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RESEARCH ARTICLE

One step bio-guided isolation of secondary metabolites from the endophytic fungus *Penicillium crustosum* using high-resolution semi-preparative HPLC

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Abstract: An endophytic fungal strain *Penicillium crustosum* was isolated from the seagrass *Posidonia oceanica* and investigated to identify its antimicrobial constituents and characterize its metabolome composition. The ethyl acetate extract of this fungus exhibited antimicrobial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) as well as an anti-quorum sensing effect against *Pseudomonas aeruginosa*. The crude extract was profiled by UHPLC-HRMS/MS and the dereplication was assisted by feature-based molecular networking. As a result, more than twenty compounds were annotated in this fungus. To rapidly identify the active compounds, the enriched extract was fractionated by semi-preparative HPLC-UV applying a chromatographic gradient transfer and dry load sample introduction to maximise resolution. The collected fractions were profiled by ¹H-NMR and UHPLC-HRMS. This method led to the unambiguous identification of eight known secondary metabolites as well as the determination of their bioactivity using a single purification step.

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1. INTRODUCTION

Antimicrobial resistance (AMR) is a major public health concern, with a 700.000 annual death toll (1). Indeed, the bacterial resistance to last-resort antibiotics, is one of the most prevalent and serious dangers to patient safety. Among the several resistant bacteria that are reported today, particular attention should be given to “ESKAPE pathogens” (2). ESKAPE is an acronym for (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species), a group of well-characterized Gram-positive and Gram-negative bacteria that cause serious nosocomial infections mainly for immunocompromised hospitalized patients (2). Another antibiotic resistant microorganism is *Cutibacterium acnes* a Gram-positive

bacterium mainly responsible for acne vulgaris and postoperative and device-related infections (3, 4).

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Unfortunately, the efficacy of the current drugs is inadequate to overcome these infections (2). Thus, more effective treatments are urgently needed to counter this issue.

Classic treatment for bacterial infection relies mainly on the use of antibiotics that either kill or inhibit the growth of bacteria (5). However, most of these antibiotics have become ineffective by now. Thus exploring new drug targets such as the quorum sensing (QS) in resistant bacteria is an option (6). QS is a way of communication between bacteria of the

same species, based primarily on their sensitivity to autoinducers (AIs). The QS relies on the synthesis, release, and uptake of the AIs in the bacterial population. AIs regulate gene expression in the colony, modulating virulence and biofilm formation (7). Therefore, blocking the QS gene pathways result in a significant reduction in bacterial pathogenicity and resistance (6, 7).

In this regard, natural products (NPs) have always significantly contributed to the fight against resistant pathogens (8). Among the different sources of NPs, the search for bioactive molecules within extracts of endophytic fungi is a promising pathway to find molecules that can be used generally to treat human diseases and particularly those caused by bacteria (9).

In this study, *Posidonia oceanica* (L.) Delile (Posidoniaceae) was used as a source of fungal endophytes. *P. oceanica* is a seagrass which grows only in the Mediterranean basin. This vascular plant hosts a wide variety of microorganisms including bacteria, fungi and microalgae. The endophytic fungus *Penicillium crustosum* Thom (Aspergillaceae) was isolated from the leaves *P. oceanica* in addition to other 32 fungal endophytes. *P. crustosum* is an ubiquitous fungal species, which is isolated frequently from various substrates in cold and temperate regions (10, 11). This fungus has been found in terrestrial plants, soil, sea sediments, and the Antarctic Ocean (10-15). Secondary metabolites produced by *P. crustosum* have shown a wide variety of chemical structures and several biological activities (16, 17). In this study, based on the bioactivity potential of this strain, the ethyl acetate extract of *P. crustosum* was investigated.

The identification of the compounds present in a natural extract can be performed by an MS data-dependent analysis (DDA) which provides both MS and MS/MS data on ideally all detected features in an metabolite profile of an extract (18). This preliminary putative identification of the metabolites provides an early assumption for the identity of the detected molecules. Thanks to the dedicated MS data mining software, which allows a fully automated dereplication workflow. Furthermore, the development of molecular networking (MN) to organize HRMS/MS data has proven very useful in facilitating metabolite annotation (19, 20). At this level, however, the unambiguous identification of individual metabolites is still a limitation, and isolation is required for *de novo* structural elucidation using nuclear magnetic resonance (NMR).

