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Antimicrobial compound from *Trichoderma harzianum*, an endophytic fungus associated with ginger (*Zingiber officinale*)

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ABSTRACT

Background: Genus *Trichoderma* of fungal kingdom are largely used as biological control agents due to broad-spectrum activity against plant pathogens.
Objective: This study aimed to investigate *Trichoderma harzianum*, an endophytic fungus obtained from ginger (*Zingiber officinale*) leaves.
Methods: The chemical structures of the isolated compounds were deduced on the basis of UV, ^{1}H NMR and MS data analyses, as well as comparison with literature.
Results: Two known tetramic acid derivatives were isolated from this fungus, including harzianic acid (A) and isoharzianic acid (B). Compound B inhibited the growth of a corn pathogenic fungus, *Ustilago maydis*, with inhibition zone diameter (39 ± 0.33 mm) larger than nystatin (29 mm). Additionally, iso-HA (B) revealed antibacterial effect towards *Staphylococcus aureus* with MIC value of 25 µM. However, both compounds showed no cytotoxicity against human cervical and ovarian cancer cell lines.
Conclusion: *T. harzianum* produced antimicrobial compound like iso-HA which has potential application either in agricultural or health.

Latar Belakang: *Jamur dari genus Trichoderma paling sering dimanfaatkan sebagai agen biokontrol karena bersifat antagonis terhadap patogen tanaman.*
Tujuan: *Penelitian ini bertujuan untuk mengisolasi senyawa bioaktif dari fungi endofitik Trichoderma harzianum yang berpotensi sebagai antimikroba.*
Metode: *Struktur kimia senyawa murni ditentukan dari analisis spektra UV, ^{1}H-NMR dan MS, serta dari perbandingan dengan literatur.*
Hasil: *Hasil investigasi strain jamur T. harzianum yang diisolasi dari daun jahe (Zingiber officinale) diperoleh dua senyawa turunan asam tetramat, yaitu harzianic acid/HA (A) dan isoharzianic acid/iso-HA (B). Senyawa B dapat menghambat pertumbuhan jamur Ustilago maydis dengan diameter zona hambat (39 ± 0,33 mm) lebih besar dibandingkan nistatin (29 mm). Selain itu, iso-HA (B) menunjukkan aktivitas antibakteri terhadap Staphylococcus aureus dengan kadar hambat minimum sebesar 25 µM. Namun demikian, kedua senyawa tersebut tidak menunjukkan efek sitotoksik pada sel-sel kanker leher rahim dan ovarium.*
Kesimpulan: *Spesies jamur endofit T. harzianum yang mengandung senyawa antimikroba seperti iso-HA berpotensi untuk diaplikasikan baik pada bidang pertanian ataupun kesehatan.*

INTRODUCTION

The fact that over 80% of the natural remedies available to the market originate from medicinal plants and their endophytes has raised the question whether the plant or rather the fungal endophytes are the real contributors of several bioactive metabolites producers.[1] On the other hand, approximately 25% of known medicinal plants are on the verge of extinction due to indiscriminate exploitation, including habitat destruction and overharvesting as reported by the International Union for Conservation of Nature and the World Wildlife Foundation.[2] Interestingly, some endophytic fungi are able to produce the same or similar bioactive metabolites as those known from their host plants.[3] These facts have shifted the focus of scientists from plants to fungal endophytes and triggered a recent strategy to investigate endophytes as a major source of bioactive compounds with promising pharmaceutical application.[4,5]

Medicinal plants and their endophytes are important resources for discovery of natural products. Endophytes are endosymbiotic microorganisms (fungi, bacteria, and actinomycetes) that colonize the intra- and/or intercellular tissue of plants, from which they can be readily isolated and cultivated on commonly used microbial or plant growth media.[6] Ginger rhizome which contains gingerol and paradol is traditionally used as antiemetic and in the treatment of gastritis, influenza, malaria, cholera, as well as anorexia.[7] Essential oil and oleoresin in ginger containing mono- and sesquiterpenoids, also phenolic compounds, and its derivatives provide a broad spectrum of antimicrobial activity.[8]

