedens</i>											
51	E	D	E	-	-	-	D	A	-	-	-
211	C	C	C	B	-	-	B	A	-	-	-
215	E	E	E	A	-	-	C	A	-	-	A
<i>N. pusilla</i>											
3	E	E	H	B	-	-	C	A	-	-	-
7	E	D	E	A	-	-	C	A	-	-	-
25	C	B	D	-	-	-	B	A	-	-	-
34	D	C	F	-	-	-	D	A	-	-	-
35	C	B	D	-	-	-	C	A	-	-	-
49	G	E	H	-	-	-	G	A	A	-	A
166	C	C	B	B	-	-	B	A	-	-	-
212	E	D	E	D	-	-	C	A	-	-	-
<sup>a</sup> <i>B.s</i> , <i>Bacillus subtilis</i> (DSM 10); <i>S.a</i> , <i>Staphylococcus aureus</i> (Newman); <i>M.l</i> , <i>Micrococcus luteus</i> (DSM 1790); <i>M.s</i> , <i>Mycobacterium smegmatis</i> (ATCC 700084); <i>Ps.a</i> , <i>Pseudomonas aeruginosa</i> (PA14); <i>E.c</i> , <i>E. coli</i> (DSM 1116); <i>E.c T</i> , <i>E. coli</i> (TolC mutant); <i>C.v</i> , <i>Chromobacterium violaceum</i> (DSM 30191); <i>C.a</i> , <i>Candida albicans</i> (DSM 1665); <i>Pa</i> , <i>Pichia anomala</i> (DSM 6766); <i>M.h</i> , <i>Mucor hiemalis</i> (DSM 2656).											
<sup>b</sup> Growth inhibition after the C row indicates the susceptibility of test microorganisms											

## Secondary metabolites

The HPLC fractionation and LC-MS analysis detected nine secondary metabolites in 10 out of the eleven extracts. Five metabolites were common between different isolates and the other four were produced each by a single strain (Table 4). Also, LC-MS analysis showed two new derivatives of nannochelin and

myxoprincomide in this study. Myxopyronin, myxoprincomide, and pyrroazol were found exclusively in four strains of *N. pusilla*; on the other hand, myxothiazol and germacrane were solely produced by *N. exedens* 215. Althiomycin, the potentially new nannochelin and, nannochelin were the most frequented metabolites in the mass spectrums of extracts, respectively. The most fruitful stain was 215 of *N. exedens* that produced four different metabolites, while 7 and 211 each produce one metabolite. Isolates 34, 35, 51, and 166 from both species have the same profile of secondary metabolites although, they were from distinct locations and phylogenetically not related. Strain 25 antimicrobial activity was related to fatty acids, as proved by double peaks in 17 min of the chromatogram. Hierarchical clustering of detected metabolites by species of *Nannocystis* visualized in the heatmap graph (Fig. 5). There was a strong correlation between nannochelin, germacrane and, myxothiazol with *N. exedens* strains though althiomycin, pyrroazol, and potentially new compounds production were more related to the *N. pusilla* strains exclusively, a new myxoprincomide identified only in the extract of the *N. pusilla* 3. There is no report of secondary metabolites from *N. konarekensis*.

Table 4  
List of secondary metabolites produced by each strain of *Nannocystis* sp.

	<i>N. pusilla</i>	<i>N. exedens</i>	<i>N. konarekensis</i>
Althiomycin	34, 35, 166	51	-
Germacrane	-	215	-
Myxopyronin	7, 212	-	-
New myxoprincomide	3	-	-
Myxothiazol	-	215	-
Nannopyrazinone	3	215	-
Nannochelin	49	211, 215	-
New nannochelin	34, 35, 166	51	-
Pyrroazol	49	-	-

## Discussion

Among the cultivable myxobacteria, *Nannocystineae* is the less abundant and more diverse group. Based on a recent metagenomics study, this suborder contains 11 families, 157 genera, and 309 species that only a small number of them (nine species) held in pure cultures (Liu et al. 2019).