In the present article, we proposed a one step-isolation workflow suitable for microbial extracts where a limited amount of material is usually obtained from cultures. We applied this method to a fungal endophyte extract of the marine-derived *P. crustosum* that showed interesting anti-microbial activities.

2. MATERIALS AND METHODS

Isolation and identification of the strain

The endophytic fungal strain *P. crustosum* Thom was isolated from leaves of *Posidonia oceanica* (L.) Delile

(Posidoniaceae). The plant was collected from the Mediterranean Sea in 2014 with authorization from the French regional directorates for environmental protection. The isolation of the endophytes and the taxonomical identification procedures have been described in Alfattani *et al.* (2021) (21). Fungal strains were characterized by amplification and sequencing of their internal transcribed spacers 1 and 2 (ITS) plus the 5.8S of ribosomal DNA (rDNA), using the primers ITS1F and ITS4 (5'-CTTGGTCATTTAGA GGAAGTAA-3' and 5'-TCCTCCGCTTATTGATATGC-3' respectively); (<http://sites.biology.duke.edu/fungi/mycolab/primers.htm>). Polymerase chain reaction (PCR) used the following program: Initial denaturation at 95 °C for 2 min; 20 cycles including 30 s of denaturation at 94 °C, 30 s of primer annealing at 55 °C, 30 s of extension at 72 °C, and a final extension at 72 °C for 10 min. Purification and Sanger's sequencing of the PCR products were performed by Fasteris Sa (Geneva, Switzerland; <https://fasteris.com/>). Sequencing was performed in both directions using the same primers as for amplification and using Sanger's method. The obtained ITS sequences were deposited in GenBank. Once trimmed, a first sequence similarity search (BLAST®; <https://blast.ncbi.nlm.nih.gov/>) in GenBank (National Center for Biotechnology Information; (22) was performed using the "blastn" (Megablast) option excluding "uncultured/environmental sample sequences". A second similarity search was done limiting the search to "sequences from type material". When blast top scores were equally high for several GenBank sequences we favored sequences published in taxonomic studies and/or sequences that were obtained from type material. The method used for the identification of the fungal isolates followed with higher cut-offs (23).

The fungus *P. crustosum* has been successfully cultivated in yeast malt glucose (YMG) solid medium in small scale (5 Petri-dishes). The culture material was agitated with hexane to remove lipophilic compounds (3 x 100 mL). Next, the culture was agitated with ethyl acetate three times overnight (3 x 100 mL). The accumulation of the three extractions yielded 13.3 mg of dry material.

Up-scale cultivation of *Penicillium crustosum*

To obtain sufficient material of crude extract for purification of metabolites, the culture of *P. crustosum* was scaled up to 100 Petri dishes containing 25 mL of yeast malt glucose (YMG) dissolved in agar. Upon daily observation of the growth progress, most of the plates were fully occupied by the fungus in 28 days. The extraction procedure was performed by the agitation of the culture in hexane (3 x 500 mL) to remove highly lipophilic compounds then by ethyl acetate 3 x 500 mL) to capture secondary metabolites. The latter step was repeated 3 times and the extracts were combined. The extraction of 100 Petri dishes yielded 240 mg of crude ethyl acetate extract.

UHPLC-DAD-HRMS/MS

UHPLC-HRMS/MS analysis was performed on a Waters Acquity UHPLC system interfaced to a Q-ExactiveTM Focus Orbitrap (Thermo Scientific, Bremen, Germany)

supplemented with heated electrospray Ionization source (HESI-II). The system was controlled by Xcalibur v. 3.1 (Thermo Scientific). The HESI-II parameters were as follows: source voltage, 3.5 kV (+); sheath gas flow rate (N_2), 52 units; auxiliary gas flow rate, 13.75 units; spare gas flow rate, 2.75; capillary temperature, 320 °C, S-Lens RF Level, 50. The DAD wavelength absorbance was set between 210 to 450 nm. The crude extract (conc. 4 mg/mL, vol. 2 μ L) was injected into an Acquity BEH C18 column (100 x 2.1 mm i.d., 1.7 μ m, Waters, Milford, MA, USA) for 13 minutes. The mobile phase was H_2O (A) and MeCN (B), both containing 0.1% FA. Gradient mode was as follows: 2% to 100% of B in 11 min followed by 2 min of 100% B. The separation was conducted at room temperature and the flow rate was 0.5 mL/min.

