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Cytotoxic and antibacterial substances against multi-drug resistant pathogens from marine sponge symbiont: Citrinin, a secondary metabolite of *Penicillium* sp.

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PEER REVIEW

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Comments
This article describes the isolation and identification of citrinin from symbiotic fungus *Penicillium* sp. associated with marine sponge. The study has been conducted in a well organized manner and presented in a convincing way. The cutting edge tools (NMR, HRLCMS *etc.*) employed in the study provides very credible data. This finding can further accelerate the search for alternative drugs in treating certain dreadful diseases.
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ABSTRACT

Objective: To Isolate, purify, characterize, and evaluate the bioactive compounds from the sponge-derived fungus *Penicillium* sp. FF001 and to elucidate its structure. **Methods:** The fungal strain FF001 with an interesting bioactivity profile was isolated from a marine Fijian sponge *Melophlus* sp. Based on conidiophores aggregation, conidia development and mycelia morphological characteristics, the isolate FF001 was classically identified as a *Penicillium* sp. The bioactive compound was identified using various spectral analysis of UV, high resolution electrospray ionization mass spectra, 1H and 13C NMR spectral data. Further minimum inhibitory concentrations (MICs) assay and brine shrimp cytotoxicity assay were also carried out to evaluate the biological properties of the purified compound. **Results:** Bioassay guided fractionation of the EtOAc extract of a static culture of this *Penicillium* sp. by different chromatographic methods led the isolation of an antibacterial, anticryptococcal and cytotoxic active compound, which was identified as citrinin (1). Further, citrinin (1) is reported for its potent antibacterial activity against methicillin-resistant *Staphylococcus aureus* (*S. aureus*), rifampicin-resistant *S. aureus*, wild type *S. aureus* and vancomycin-resistant *Enterococcus faecium* showed MICs of 3.90, 0.97, 1.95 and 7.81 μg/mL, respectively. Further citrinin (1) displayed significant activity against the pathogenic yeast *Cryptococcus neoformans* (MIC 3.90 μg/mL), and exhibited cytotoxicity against brine shrimp larvae LD_{50} of 96 μg/mL. **Conclusions:** Citrinin (1) is reported from sponge associated *Penicillium* sp. from this study and for its strong antibacterial activity against multi-drug resistant human pathogens including cytotoxicity against brine shrimp larvae, which indicated that sponge associated *Penicillium* spp. are promising sources of natural bioactive metabolites.

KEYWORDS
Penicillium sp., Sponge derived, Citrinin, Multi-drug resistant, Antimicrobial, Cytotoxic activity

1. Introduction

The demand for new antibiotics continues to grow due to the rapid emergence of multiple drug resistant human pathogens particularly methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococci*, cephalosporin-resistant *Klebsiella pneumonia*, fluoroquinolone-resistant *Pseudomonas aeruginosa*, multidrug resistant Gram-negative bacteria and extensively drug resistant tuberculosis causing life threatening infections[1]. Although considerable progress is being made within the fields of chemical synthesis and engineered biosynthesis of active compounds, nature still remains the richest and the most versatile source for new antibiotics[2]. Microbial natural products are an important source and play a significant role in the discovery and understanding of cellular pathways that are essential in the drug discovery process. In the search for new bioactive natural products from marine organisms, increasing attention is being given to microorganisms such as bacteria and fungi[3-5]. Particularly sponge-derived fungal cultures

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have repeatedly been shown to be interesting sources of new bioactive secondary metabolites previously unknown from terrestrial strains of the same species[6–8]. On the other hand, interesting biological activity from previously reported terrestrial or marine environmental strains are also gaining attention in the pharmaceutical industry. In continuation of our studies on sponge-derived fungi we now report on the secondary metabolite of a strain of *Penicillium* sp. FF001 that we obtained from the marine sponge *Melophlus* sp. The present study is aimed at the isolation of a bioactive compound showing antibacterial, anticryptococcal and cytotoxic activities from the strain *Penicillium* sp., which was isolated from the Fijian marine environment.