The first study on the myxobacteria of Iran concluded that with an average of 7.4 species per sample, this region is one of the most fruitful places for the isolation of myxobacteria (Dawid 2000). This study attributed this finding to the warm summers and humid winters of Iran. Also, it reported *N. exedens* in

58% of the collected soil samples. The recent study on 62 collected specimens of various types from Iran could not isolate any *Nannocystis* species, though it used innovative culture media that seem well not suited for *Nannocystis* isolation (Saadatpour and Mohammadipanah 2020).

In the present study, *Nannocystis* species were retrieved from 22 % of sampling sites. They formed 14% of all isolated myxobacteria and included a new species (Mohr 2018). The isolation on ST21 agar was a more efficient method compared to WCX agar because this medium is more enriched and nutritious.

*N. exedens* strains grown mainly from rotten plants of the arid climates, as was suggested previously (Dawid 2000). In contrast, the isolation of *N. pusilla* strains was more successful from the topsoil layer of northern forests and Zagreus Mountains that have much more vegetation because of increased humidity. *N. konarekensis* again shows more affiliation to hot and dry places. The difference in habitats results in the difference in purification techniques; *N. pusilla* was mainly purified on media containing antibiotic solutions due to the more contaminations of samples, though purification of *N. exedens* was done by several subcultures. The physiology of *Nannocystis* species closely resembles together and needs careful examination to distinguish between strains. On the other hand, phylogenetic analysis based on partial sequences of 16S rDNA discriminates clearly between strains of *Nannocystis* species. Although, genetic markers such as *rpoB* (Ogier et al. 2019) or genotyping methods like semi-nested PCR-DGGE (Li et al. 2014), MLSA (Lalucat et al. 2020), and comparative genomics (Livingstone et al. 2018; Chambers et al. 2020) had a better discriminatory power to resolve taxonomy at the species level. These findings emphasize the importance of attention to morphological aspects of swarms and fruiting bodies. Therefore, the polyphasic approach is the most suitable for the identification and classification of *Nannocystis*.

Seven out of ten secondary metabolites producers have had OSMAC (one strain many compounds) potentiality (Table 4) that synthesized compounds from different structural classes. This phenomenon was previously reported from *N. pusilla* strains (Bader et al. 2020), but this study revealed both species had such ability. The most notable was *N. exedens* 215 production of the metabolites in the full spectrum of bioactivity from unknown to cytotoxic. Despite their outstanding capability to produce metabolites from different structural classes, strains of this study had a narrow range of bioactivity on Gram-positive bacteria and the *E. coli* TolC mutant.

Nannochelins and althiomycin were the most abundant secondary metabolites identified in both *N. pusilla* and *N. exedens* strains. Up to 70% of the isolates had produced a type of nannochelins. These compounds are citrate-hydroxamate siderophores with the activity on Gram-positive bacteria and weak inhibition of fungi by a still unknown mechanism (Kunze et al. 1992). Althiomycin is a sulfur-containing cyclic peptide, previously isolated from several myxobacteria in *Cystobacterineae* and *Streptomyces* that elicits broad antibacterial activity by inhibiting peptidyltransferase reactions [5,25]. All althiomycin producing strains except *N. pusilla* 166 inhibited Gram-positive test microorganisms. Also, strains 34 and 51 inhibited TolC mutant *E. coli* in a somewhat weaker manner.

Nannopyrazinones (nannozinone) and pyrroazols are rare bioactive molecules of N-containing heterocyclic compounds with a weak antibacterial and antifungal activity. Pyrroazols have been identified in extracts of both *N. pusilla* and *N. exedens* broths (Jansen et al. 2014a). LC-MS analysis of only one strain (49) of *N. pusilla* showed the peak of pyrroazol consistent with its very weak inhibition of *M. hiemalis*. Nannopyrazinones were reported from *N. pusilla* (Jansen et al. 2014b) with an additional slight cytotoxicity effect on the L929 cell line, while analysis indicated these compounds in one strain of each species (3 and 215) and therefore it may contribute to the lethal effect of strain 215 on L929 cells (Herrmann et al. 2017).