MS data analysis and molecular networking

The MS (.RAW) format files were converted to (.mzXML) by MSConvert software. The converted files were treated by MZMine software v. 2.53. In both modes, MS1 and MS2 for each scan were detected at threshold of $1E7$ and 0 respectively. The ADAP module was used to build chromatograms by connecting data points from mass lists. The chromatogram was deconvoluted into individual peaks by application of “wavelet” algorithm. In case of presence of isotopic pattern, isotopes were grouped to the most intense ion. The extracted ions were aligned in a table and represented as features that demonstrate m/z , RT and peak area. The parameters for each mentioned treatment were adjusted according to Rutz, *et al.* (2019) (24). MNs were built online with the GNPS (25) and visualized by Cytoscape v3.8. (26) The GNPS parameters were adjusted according to Houriet *et al.* (2020) (27).

Analytical HPLC-UV-ELSD

Analysis was conducted on an HP 1260 system equipped with a diode-array detection unit (Agilent Technologies, Santa Clara, CA, USA) using an InterChim® HQ C18 column (250 x 4.6 mm i.d., 10 μ m, Moluçon, France). The detection was performed by DAD and ELSD. The DAD parameters were set as follows: UV wavelength at 210, 254, 280, and 366 nm, five spectra acquired per peak, the threshold was 5 mAU. The ELSD parameters were set as follows: pressure 3.4 bar, 45 °C, split to provide a 500 μ L/min flow rate, gain 8. The HPLC conditions were as follows: mobile phase H_2O (A) and MeOH (B) both containing 0.1% FA. The flow rate was 1 mL/min, injection volume 20 μ L, separation temperature was 25 °C and sample concentration was 10 mg/mL dissolved in MeOH. The slope of the mobile phase was set as follows: gradient flow of 35% to 100% of B in 80 min followed by 10 min washing by 100% B.

Semi-preparative HPLC-UV

The enriched extract was purified with an Armen Modular spot prep II (Saint-Avé, France). 40 mg of extract was mixed in the stationary phase (C18 Zeoprep® 40-63 μ m) in a proportion of 1:5 then introduced in a dry-load cell according our previously published method (28). The

separation was performed through an HQ C18 column (10 μ m, 250 x 21.2 mm, i.d., Interchim, Montluçon, France) The mobile phase was composed of MilliQ water (A) and HPLC grade methanol (B), both containing 0.1% F.A. The gradient slope was set as follows: 35% of B was gradually increased to 100% at time point 81.10 min. This was followed by 11.22 min isocratic at 100% B. Fractions were collected by the UV observation of the response of the peaks at 254 and 366 nm. The fractions were evaporated by a high-performance evaporation system (HT-4X Genevac®, Stone Ridge, NY, USA). The semi prep. HPLC separation yielded 24 fractions, which were analyzed by NMR and UHPLC-HRMS. Fraction F1 yielded compounds 1 and 2 (0.5 mg), F7 yielded compound 3 (0.3 mg), F12 yielded compound 4 (0.1 mg), F15 yielded compound 5 (0.5 mg), F18 yielded compound 6 (0.7 mg), and F19 yielded compounds 7 and 8 (1.7 mg).