In the course of our screening for secondary metabolites for anti-infective agents from plants and associated endophytes, we investigated an endophytic fungus *Trichoderma harzianum* isolated from leaves of *Zingiber officinale* (Zingiberaceae). Reportedly, this fungus has been broadly applied for controlling diseases caused by fungal pathogens in Zingiberaceae crops.[9] Recently, we reported a novel epidithiodiketopiperazine and one bioactive compound isolated from the same endophytic fungus.[10] Interestingly, nine new metabolites belonging to indole diterpenoids along with one cytotoxic compound obtained from a fungal endophyte associated with roots of ginger collected from the same biological source.[11] In this study we report two fungal metabolites belonging to tetramic acid derivatives and bioactivity tests against a panel of human pathogenic bacteria, plant pathogenic fungi, and certain tumor cell lines.

METHODS

Instrumentation

High Performance Liquid Chromatography (HPLC) analysis by a Dionex p580 DAD3000RS (Dionex Softron, Munich, Germany) with an LPG-3400SD pump coupled with a photodiode array detector (UVD340S), using routine detection channels at 235, 254, 280, and 340 nm wavelengths. Semi-preparative RP-HPLC was performed using a Merck Hitachi system (Eurosphere-100C18, 300×8 mm, pump L-7100; UV detector L-7400; Merck KGaA, Darmstadt, Germany). ^{1}H NMR spectra were measured on Bruker Avance III 300 MHz NMR spectrometer (Bruker BioSpin, Rheinstetten, Germany). Low and high resolutions mass spectra were recorded on HP110 Agilent Finnigan LCQ Deca XP Thermoquest mass spectrometer and a UHR-TOF maxis 4G mass spectrometer (Thermo Fisher Scientific, Bremen, Germany), respectively. Specific optical rotation (SOR) was performed by a Jasco P-2000 digital polarimeter (Jasco International, Tokyo, Japan).

Materials

The fungus *T. harzianum* was isolated from healthy and fresh leaves of ginger (*Z. officinale*), collected in May 2016 at Kalibagor Banyumas regency, Central Java, Indonesia. Eight strains of bacteria were used in bioassay, including three Gram-positive bacteria (*Staphylococcus aureus*, *Enterococcus faecium*, and *Mycobacterium tuberculosis*) and five Gram-negative bacteria (*Acinetobacter baumannii*, *Enterobacter aerogenes*, *Escherichia coli*,

Klebsiella pneumoniae, and *Pseudomonas aeruginosa*). Moreover, *Ustilago maydis* AB33 and *Saccharomyces cerevisiae* ESM356-1 were subjected in antifungal assay. Cytotoxicity assay employing the cervical cancer HeLa and human ovarian cell lines A2780 cisplatin-sensitive.

Fungal isolation and identification

Isolation of fungus was achieved by the dilution plate method employing isolation medium (15 g/L bacto agar, 15 g/L malt extract in distilled water, at pH 7.4–7.8) supplied by streptomycin sulfate (0.25 g/L) and chloramphenicol (0.20 g/L) in order to inhibit growth of actinomycetes and bacteria. Fungal identification was performed according to a molecular biology protocol by deoxyribonucleic acid (DNA) amplification and sequencing of the internal transcribed spacer (ITS) region, followed by nucleotide blast (BlastN) search in the NCBI database resulting in the GenBank accession number MK213940.[10] Voucher strain was deposited at the research group leaders' laboratory (P.P.).