2. Materials and methods

2.1. General experimental procedures

The UV spectrum was acquired in spectroscopy grade methanol using a PerkinElmer *Lambda* 35 spectrophotometer. NMR experiments were performed on Varian Inova spectrometer 300 MHz. The chemical shifts were expressed in δ (ppm) and referenced to the residual $CHCl_3$ (δ_C 77.2 ppm, and δ_H 7.26 ppm) in $CDCl_3$. High resolution electrospray ionization mass spectra (HRESIMS) were acquired using an Agilent 1100 series separations module equipped with an Agilent G1969A MSD (mass spectroscopy detector) in positive ion mode. Analytical HPLC was performed using a Waters 515 pump connected to a 2487 UV-Vis detector. TLC analyses were carried out on pre-coated TLC sheets (ALUGRAM SIL G/UV_{254}, Germany) and column chromatography on silica gel (Labchem, USA). The R_f values were determined on 20 cm×20 cm plates, the evaluation length being 10 cm. Compound was visualized either by observing under a UV lamp at 254 nm and 365 nm or by spraying with $KMnO_4$ followed by heating. Analytical grade solvents were utilized for chromatographic analysis. Riedel-de Haen, Chromasolv high purity LCMS grade solvents were used for HRLCMS.

2.2. Sponge material

The sponge *Melophlus* sp. was collected by hand using SCUBA at a depth of 10 m from Cicia, Lau group, Fiji Islands (17°47′33′′ S, 179°23′94′′ W) in September 2008 during a three-week biodiversity expedition in the central Lau group. The sponge material was transferred into a sterilized bag immediately after harvesting and was transported cooled to the nearby laboratory. The isolation of fungi was subsequently carried out. The sponge was identified by Prof. John Hooper, Queensland Museum, Australia. A voucher specimen (G-0634) has been preserved at the Marine Reference Collection, The University of the South Pacific, Fiji Islands and at Georgia Institute of Technology, USA.

2.3. Fungal material and cultivation conditions

The sponge sample was rinsed three times with sterile seawater to eliminate nonspecific fungal propagules that stick to the sponge surface from the seawater, and the surface of the sponge tissue was subjected to surface sterilization using 70% ethanol under aseptic conditions. The surface sterilized sponge tissue was then cut into small pieces of approximately 0.1 cm^3, which were either placed directly onto agar plates or homogenized and diluted with membrane-filtered seawater. The resulting homogenate was diluted with sterile seawater at three dilutions (1:10, 1:100, and 1:1 000). For fungi cultivation, small pieces of sponge tissue or 100 μL of each dilution was plated onto the three agar plates of the following media: potato dextrose agar (PDA) medium (PDA, Difco^{TM}), Rose bengal agar (RBA) (peptone 5 g, glucose 10 g, KH_2PO_4 1 g, $MgSO_4.7H_2O$ 0.5 g, Rose bengal 0.033 g, agar 18 g, seawater 1 L, pH 6.8) medium, and Czapek-Dox agar (CDA) ($NaNO_3$ 3 g, KCl 0.5 g, K_2HPO_4 0.1 g, $MgSO_4.7H_2O$ 0.5 g, $FeSO_4$ 0.01 g, sucrose 30 g, agar 18 g, seawater 1 L, pH 6.8) medium. A broad spectrum (bacteriostatic and bacteriocidal) antibiotic penicillin-streptomycin (Sigma; lyophilized) (100 mg/mL) was added in the media to avoid Gram negative and Gram positive bacterial contamination. The plates were incubated at 28 °C for 3-4 weeks until the morphology of fungi could be distinguished. Pure cultures were used for morphological identification by light microscopy. Fungal isolates were stored as agar slant cultures at 5 °C, and additionally were preserved at -80 °C using cryobank vials.

2.4. Fungal identification

The morphology of fungal strain was studied under the Olympus BX50 light microscope (200-400× magnification). The morphological traits (*e.g.*, morphology and arrangement of spores and mycelia structure) of the pure fungal colonies were examined by microscopy. The observed specimens are converted to figures with the aid of a camera lucida attached to the microscope. Images were captured with a CCD digital camera (CoolSnapPro) integrated with the microscope. Micro- and macromorphological features, reverse and surface coloration of colonies grown on PDA, RBA and CDA media were also observed. In addition, the isolated sponge associated fungus was identified by classical taxonomy as well as using standard monographs[9]. The identified culture of fungal strain was deposited at the marine microbial collection of the Centre for Drug Discovery and Conservation, The University of the South Pacific with the accession number FF001.