Minimum inhibitory concentration test

Two strains were used to evaluate the potential MIC of the extract and the fractions, Methicillin-resistant *Staphylococcus aureus* (MRSA, ATCC 33591, gram-positive) and *Pseudomonas aeruginosa* (ATCC 27853, gram-negative). The results were determined in triplicates according to Wiegand, *et al.* (2008) in Mueller-Hinton medium (MH) (29). After the incubation of the inoculated 96-well plates at 37°C for 24h, iodinitrotetrazolium chloride (INT, Sigma-Aldrich) was added to each well at a final concentration of 0.2 mg/ml and incubated for 20 minutes) (30). Controls were gentamicin for *P. aeruginosa* and chloramphenicol for *S. aureus*.

Anti-quorum sensing assay for *Pseudomonas aeruginosa*

The test was carried out following the methodology described by Hentzer, *et al.* (2002) (31) using *P. aeruginosa* PAO1 strain. The *pqsA-gfp* reporter was constructed using the primers 5'-GCTCTAGATCGAGCAAGGGTTGTAACGGTTTTTG-3' and 5'-GCTGCTGCATGCGACAGAACGTTCCCTCTTCAGCGA-3' to amplify *pqsA* gene promoter and cloned by usual molecular methods into XbaI-SphI sites of *lasB-gfp* plasmid in place of the *lasB* promoter. Reporter PAO1 strains containing the *lasB-gfp* or the *pqsA-gfp* plasmid were grown at starting OD600 of 0.05 in PTSB (5% peptone, 0.25% trypticase soy broth) supplied with gentamicin 50 μ g/mL and each sample at 128 μ g/mL. Azithromycin 2 μ g/ml was used as the positive control. Plates were incubated at 37°C, 160 rpm. After 15 hours fluorescence at 480/520 nm was measured using a microplate reader (SynergyHT BioTek) and normalized by the OD600. Results are represented as the percentage of fluorescence compared to the solvent control (DMSO) fixed at 100%.

Description of the isolated compounds

The spectral data of all described compounds can be found in the supplementary materials (Figure S1-S29).

3. RESULTS AND DISCUSSION

The endophytic fungal strain *P. crustosum* was isolated from the leaves of *P. oceanica*. The fungus *P. crustosum* has been successfully cultivated in yeast malt glucose (YMG) solid medium in a small scale. The culture material was defatted with hexane to remove lipophilic compounds and extracted with ethyl acetate (see material and methods). The ethyl acetate extract was submitted to a series of antibacterial assays.

The minimum inhibitory concentration (MIC) of the ethyl acetate crude extract was determined against methicillin-resistant *Staphylococcus aureus* (MRSA, Gram-positive, ATCC 33591) (32), *Cutibacterium acnes* strain (Gram-positive, ATCC 11828) (33), and *Pseudomonas aeruginosa* (Gram-negative, ATCC 27853) (34). As a result, the ethyl acetate extract presented an antibacterial activity against MRSA at 64 µg/mL but no activities were observed against *P. aeruginosa* or *P. acnes*.

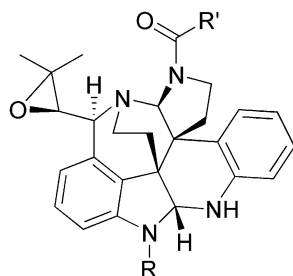
Furthermore, the level of quorum sensing (QS) inhibition was evaluated against *P. aeruginosa*. For this, reporter assays were performed based on a percentage of fluorescence reduction (FR) at a concentration of 128 and 32 µg/mL for two reporter genes, (*pqsA* and *lasB*) (31). The *pqsA* gene is involved in the synthesis of *Pseudomonas* quinolone signal (PQS) while the *lasB* gene encodes a major elastase enzyme. As a result, for the gene *pqsA*, the ethyl acetate extract displayed nearly 82% FR at a concentration of 128 µg/mL and 65% FR at a concentration of 32 µg/mL. For the gene *lasB*, the crude extract exhibited 81% FR at a concentration of 128 µg/mL and 65% FR at a concentration of 32 µg/mL. These preliminary bioassay findings motivated us to pursue further investigation to identify the molecules responsible for those activities.