Fermentation, extraction, and isolation

The fungal strain was cultured on solid rice medium, which was prepared by autoclaving 100 g of rice and 110 mL of water in a 1 L Erlenmeyer flask. Large scale fermentation was subjected in five flasks each fungus for 14 days at room temperature under static condition. The fungal cultures were diced and extracted with ethyl ethanoate/ EtOAc (5 L). The crude extract of *T. harzianum* (11.34 g) was partitioned by liquid-liquid extraction using 90% methanol-H_2O (MeOH-H_2O) and n-hexane to yield MeOH fraction (4.40 g), then subjected to vacuum liquid chromatography (VLC) using a step gradient of n-hexane/EtOAc, followed by dichloromethane or DCM/MeOH, to yield some fractions. Fraction 4 (V4, 296 mg), eluted with n-hexane/EtOAc (40/60), were subjected to a size exclusion chromatography using a Sephadex LH-20 column (100×2.5 cm) with MeOH as eluting solvent, followed by monitoring the eluted subfractions employing thin liquid chromatography (TLC). This subfraction (HTH-V4-SD2) was selected for further purification via semi-preparative RP-HPLC using gradient elution of water and methanol with 5 mL/min flow rate yielding the compounds A (19.24 mg) and B (16.98 mg).

Biological activity evaluation

The antibacterial assay was performed using the broth microdilution method following the recommendations of the Clinical and Laboratory Standards Institute (CLSI) 2012 as described in the previous study.[10] Meanwhile, the antifungal assay was carried out using the agar diffusion (Kirby-Bauer) method adopted from Harwoko et al. (2021).[10] Cytotoxicity screening was evaluated using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described.[10] Moxifloxacin, nystatin and nourseothricin (clonNAT), as well as cisplatin were used as positive controls in the respective bioassays.

RESULTS

The fungal strain *T. harzianum* MK213940 isolated from leaves of *Z. officinale* was fermented on solid rice medium to yield two known compounds (A and B) during chromatographic analysis. During preliminary analysis of the crude EtOAc extract by HPLC with diode-array detection (HPLC-DAD), a series of peaks with ultraviolet (UV) absorption spectra at 360 and 363 nm were detected. These matched the characteristics of harzianic acid in the *in-house* UV spectral database. Chromatographic separation of the fungal extract led to the isolation of tetramic acid derivatives. The chemical structures (Fig. 1) were determined on the basis of proton nuclear magnetic resonance (^{1}H NMR) and high resolution electrospray ionization mass spectrometry (HRESIMS) data analysis, as well as in comparison with literature.

Compound A was a yellowish gel and assigned as $C_{19}H_{27}NO_6$ for its molecular formula, indicating 6 degrees of unsaturation. The SOR value $[\alpha]^{20}D$ of A was +14.5 in MeOH. The ^{1}H NMR data of A (Table 1) was identical to that of harzianic acid,

a known fungal metabolite found in the same strain *T. harzianum* firstly obtained from water and later found in the composted hardwood bark.[12,13]

Compound B was obtained as yellow gel, but its SOR value $[\alpha]^{20}D$ was +140.9 in MeOH. The HRESIMS data exhibited a protonated molecular ion peak at *m/z* 366.1915, corresponding to $C_{19}H_{27}NO_6$ (6 degrees of unsaturation). The ^1H NMR data of B (Table 1) was identical to isoharzianic acid, a secondary metabolite biosynthesized by the same fungal strain *T. harzianum* isolated from composted hardwood bark.[14]

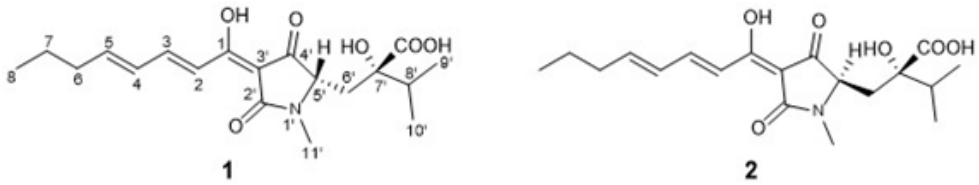

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Figure 1. Structures of (5'*S*,7'*S*)-harzianic acid (1, compound A) and (5'*R*,7'*S*)-isoharzianic acid (2, compound B)

Table 1. ^1H NMR spectral data of the isolated compounds [in CD_3OD].