In addition to nannopyrazinone, *N. pusilla* 3 secreted a potentially new derivative of myxoprincomide to the fermentation broth that is a linear peptide with unusual amino acids. It was discovered in *M. xanthus* DK 1622 broth mass spectrum by metabolome mining and statistical evaluations (Cortina et al. 2012). There is no report of biological activity or mechanism for this metabolite but, increased bioactivity of *N. pusilla* 3 than the reported bioactivity of nannopyrazinones may be due to the synergism between these metabolites.

Strains 7 and 212 of *N. pusilla* produced a single metabolite, myxopyronin, with the  $\alpha$ -pyrone structure that inhibits bacterial RNA polymerase by binding to the “switch region” of the molecule (Mukhopadhyay et al. 2008). Myxopyronin was initially reported from *M. fulvus* and exhibited a broad antibacterial activity against both high and low G + C Gram-positive and some Gram-negatives (Irschik et al. 1983). Screening of bioactivities of these strains (7 and 212) support the identification based on LC-MS with inhibition of all tested Gram-positive bacteria, *M. smegmatis* and, TolC mutant of *E. coli*.

Germacrane produced by *N. exedens* 215 is a cyclodecane sesquiterpene previously reported from *N. exedens* (Reichenbach, H.; Höfle 2000). Its biological importance for producing strains is not understood, but derivatives of germacrane in medicinal plants show a broad range of antimicrobial activity and cytotoxicity (Zhang et al. 2018).

Only *N. exedens* 215 with the production of myxothiazol displayed toxicity to L929 fibroblasts. Myxothiazol was reported for the first time from *Myxococcus fulvus* strain Mx f16 and structurally belongs to the bithiazole compounds that inhibit respiratory complex III [33,34]. In addition to cytotoxicity, this antibiotic has antifungal activity as seen by slight inhibition of *M. hiemalis* by *N. exedens* 215.

The heatmap clearly shows the distinct distribution of metabolites between species of *Nannocystis*. Also, *Nannocystis* in this study shares three metabolites with *Myxococcus* from the *Cystobacteriinea* suborder. Hoffmann and Krug reveal a correlation between chemical diversity and the taxonomy of myxobacteria; in this study, the profile of known and unknown metabolites of each taxon above the genus rank differs meaningfully from other taxons. Likewise, they found such a correlation between four species of *Myxococcus* and their metabolites, however, in a less clear state (Hoffmann et al. 2018). Another relevant finding of Hoffmann was near relatedness in secondary metabolites profiles of *Myxococcus* and *Nannocystis* genera in unknown and known metabolites (Hoffmann et al. 2018).

In conclusion, this study presents Iran as an excellent source for the isolation of *Nannocystis* strains with a high potential for secondary metabolites production. Especially, extensive sampling of dry and remote deserts and islands can result in the identification of new species that increase the chance of encountering new metabolites. According to the production of several natural products by most strains in our collection, novel secondary metabolites may be found by in-depth analysis of MS data and genomes for cryptic biosynthetic pathways. Our findings also emphasize the importance of well-established protocols for the isolation and cultivation of this genus.

## **Declarations**

# **Declarations**

## **Funding**

The authors did not receive support from any organization for the submitted work.

## **Conflicts of interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## **Availability of data and material**

All sequences were deposited in Genbank and are accessible through accession numbers KX572697, KX572719-21, KX572723-24, KX572736,58,88,91, and KX57572856. *N. konarekensis* type strain is held by DSMZ culture collection under DSM 104509<sup>T</sup> catalog number.