To obtain a survey of various secondary metabolites produced by *P. crustosum*, the ethyl acetate crude extract was submitted to metabolite profiling by UHPLC-HRMS/MS in positive (PI) and negative (NI) ionization modes. It is worth noting that the metabolome of this fungal species was not completely described in the literature, where 38 compounds were reported in the dictionary of natural products (DNP) (35). The HRMS/MS spectra of all features were obtained by data-dependent analysis (DDA). In addition, a feature-based molecular network (FBMN) (36) was built to arrange the extracted ions (precursor ions) into clusters based on MS/MS fragmentation similarity. In this regard, FBMN is a robust tool that provides a reduction of spectral redundancy and distinguishes the possible isomers compared to the classical MN (36). In positive ionization (PI) mode, 620 features (nodes) were arranged into 46 clusters in addition to single nodes. In negative ionization

(NI) mode, 454 nodes were arranged into 34 clusters. The precursor masses and their associated MS/MS spectra were matched against experimental data from GNPS as well as predicted spectra obtained by *in silico* MS/MS fragmentation database (ISDB-DNP) restricted to fungal metabolites (20).

For each precursor mass, possible structure candidates were listed according to MS/MS similarity with a maximum of top 50 candidates reported to occur in fungi. To increase the level of confidence in annotation, a reranking step based on taxonomy was performed. It takes into consideration the matching with the biological source reported in DNP at the level of species > genus > family, resulting in a maximum of top 5 candidates (24). In addition, annotated nodes were given a match score between 0-1 based on their fragmentation pattern similarity with the predicted ones. As a result, in PI mode, 140 nodes were annotated by compounds that occur in fungi, 75 of them were found in the family Trichocomaceae, 39 of them present in genus *Penicillium*, and 7 features reported in species *P. crustosum*. In NI mode, 142 features were annotated, 60 of them belonging to Trichocomaceae, 23 of them were reported in the same genus and 3 of them have been found in *P. crustosum*. The annotated metabolites corresponding to the most intense MS peaks detected in both PI (threshold > 5E⁷) and NI (threshold > 1E⁷) are presented in table 1. Their annotation was further refined by the structural consistency of corresponding clusters.

Fig. (1). Structures of communesins A-D (37). Those compounds were putatively identified by UHPLC-HRMS/MS. Communesins A and B were lately isolated in this study.



Communesin A	R=CH ₃	R'=CH ₃
Communesin B	R=CH ₃	R'=pentadienyl
Communesin C	R=H	R'=pentadienyl
Communesin D	R=CHO	R'=pentadienyl

As a result, a series of indole alkaloids (communesin A-D) (37) was dereplicated by HRMS/MS and observed in the MN in clusters P-15 and N-8. Communesin derivatives are highly complex structures composed of seven interconnected rings, multiple amine linkages, and five stereocenters (Figure 1). Interestingly, communesins A-C were previously reported in a *Penicillium* strain associated with a Mediterranean sponge (38). Moreover, the analysis of the PI mode through MN revealed the presence of series of chaetoglobosins (CGs). Seven members of this series were putatively identified through the MS/MS dereplication assisted with molecular networking. The identified CGs were as follows: chaetoglobosin A, chaetoglobosin P, chaetoglobosin W, cytoglobosin G, prochaetoglobosin IV, chaetoglobosin J, 21,22-dihydro and 20-dihydroprotochaetoglobosin III (Figure 2). CGs are fungal metabolites belonging to cytochalasan alkaloids which have been found in various fungal genera but mainly in *Chaetomium*. Several studies have reported the presence of CGs in *Penicillium* endophytic fungi associated with marine and mangrove plants (39-42). Recent reports have demonstrated that CGs possess a broad range of biological activities, including antibacterial (43), antifungal (44), antiviral (45), and antitumor activity (46). Later on, we will

describe the isolation of chaetoglobosin B from the ethyl acetate extract of this fungus.