Position	Harzianic acid (A)	Isoharzianic acid (B)
	δ_H (mult., J in Hz) ppm	δ_H (mult., J in Hz) ppm
2	7.08 (d, 15.4)	7.09 (d, 15.4)
3	7.53 (m)	7.44 (dd, 15.4, 9.3)
4	6.39 (m)	6.36 (m)
5	6.39 (m)	6.36 (m)
6	2.03 (m)*	2.22 (m)*
7	1.50 (m)	1.50 (m)
8	0.95 (m)	0.95 (m)
5‘	3.82 (dd, 9.0, 2.5)	3.71 (m)
6‘a	2.24 (m)	2.22 (m)*
6‘b	2.34 (dd, 14.8, 2.6)	2.43 (dd, 14.8, 2.8)
8‘	2.00 (m)*	1.90 (m)
9‘	0.95 (m)	0.95 (m)
10‘	0.95 (m)	0.87 (m)
11‘	2.94 (s)	2.92 (s)

*Overlapping signals; mult: multiplicity, J: coupling constant, s: singlet, d: doublet, dd: doublet of doublets, m: multiplet

The isolated compounds were assessed for their antibacterial, antifungal, and cytotoxic properties as presented in Fig. 2 and Table 2. Noteworthy, compound B displayed inhibitory effect against *U. maydis*, showing zone of inhibition of 39 mm at 100 µg/disk. Meanwhile, nystatin 10.8 mM and clonNat (nourseothricin) 39.8 mM possessed inhibition diameters of 29 and 14 mm, respectively (Fig. 2). Furthermore, isoharzianic acid (B) showed antibacterial potential towards *S. aureus* with MIC value of 25 µM (Table 2). However, none of the isolated compounds was toxic against A2780 sens cancer cell lines up to concentration of 100 µM.

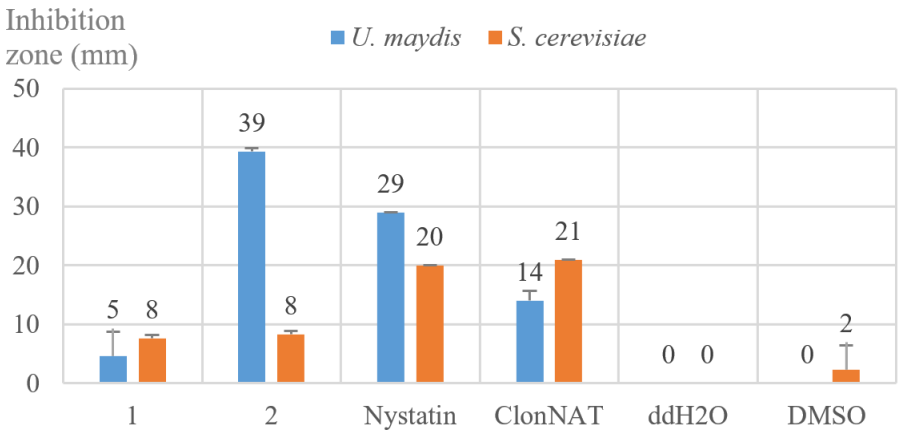

Figure 2. Growth inhibition of compounds A and B against plant pathogenic fungi in comparison with positive and negative controls

Table 2. Biological activity of the isolated compounds.

Compound	IC_{50} (µM)		MIC (µM)
	HeLa	A2780 sens	*S. aureus*
Harzianic acid (A)	n.t.	> 100	> 50
Isoharzianic acid (B)	> 100	> 100	25
Cisplatin[a]	-	1.0	-
Moxifloxacin[b]	-	-	3.9

*n.t.: not tested; a–b: positive controls in cytotoxicity (a) and antibacterial (b) assays.