2.5. Extraction and isolation

The fungal strain was cultured on PDA at 28 °C for 10 d. Further, *Penicillium* sp. was mass cultivated (5 L) in modified Wickerham-medium (yeast extract 0.3%, malt extract 0.3%, peptone 0.5%, glucose monohydrate 1.0%, sodium chloride 3.0% and membrane filtered (0.4 μm) natural seawater 1 L), and the final pH of the medium was adjusted to 6.8 before sterilization. Agar plugs were used to inoculate from the mother culture and about 30 discs (9 mm) were inoculated in 3 L glass Fernbach flasks containing 1 L modified Wickerham-medium (MWM). After inoculation, the flasks were incubated for 21 d at 28 °C in static conditions. The

culture medium along with mycelia was extracted twice with ethyl acetate by sonicating for 20-30 min. The ethyl acetate phase was concentrated in vacuo to give a crude extract (2.53 g). The crude EtOAc extract was subjected to silica gel TLC using dichloromethane: methanol (9:1; v/v) and exhibited 7 visible spots under UV with 254 nm and 365 nm representing different R_f values. Further, the crude fungal extract showed significant antibacterial activity against methicillin-resistant *S. aureus*, wild type *S. aureus*, vancomycin-resistant *E. faecium* and it displayed cytotoxicity at 180 ppm. Therefore, the active crude extract was fractionated using vacuum liquid chromatography on silica gel (220-230 mesh size) using hexane-dichloromethane-methanol stepwise gradient elution for separation of bioactive compounds. A total of 12 fractions were collected and subjected for silica gel TLC and bioassay which yielded three sub-fractions combined based on similar retention time on TLC. The bio-active fraction (803.5 mg; F_1F2) was further separated by reverse phase-C18 silica gel column with stepwise gradient elution of 10-100% $MeOH_{(aq)}$ to yield eight fractions. The sixth (F_2F6) and seventh (F_2F7) fraction [60% $MeOH_{(aq)}$] left to stand overnight showed the presence of crystals. The crystals were separated and further purified by re-crystallization. The crystals were washed twice with hexane, dissolved in a minimum amount of 100% ethyl acetate and left to stand overnight under low temperature at 4 °C. Fine pure crystals (1; 102 mg) were separated from the liquid by filtration. The overall isolation scheme of compound 1 is shown in Figure 1. The pure crystal (1) was subjected to reverse phase (C18 column; 4.6 mm× 250, 10 μm) analytical Waters 515 HPLC system to analyse the purity.

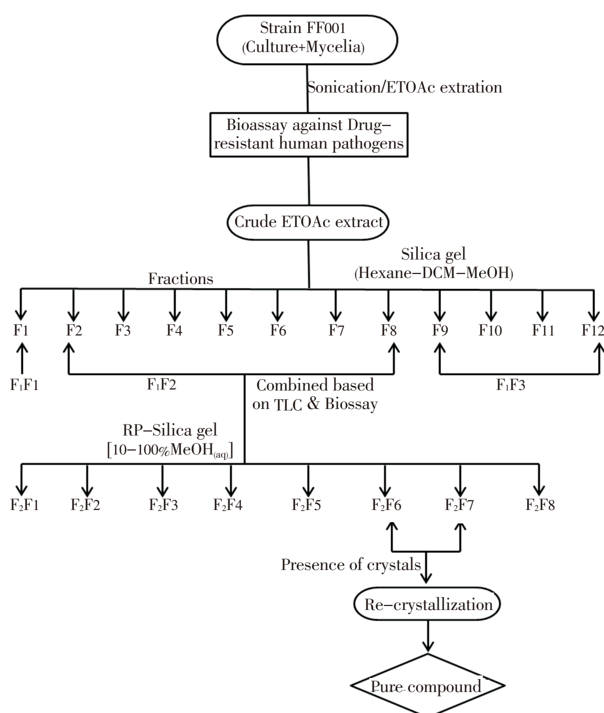

Figure 1. Scheme for the isolation of compound 1.