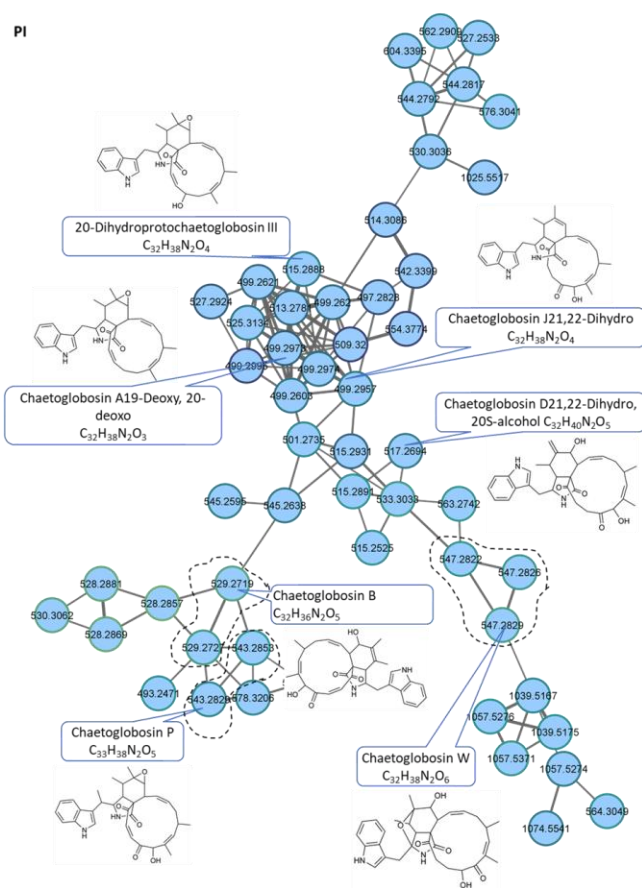


Fig. (2). Detailed view of MN cluster P-5 (positive ionization). The MN revealed the presence of series of chaetoglobosins that were putatively identified by UHLC-HRMS/MS dereplication. Isomers are circled by blue dashed lines.

In NI, cluster N-6 highlights the presence of polyketides that possess a substructure of benzofuranone such as porriolide and rubralide C (Figure S30). Those two compounds are frequently isolated from *Penicillium* and *Aspergillus* species (47, 48). Moreover, we could also dereplicate the well-known mycotoxin (patulin) which is a secondary metabolite produced by molds such as *Penicillium* and *Aspergillus* that are common contaminants to fruits and vegetables (supplementary information, Figure S30) (49).

Culture scale-up, enrichment and semi-preparative HPLC fractionation

To obtain sufficient material of crude extract for purification of metabolites, the culture of *P. crustosum* was scaled up to 100 Petri dishes. As for the extraction on small scale, the material was defatted by hexane and extracted with ethyl acetate (see experimental section). The ethyl acetate extract was first analyzed by reversed-phase HPLC-UV-ELSD using a C18 column (250 x 4.6 mm i.d., 10 µm, Moliçon, France). The conditions were

optimized and geometrically transferred to semi-preparative conditions using the same stationary phase to ensure the same selectivity (50, 51). The extract was introduced to the semi-preparative HPLC column through the dry load method to ensure the optimal chromatographic resolution (28). The semi-preparative HPLC separation was monitored by UV detection at 254 nm. Based on this, an excellent resolution was obtained, which allowed to a precise collection of fractions containing eight compounds (1-8) (Figure 3).

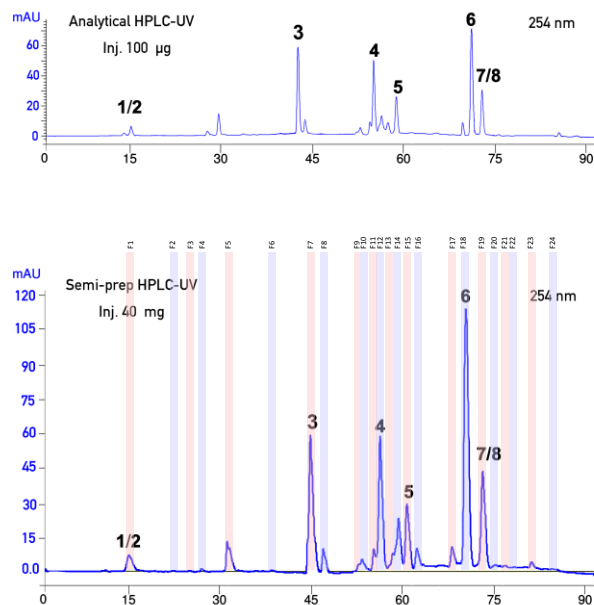


Fig. (3). A) Analytical HPLC-UV analysis of the ethyl acetate extract of *P. crustosum* at 254 nm. B) Semi-prep. HPLC-UV analysis at 254 nm was obtained after gradient transfer. The marks on the chromatogram represent the 24 fractions collected.