DISCUSSION

The spectroscopic data including UV, MS and H-NMR of compounds A and B possessed high similarities, as well as similar to earlier reports, suggesting both shared the same planar structure.[12–14] However, the absolute configuration of A differed from B as they have two chiral centers at positions of C-5' and C-7'. Reportedly, the stereochemistry of the same compounds was established by X-ray crystallography and total synthesis.[13,15] The SOR value of A ($[\alpha]^{20}D$ +14.5) was found to be identical with harzianic acid ($[\alpha]^{20}D$ +19.6).[12] While, the SOR value of B ($[\alpha]^{20}D$ +140.9) was similar to a synthesized isoharzianic acid ($[\alpha]^{20}D$ +118.0).[15] Comparison of the SOR values with the related compounds, along with biogenetic considerations, confirmed that the stereochemistry of A was 5'*S*,7'*S* and of B was 5'*R*,7'*S*.(15) Thus, the isolated compounds were identified as (*S*,*S*)-harzianic acid (A) and (*R*,*S*)-isoharzianic acid (B) belonging to tetramic acid derivatives, of which both are diastereoisomer. This class of secondary metabolite was biosynthetically assembled from an amino acid and an activated acyl entity via polyketide synthase pathway mixed with non-ribosomal peptide synthetase.[16]

Trichoderma-based products containing only living organisms are marketed worldwide as biological control agents against various agricultural pathogens.[17] For example in Indonesia, *T. harzianum* had been successfully applied for controlling *Fusarium* wilt disease in ginger and aromatic ginger.[18,19] The potent antifungal properties of both isolated compounds have been previously described in the literature, supporting their potential for biopesticide and biofertilizer.[13,14]

A single compound from this fungus like isoharzianic acid (B) was rarely reported so far to inhibit the growth of *U. maydis*, a model phytopathogenic fungus, which can infect corn and causes crop yield losses and stunted growth.[20] Moreover, compound B revealed moderate antibacterial effect towards *S. aureus*. Similarly, three of six unidentified endophytic fungi isolated

from ginger rhizome exhibited antibacterial activity towards *S. aureus* dan *E. coli*.[21] In addition, mangrove-derived fungus *T. koningiopsis* SaKB1 containing terpenoids possessed the same antibacterial potential.[22] In contrast, harzianic acid (A) was inactive against the tested microbes. Even though applications of *Trichoderma* strains and their bioactive metabolites including harzianic acid reported to improve productivity and nutritional content of soybean.[23] Regardless, cytotoxic properties of the isolated compounds were scarcely found in our literature search.

In summary, isoharzianic acid (B) has the potential to be developed as biofungicide in agricultural or as antibiotic in medical application. Further studies are needed to diversify the fungal secondary metabolites as exemplified by implementing advance strategies such as One Strain Many Compounds (OSMAC) and co-cultivation methods, or genetic manipulation of biosynthetic and regulatory genes.[24] Currently, bioinformatic approaches for fungal omics are also possible to be performed in screening of promising target compounds.[25]

CONCLUSION

Chemical investigation of a fungal endophyte *T. harzianum* MK213940 associated with ginger leaves resulted in the isolation of two tetramic acid derivatives, namely harzianic acid (A) and its stereoisomer isoharzianic acid (B). To the best of our knowledge, antimicrobial activity of compound B against a Gram-positive bacterium *S. aureus* (MIC 25 μM) and a corn smut fungus *U. maydis* (MIC < 27 mM) was firstly reported in this study. However, both compounds were not significantly cytotoxic to human cervical cancer (HeLa) and ovarian cancer (A2780 sens) cell lines. Hence, this fungal strain produces antimicrobial compound with potential agricultural and pharmaceutical applications.

CONFLICT OF INTEREST

There is no conflict of interest.

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