Citrinin (1): Lemon yellow crystals, UV (MeOH) λmax 213, 253, and 321 nm; molecular formula $C_{13}H_{14}O_5$; HRESIMS (*m/z*): 251.0936 $[M+H]^+$ (calcd. for $C_{13}H_{15}O_5$, 251.0914) and 273.0743 $[M+Na]^+$; ^1H NMR (300 MHz, $CDCl_3$, *J* in Hz): δ8.23 (^1H, s, H-1), δ4.77 (^1H, m, H-3), δ2.97 (1H, m, H-4), δ2.01 (3H, s, H-13), δ1.34 (3H, d, *J* = 6.7, H-11), δ1.22 (3H, d, *J*=7.2, H-12); ^{13}C NMR (75 MHz, $CDCl_3$): δ183.9 (C-8), δ177.3 (C-6), δ174.6 (C-14), δ162.9 (C-10), δ139.2 (C-1), δ123.2 (C-5), δ111.5 (C-7), δ81.8 (C-3), δ34.7 (C-4), δ18.7 (C-12), δ18.4 (C-11), δ9.6 (C-13).

2.6. Antimicrobial assay

The minimum inhibitory concentrations (MICs) of the compound 1 against different test organisms were determined by the broth dilution method. The isolated compound 1 was dissolved in MeOH at 10 mg/mL and diluted further to give required the concentrations (μg/mL) 500.00, 250.00, 125.00, 62.50, 31.25, 15.62, 7.80, 3.90, 1.90, 0.97, 0.48, 0.24, 0.12, 0.06, 0.03 and 0.01. The diluted solutions (100 μL) were added to separate wells on a 96-well plate. An inoculum of 100 μL from 24 h old culture of each test human pathogens, methicillin-resistant *Staphylococcus aureus* (MRSA, ATCC10537), wild type *S. aureus* (WTSA), rifampicin-resistant *S. aureus* (RRSA), vancomycin-resistant *Enterococcus faecium* (VREF, ATCC12952) and *Cryptococcus neoformans* (CN, ATCC32045) were inoculated separately in each well. The antibacterial agent vancomycin (MRSA, WTSA and RRSA) and rifampicin (VREF) and antifungal agent nystatin (CN) were used as positive controls and the solvent MeOH was included in the bioassay as negative control. The cultures were incubated for 24 h at 37 °C. Replicates were maintained for each treatment. The MIC was defined as the lowest concentration of the pure compound/antibiotics showing no visible growth after the incubation time.

2.7. Brine shrimp cytotoxicity assay

Brine shrimp (artemia salina) eggs were hatched in 150 mL of filtered seawater under constant aeration for 48 h at (27±2) °C. After hatching, active nauplii free from egg shells were collected and used for the assay; 100 μL of seawater containing ten to fourteen nauplii were placed in each 96-well plate. A hundred microliters of compound 1 was added in the respective wells at 250 ppm, 125 ppm and 62.5 ppm concentrations in triplicate and maintained at room temperature for 24 h at (27±2) °C. Filtered seawater was used as negative control. The LD_{50} value was calculated using the Reed-Muench method[10].

3. Results

The fungal strain, FF001 was isolated from a marine Fijian sponge *Melophlus* sp. The macroscopic features of the fungus on CDA, after 7 d of growth at 28 °C, colonies were 35-39 mm in diameter, showed good sporulation, were mainly pale greenish black at the centre and had a slightly white periphery with a regular margin, whilst the reverse was dominated by a pale yellow color. After 14 d of growth at 28 °C, colonies were 69-73 mm in diameter, displayed very good sporulation, were dark greenish black and dominated by a deep yellow reverse. On PDA, after 7 d of growth at 28 °C, colonies were 47-49 mm in diameter, showed good sporulation, had dark greenish black mycelia

with a regular margin and a reverse dominated by a pale yellow color. After 14 d of growth at 28 °C, colonies showed 86–88 mm in diameter, presented good sporulation, were dominated by dark greenish black mycelia, and a reverse that was dark yellow in color. On RBA, after 7 d of growth at 28 °C, colonies were 45–49 mm in diameter, displayed good sporulation, were slight greenish black mycelia with a regular margin whilst the reverse was dominated by pale yellow. After 14 d of growth at 28 °C, colonies were 81–84 mm in diameter, exhibited abundant sporulation, had black mycelia, and with a reverse dominated by yellow in colour.