All the isolated compounds were submitted to nuclear magnetic resonance (NMR) and high resolution mass spectrometry (HRMS) analysis for structural identification. Based on this analysis the mycotoxin patulin (1) (52) and its less toxic breakdown derivative desoxypatulic acid (2) (53) were isolated in mixture. Compound 3 was identified as gentisic acid (54). The identification of communesin A and B (37, 55) confirmed the efficacy of the dereplication of this class of compounds. The cytochalasan, chaetoglobosin B, was also confirmed as compound 6 (56). The presence of roquefortine C (4) in fraction 12 was difficult to confirm due to the small amount isolated and the presence of two structurally related molecules in chemical exchange in this fraction. All previous NMR spectra of roquefortine C being recorded in CDCl₃ (57, 58), it was difficult to compare them with our data in DMSO-*d*₆. In the same fraction, communesin B (7) and 1,4-dimethyl-3,6-bis(phenylmethylene)-2,5-piperazinedione (8) could also

be identified (59). This compound is the N-dimethyl derivative of piperafizine B previously isolated from several *Streptomyces* species (60, 61). The N-methyl form piperafizine A was isolated from *Streptoverticillium aspergilloides* and a marine-derived *Streptomyces* sp. but this is the first time that a N-dimethyl derivative has been identified from natural sources.

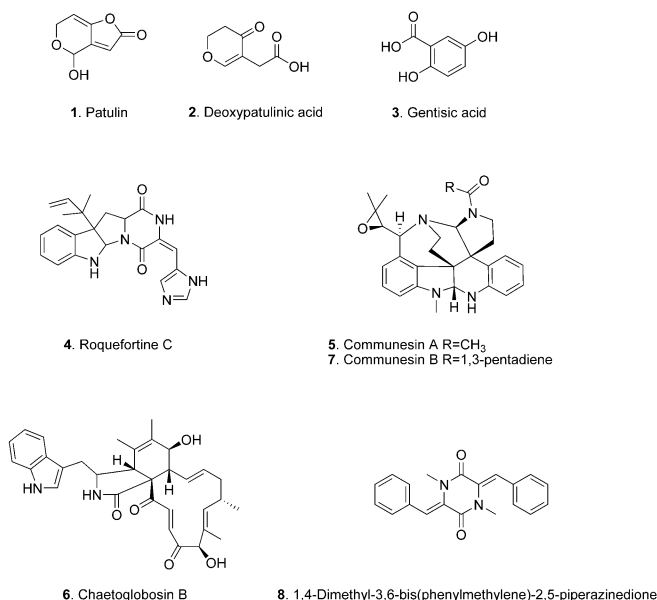


Fig. (4). Structures of isolated compounds **1-8** from the ethyl acetate extract of *P. crustosum*.

The isolation of these compounds confirmed the hypothesis of their presence based on the metabolite profiling results (Supplementary material, Figure S30 and Table S1). Compound **6** was identified as cytochalasan chaetoglobosin B (56). Structurally related cytochalasan derivatives were putatively annotated by UHPLC-HRMS/MS (Figure 2) and (Table S1). By locating this molecule in MN, four analogues of compound **8** were dereplicated by matching their HRMS/MS spectra with the ones found in ISDB-DNP (Table S1). Piperafizine B and its N-Me derivative piperafizine A were properly dereplicated by HRMS/MS. Both were originally isolated from *Streptoverticillium aspergilloides* (62) and a marine-derived *Streptomyces*. Their analog 3-benzyl-6-benzylidene-2,5-piperazinedione putatively identified by MN has been isolated before from *Streptomyces* as well (60, 61). Coelomycin is a related analog, which showed a great antibacterial activity against *Staphylococcus aureus* (63). It is produced by an unidentified coelomycete fungus isolated from the leaf of *Juniperus phoenicea*.