## **Code availability**

Not applicable

## **Ethics approval**

Not applicable

## **Consent to participate**

Not applicable

## **Consent for publication**

Not applicable

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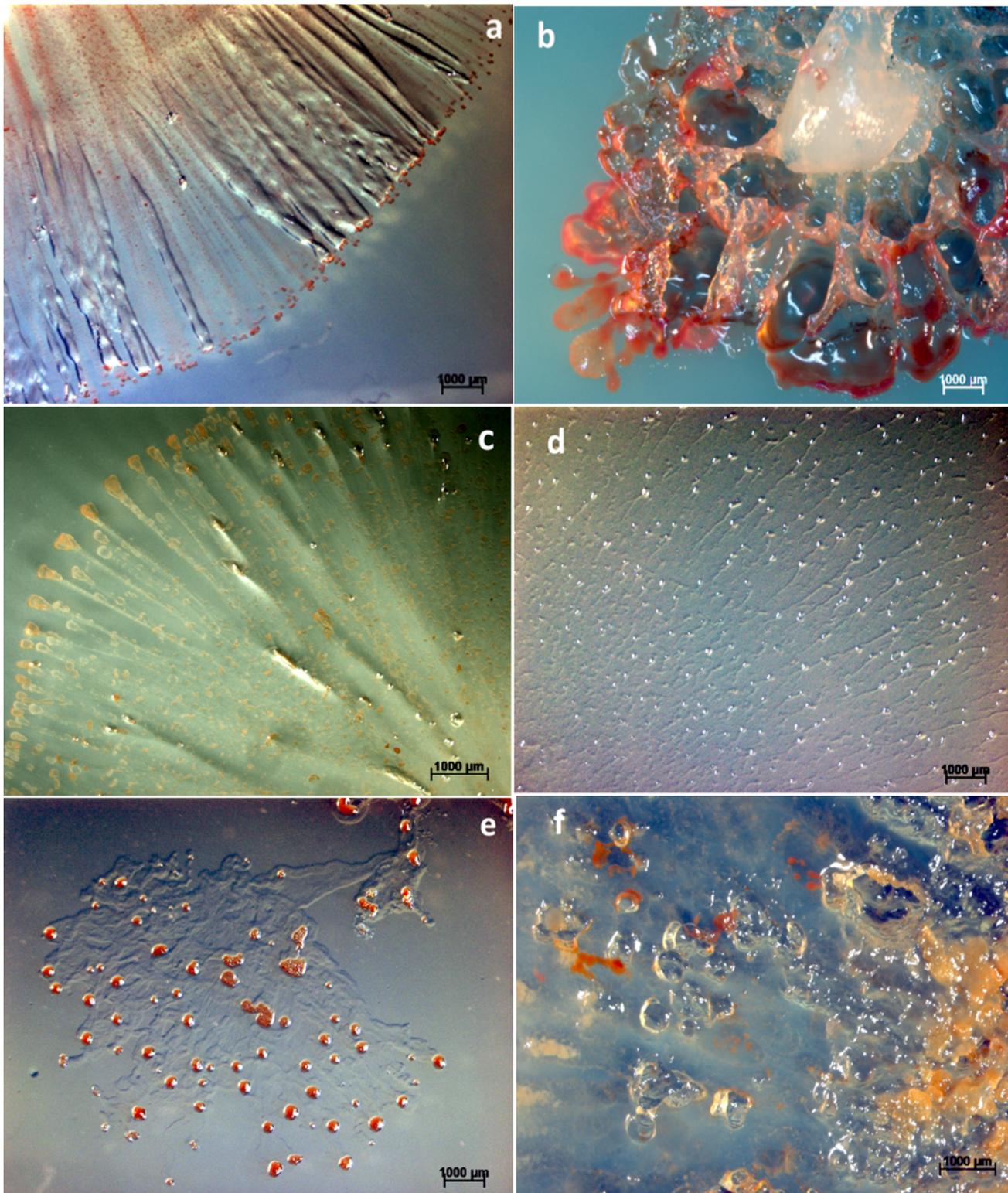
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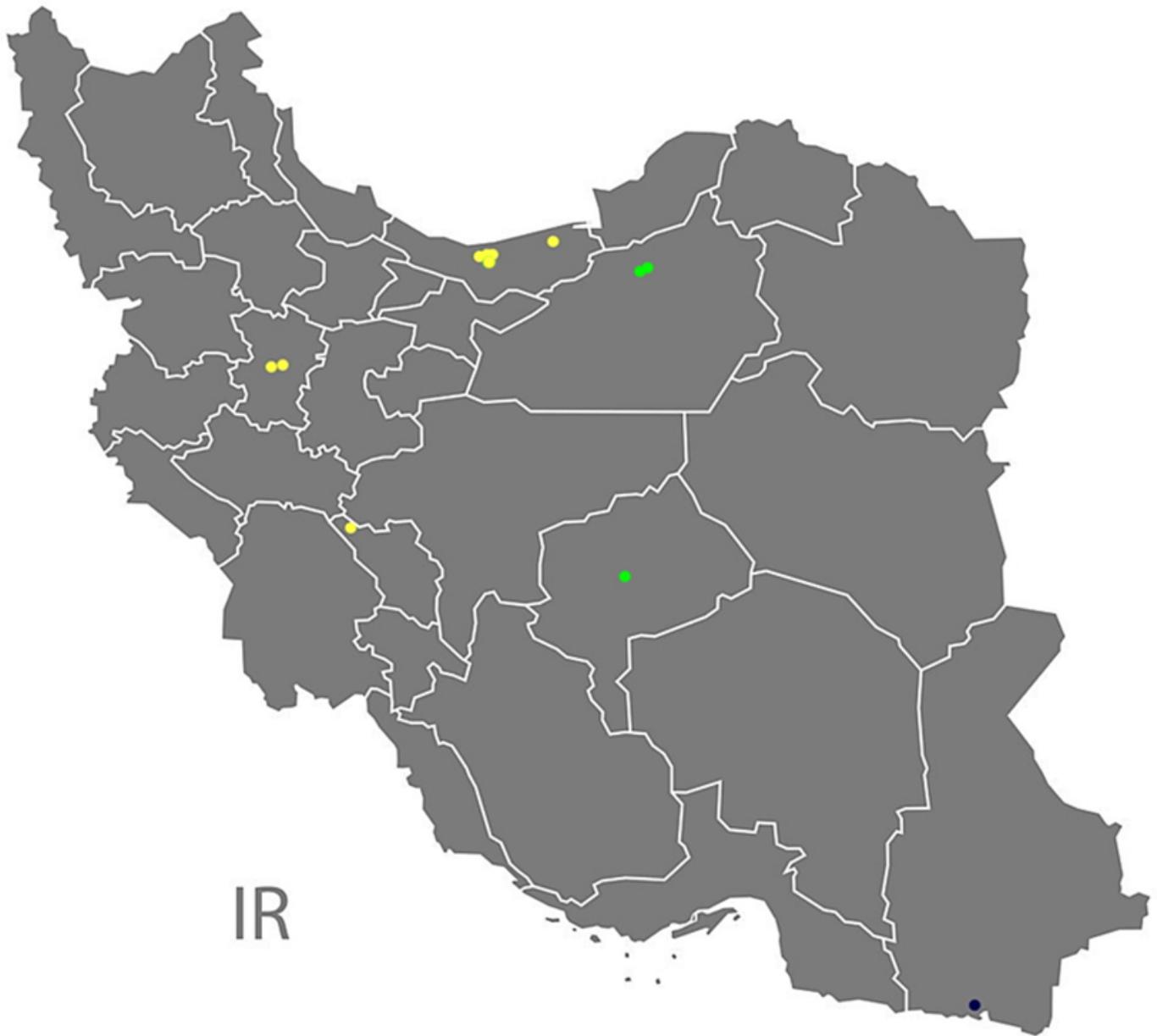
## Figures