Taxonomic description for FF001: *Penicillium* sp. (*dravuni*) or *simplicissimum* (*oudem*) Thom.: Colonies on PDA are thick, greenish black, reverse colourless to yellow, vegetative hyphae thin, hyaline, up to 19–20 μm, conidiophores arising mostly from the substratum, long and ascending or short and arising from vegetative hyphae or on side branches at lower levels on the ascending (Figures 2A–2C). Morphology structure and type of conidiophores branching as simple and one stage branched was conidiophores with wall roughened, variable in size, ranging from 400–700 μm or more in length and 2–3 μm wide in larger structures or very short, being less than 45.0 ×2.5 μm arising as lateral branches. *Penicilli*: usually not abundantly produced, asymmetric, strongly divaricated, rarely showing tube branches, but commonly consisting of more or less well-defined terminal clusters of 2–4 divergent *metulate* bearing verticles of phialides (appearing essentially monoverticulate) (Figures 2A–2G). *Metulate*: variable and often unsatisfactorily identifiable, 9.0–20.0×2.5–3.0 μm mostly 10.0–16.0×2.5 μm (Figures 2E, G). Phialides: mostly in clusters of 3–10, 7–10 μm long, 2.0–2.5 μm wide in the basal part tapering conidium producing tube 3.0×1.0 μm (Figures 1F and 1G). *Conidia*: Finally echinulate forming long, divergent to loosely tangled chains (Figures 2A and 2B).

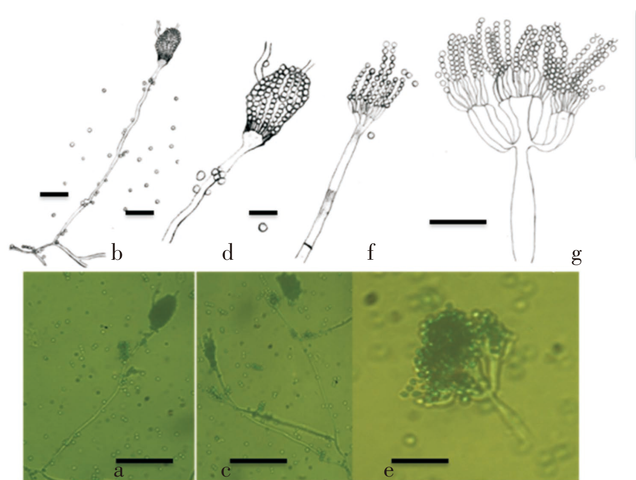

Figure 2. Morphological features of strain FF001.
A, B, C, D and F: Simple conidiophores branching; E and F: One stage branched conidiophores.

The *Penicillium* sp. was mass cultivated (5 L) in modified Wickerham-medium for 21 d at 28 °C in static conditions. After 21 d of growth in MWM, the mycelia mat were observed as good sporulation, and dominated by a green colour. The total culture medium was extracted with ethyl acetate and concentrated in vacuo to give a crude extract (2.53 g). The crude extract (active against methicillin-resistant *Staphylococcus aureus*, wild type *S. aureus*, vancomycin-resistant *Enterococcus faecium* and it displayed cytotoxicity at 180 ppm) was fractionated using different chromatography (Figure 1) and re-crystallization process yielding a major metabolite as pure crystals (compound 1; 102 mg). The pure crystals were subjected to reverse phase analytical HPLC to analysis the purity and further the pure compound 1 was submitted for NMR and other spectroscopic studies for structure elucidation (Figure 3).

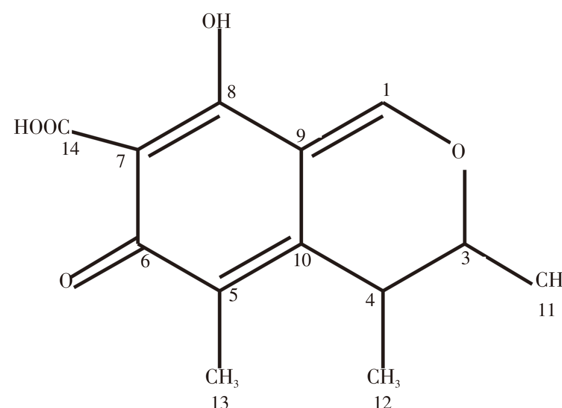

Figure 3. The structure of compound 1.