Antimicrobial activity of semi-preparative HPLC-UV fractions

To assign compounds that are responsible for the antibacterial and anti-QS activities, all of the 24 fractions (F1 to F24) obtained by semi-preparative HPLC-UV of *P. crustosum* have been tested. Although the crude extract exhibited MIC activity against MRSA at 64 µg/mL, none of the fractions showed such activity.

For anti-QS activity on *P. aeruginosa*, the mixture of the two cyclic polyketides patulin (**1**) and deoxypatulinic acid (**2**) which co-eluted in fraction 1 (F1), presented a significant level of QS inhibition as they showed fluorescence reduction of 62.26% and 62.67% at 128 µg/mL in Pqs and Las systems, respectively (Figure 5). Moreover, a mixture of communesin B (**7**) and 1,4-dimethyl-3,6-bis(phenylmethylene)-2,5-piperazinedione (**8**) which were found in fraction 19 (F19) exhibited moderate activity only on Las system as they showed fluorescence reduction of 63.11% at 128 µg/mL (Figure 5).

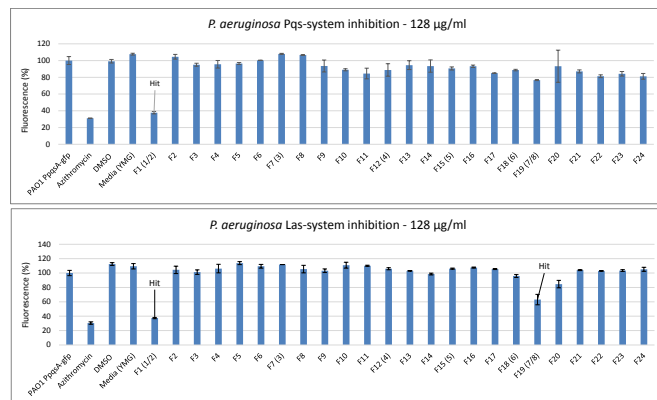


Fig. (5). Biological screening of all fractions (F1 to F24) obtained by semi-preparative HPLC. The test was performed against a QS reporter strain (*P. aeruginosa* PA01), which measured the inhibitory effect through the disruption of the two major quorum sensing systems, Pqs (upper panel) and Las (lower panel) at 128 µg/mL. Azithromycin was used as a positive control.

CONCLUSION

The use of molecular networking-assisted UHPLC-HRMS/MS dereplication allowed preliminary identification of over 20 compounds present in the ethyl acetate extract of *P. crustosum*. The use of dry loading sample introduction combined with gradient transfer significantly accelerated the isolation of the majority of compounds present in the active extract. The one-step fractionation allowed the isolation and identification of eight compounds (**1-8**). Moreover, due to the excellent resolution of the separation, it was possible to correlate biological activity to certain areas of the chromatogram. A mixture of **1** and **2** showed significant QS inhibition against *P. aeruginosa* (Pqs and Las systems), while a mixture of **7** and **8** showed moderate inhibition of *P. aeruginosa* Las system. It is important to note that patulin (**1**) is known to be a very mycotoxic compound in contrast to deoxypatulinic acid (**2**) which is a much less cytotoxic reduction compound of patulin (64). Interestingly, communesin B (**7**) was carried over for interesting anticancer activities (65). None of the isolated compounds were identified as responsible for the observed MIC against the *S. aureus* MRSA strain. This could be explained by a synergistic effect among the compounds or by the presence of minor active compounds that could not

be isolated using this one-step high resolution semi-preparative HPLC fractionation process.

AVAILABILITY OF DATA AND MATERIALS

The raw NMR data of the isolated compounds are available at the following link: <https://doi.org/10.26037/yareta:d3xkhjdlva7vmrt5yp6kwzx6y>

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CONFLICT OF INTEREST

The authors declare not having conflict of interest.

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