**Figure 1**

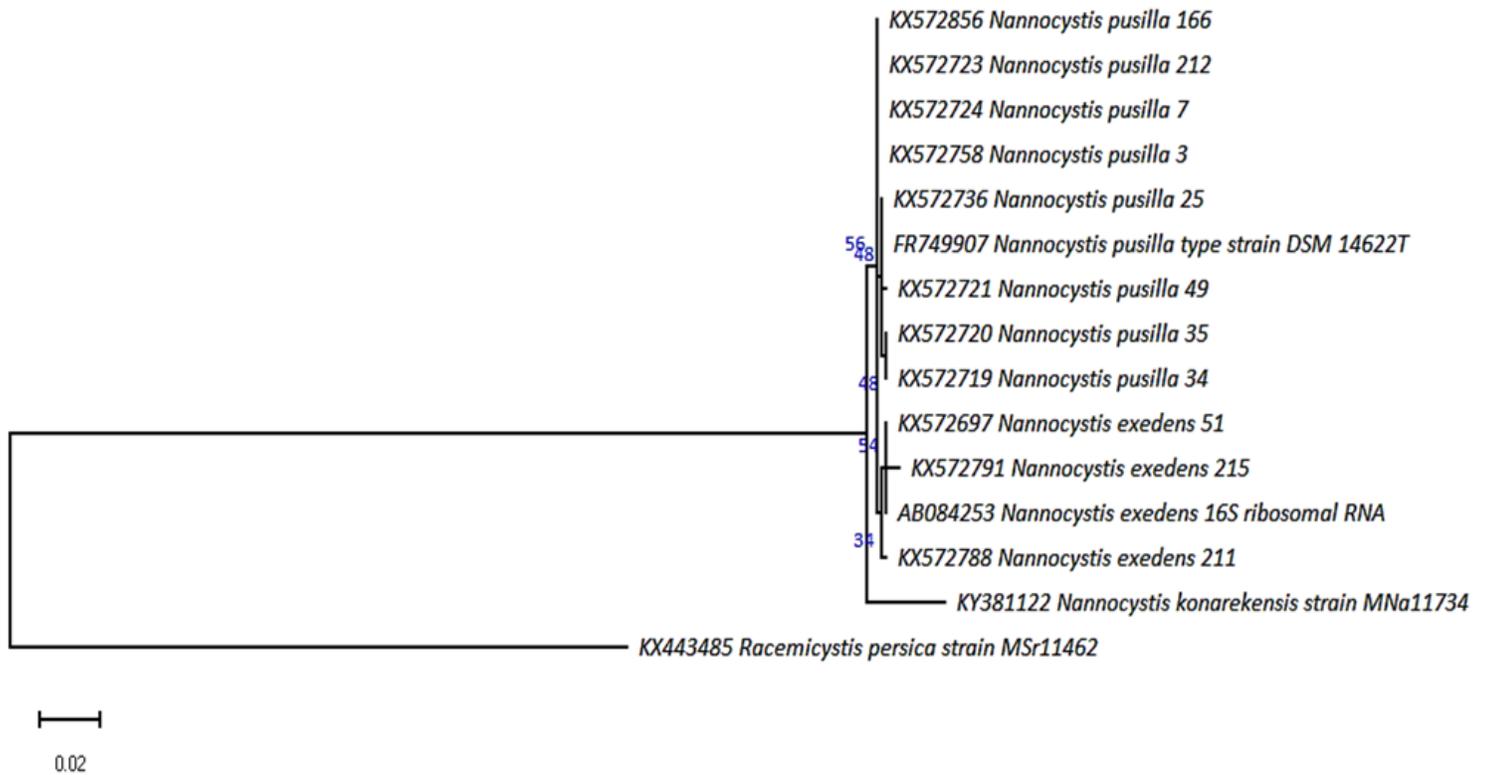
Morphology of *Nannocystis* strains on agar media. a) fan like swarm with the agar channeling of *N. pusilla* 3 on the VY agar, the single sporangioles formed on the edge and trail of swarms; b) the extensive agar degradation by *N. pusilla* 25, the sporangioles grown onto the CY medium; c) swarm of *N. pusilla* 212 on CY agar resembled *N. pusilla* 3, note the less agar channeling; d) the tiny colorless sporangioles of *N. exedens* 51 on VY agar, the agar degraded on the trail of swarming cells; e) the single and clustered

sporangioles of *N. exedens* 211 ditch CY agar but the sporangioles formed on the surface of solid media;  
f) *N. exedens* 215 pits on the CY agar with clustered orange sporangioles.