Compound 1 was isolated as lemon yellow crystals. Its molecular formula was found to be $C_{13}H_{14}O_5$ by HRESIMS [251.0936 $(M+H)^+$]. The TLC assay revealed the R_f value of 0.60 (choloroform: acetic acid; 99:1 v/v). This compound is soluble in ethyl acetate and methanol and insoluble in hexane and water. The HRESIMS also displayed the $(M+Na)^+$ ion signal at *m/z* 273.0743 establishing an error of 0.0010 amu from the calculated mass for $C_{13}H_{14}O_5Na$ (*m/z* 273.0733) (Figure 4). AntiBase®, a natural product database search of the corresponding mass revealed a match to the compound, citrinin. Further, the UV spectrum of 1 in methanol showed absorbance at λmax 213.50, 253.00 and 321.50 nm. The proton NMR spectrum (300 MHz, $CDCl_3$) has shown (Figure 5A) two methyl proton signals, one methyl protons attached to a double bond, one olefenic proton signal and two methine signals with one methine proton bearing the oxygen function. Moreover, two doublets at δ 1.22 (*J*=7.2 Hz) and δ 1.34 (*J*=6.7 Hz) are attributed to two methyl protons, two singlet signals at δ8.23 and δ 2.01 corresponds to the olefenic proton and methyl protons attached to the double bond, respectively. Furthermore, the carbon NMR spectrum (75 MHz, $CDCl_3$) showed (Figure 5B) seven quarternary carbons, one sp^2 methine carbon, two sp^3 methine carbons and three methyl carbons. These spectral data along with mass spectra and AntiBase® search clearly indicated that the data were consistent with the reported data of citrinin. Therefore, the purified compound in this study was confirmed by comparison of spectroscopic data as citrinin (1).

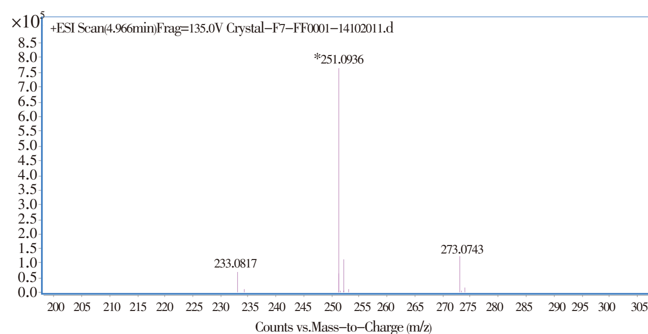

Figure 4. High resolution electron spray ionization mass spectroscopy of compound 1.

Citrinin (1) showed appreciable antibacterial activity against methicillin-resistant *S. aureus*, rifampicin-resistant *S. aureus*, wild type *S. aureus* and vancomycin-resistant *E. faecium* exhibiting MICs of 3.90, 0.97, 1.95 and 7.81 µg/mL, respectively. Further citrinin (1) displayed significant activity against the human pathogenic yeast *C. neoformans* (MIC 3.90 µg/mL) (Table 1). Moreover, citrinin (1) exhibited cytotoxicity against brine shrimp larvae LD_{50} of 96 µg/mL (Table 1).

Table 1

Biological properties of citrinin (1).

Test organism	MIC (µg/mL)
Methicillin-resistant *S. aureus*	3.90
Rifampicin-resistant *S. aureus*	0.97
Wild type *S. aureus*	1.95
Vancomycin-resistant *E. faecium*	7.81
Cryptococcus neoformans	3.90
Cytotoxicity (LD_{50})	96 µg/mL

4. Discussion

Marine-derived fungi have proven to be a rich source of structurally unique and biologically active secondary metabolites[11,12]. In the search for pharmaceutical or agrochemical lead structures, sponge-associated fungi yielding cytotoxic and other antimicrobial metabolites have received increasing attention at a rate much faster than those of other unicellular organisms[6]. In continuation of our studies on sponge-derived fungi we reported a bioactive compound from the *Penicillium* sp. FF001. Similar to our study, the conidiophores aggregation, *conidia* development and mycelia morphological characteristics were previously reported for the identification of *Penicillium* spp.[13]. Therefore, based on the classical taxonomy including micro-and macromorphological features, the isolated fungus was identified as *Penicillium* sp. Biologically active citrinin has been reported from this study based on the various spectroscopic analysis. Supportive to our results, similar spectroscopical data were previously reported for identification of citrinin[14]. Further citrinin was first isolated from the culture medium of *P. citrinum*[15]. Since then, a number of species of *Penicillium* including *P. miczynskii, P. westlingi, P. expansum, P. hirsutum, P. verrucosum, P. steckii, P. corylophilum, P. chrysogenum* and species of Aspergillus such as *A. niveus, A. terreus* and species of *Monascus rubber* and *M. purpureus* and several saprophytic fungi reported for isolation of citrinin[14,16]. The natural habitats of these citrinin producing fungi are diverse, including air, soil, rhizosphere, and water[17]. In the present study, we report the citrinin from marine environment particularly from sponge associated *Penicillium* sp.