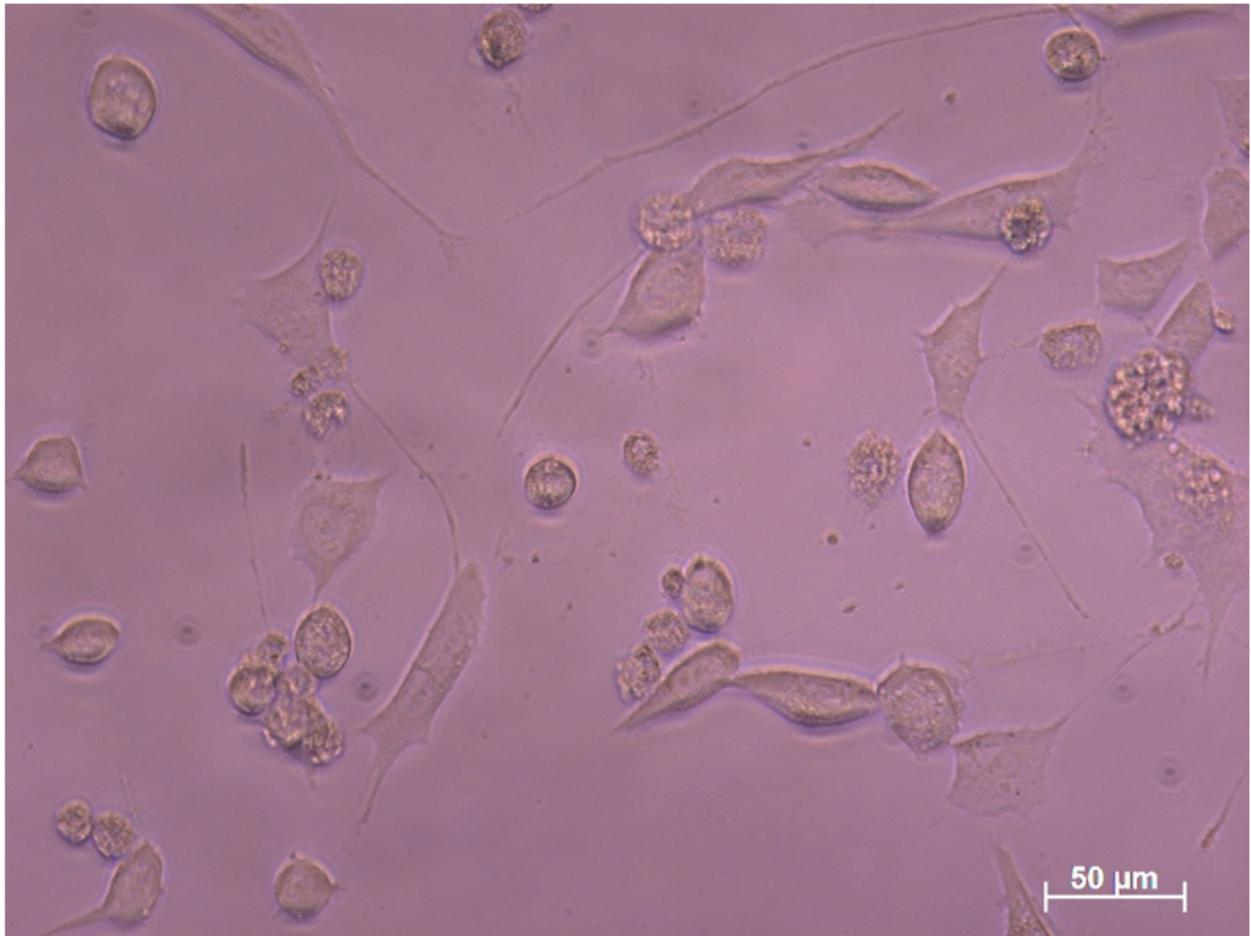
**Figure 2**

The approximate location of isolated strains in the Iran map. Yellow dots for *N. pusilla*, green dots for *N. exedens*, and navy blue for *N. konarekensis*.