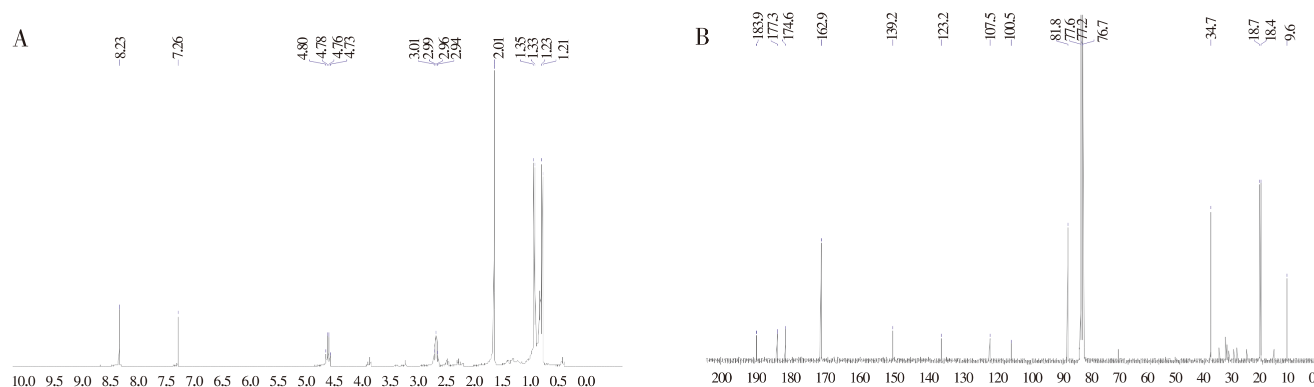

Figure 5. NMR spectra of compound 1.

A: ^{1}H NMR spectrum in $CDCl_3$ at 300 MHz; B: ^{13}C NMR spectrum in $CDCl_3$ at 75 MHz.

Citrinin has previously reported for broad spectrum antibiotic especially against Gram positive pathogens. Recently reported that citrinin was also active against Gram negative pathogens and its antifungal properties[14]. Further citrinin has also accounted to induce motility of Paenibacillus polymyxa, which is spore-forming soil bacterium, which belongs to the plant growth-promoting rhizobacteria[17]. The present study has added strength to the biological properties of citrinin as it is showed strong antibacterial activity against multi-drug resistant human pathogens.

We report herein for the isolation of citrinin from sponge associated *Penicillium* sp. from this study and described for its strong antibacterial activity against multi-drug resistant human pathogens including methicillin-resistant *S. aureus*, rifampicin-resistant *S. aureus*, wild type *S. aureus*, vancomycin-resistant *E. faecium* and *C. neoformans*. Further, it displayed cytotoxicity against brine shrimp larvae, which indicated that sponge associated *Penicillium* spp. will increase the chance to discover bioactive drug leads.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

This research was supported by the U.S. National Institutes of Health's International Cooperative Biodiversity Groups program (Grant No. NIH ICBG U01-TW007401). The authors thank the people of Cicia Island, Lau group for their hospitality and for permission to collect the sponge samples, as well as the government of Fiji for permission to perform research in their territorial waters. We are grateful to John Hooper, Queensland Museum, Australia for identification of the marine sponge. We are thankful to Kamal Subban, Indian Institute of Science, India for taxonomical identification of the fungal strain. The authors gratefully thank Paul Jensen and Ella Zafrir-Ilan for running and processing NMR spectra. We thank Brad Carte for reading of the manuscript.

Comments

Background

The authors have isolated and identified a bioactive compound from the marine fungi *Penicillium* sp. The authors have also evaluated the antibacterial, anticryptococcal, and cytotoxicity of the identified compound.

Research frontiers

Novel and sustainable source of broad-spectrum antibacterial and cytotoxic compound 'citrinin'. The results presented are based on cutting edge tools such as NMR and HRESIMS.

Related reports

The authors have followed the standard protocols and advanced techniques for the isolation and identification of the bioactive compound. Moreover, the experiments have been arranged sequentially. The materials and methods appear to be reproducible.