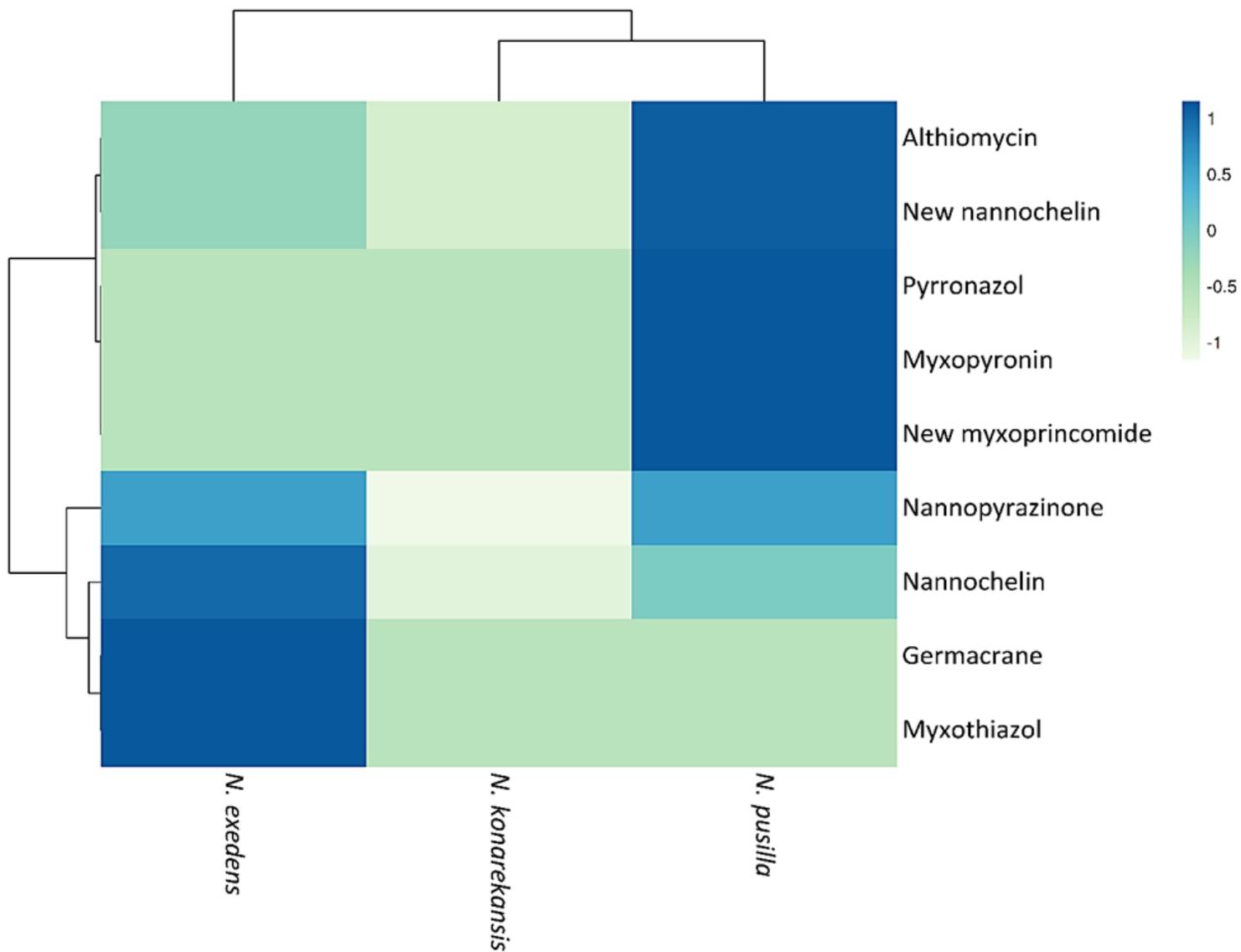
**Figure 3**

Phylogenetic relationship of strains resolved by the Maximum-Likelihood method and 100 resamplings. The accession number of isolates and bootstrap values are presented in the tree. Two type strains of *N. pusilla* (FR749907) and *N. exedens* (AB084253) included; *Racemicystis persica*, a member of the Sorangiineae, was used as the outgroup to root the tree.



**Figure 4**

The cytotoxic effect on the L929 cell line was evident through vacuolation of cytoplasm only in the experiment by extract of strain 215 of *N. exedens*.



**Figure 5**

The heatmap revealed differently distributed natural products between *Nannocystis* species. Also, some secondary metabolites are strongly grouped in the graph. Nannopyrazinone and Nannochelins were produced evenly by both *N. pusilla* and *N. exedens*.