Innovations and breakthroughs

Novel and sustainable source of broad-spectrum antibacterial and cytotoxic compound citrinin introduced by the authors.

Applications

This report finds wide applications in medicine. Few multidrug resistant bacteria shown here are in fact susceptible to citrinin and it holds promising future for the treatment of such diseases.

Peer review

This article describes the isolation and identification of citrinin from symbiotic fungus *Penicillium* sp. associated with marine sponge. The study has been conducted in a well organized manner and presented in a convincing way. The cutting edge tools (NMR, HRLCMS *etc.*) employed in the study provides very credible data. This finding can further accelerate the search for alternative drugs in treating certain dreadful diseases.

References

[1] Wright GD, Sutherland AD. New strategies for combating multidrug-resistant bacteria. *Trends Mol Med* 2007; **13**: 260-267.

[2] Pelaez F. The historical delivery of antibiotics from microbial natural products-Can history repeat? *Biochem Pharmacol* 2006; **71**: 981-990.

[3] Proksch P, Putz A, Ortlepp S, Kjer J, Bayer M. Bioactive natural products from marine sponges and fungal endophytes. *Phytochem Rev* 2010; **9**: 475-489.

[4] Rateb ME, Ebel R. Secondary metabolites of fungi from marine habitats. *Nat Prod Rep* 2011; **28**: 290-344.

[5] Subramani R, Aalbersberg W. Marine actinomycetes: An ongoing source of novel bioactive metabolites. *Microbiol Res* 2012; **167**: 571-580.

[6] Thomas TRA, Kavlekar DP, LokaBharathi PA. Marine drugs from sponge-microbe association-a review. *Mar Drugs* 2010; **8**: 1417-1468.

[7] Wiese J, Ohlendorf B, Blümel M, Schmaljohann R, Imhoff JF. Phylogenetic identification of fungi isolated from the marine sponge *Tethya aurantium* and identification of their secondary metabolites. *Mar Drugs* 2011; **9**: 561-585.

[8] Sun LL, Shao CL, Chen JF, Guo ZY, Fu XM, Chen M, et al. New bisabolane sesquiterpenoids from a marine-derived fungus *Aspergillus* sp. isolated from the sponge *Xestospongia testudinaria*. *Bioorg Med Chem Lett* 2012; **22**: 1326-1329.

[9] Pitt JI. *A laboratory guide to common Penicillium species.* 3rd ed. Australia: Food Science; 2000, p. 197.

[10] Sam TW. *Toxicity testing using the brine shrimp: Artemia salina, bioactive natural products, Detection, isolation and structural determination*. Boca Raton: CRC Press; 1993, p. 441-456.

[11] Blunt JW, Copp BR, Hu WP, Munro MHG, Northcote PT, Prinsep MR. Marine natrual products. *Nat Prod Rep* 2009; **26**: 170-244.

[12] Blunt JW, Copp BR, Munro MHG, Northcote PT, Prinsep MR. Marine natural products. *Nat Prod Rep* 2010; **27**: 165-237.

[13] Visagie CM, Roets F, Jacobs K. A new species of *Penicillium*, *P. ramulosum* sp. nov., from the natural environment. *Mycologia* 2009; **101**: 888-895.

[14] Devi P, D'Souza L, Kamat T, Rodrigues C, Naik CG. Batch culture fermentation of *Penicillium chrysogenum* and a report on the isolation, purification, identification and antibiotic activity of ctitrinin. *Indian J Mar Sci* 2009; **38**: 38-44.

[15] Hetherington AC, Raistrick H. Studies in the biochemistry of microorganisms. XI. On the production and chemical constitution of a new yellow coloring matter, citrinin, produced from glucose by *Penicillium* citrinum THOM. *Philos Trans R Soc London Ser B* 1931; **220**: 269-297.

[16] Pitt JI. Biology and ecology of toxigenic *Penicillium* species. In: De Vries JW, Trucksess MW, Jackson LS, editors. *Mycotoxins and food safety*. New York: Kluwer Academic/ Plenum Publishers; 2002, p. 29-41.

[17] Park SY, Kim R, Ryu CM, Choi SK, Lee CH, Kim JG, et al. Citrinin, a mycotoxin from *Penicillium citrinum*, plays a role in inducing motility of *Paenibacillus polymyxa*. *FEMS Microbiol Ecol* 2008; **65**: 229-237.