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Two new antileishmanial diketopiperazine alkaloids from the endophytic fungus *Trichosporum* sp.

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ABSTRACT

*The fungus Trichosporum sp. is an endophytic fungus isolated from seeds of Trigonella foenum-graecum (Fabaceae). The endophytic fungus Trichosporum sp. has been subjected to chemical and biological investigations resulted in the isolation of two new diketopiperazine alkaloid isomers identified chemically as; (6-S)-3-(1,3-dihydroxypropyl)-6-(2-methylpropyl)piperazine-2,5-dione and (6-R)-3-(1,3-dihydroxypropyl)-6-(2-methylpropyl)piperazine-2,5-dione (**1** and **2**). Three chiral centers in both of **1** and **2** were not identified due to lack of the isolated amounts. The isolated compounds were identified using different 1D and 2D NMR as well as IR and HR-MS techniques. The isolated compounds were examined for in vitro antileishmanial, antifungal, antibacterial and antimalarial activities. Compound **1** and **2** showed antileishmanial activities with IC_{50} values of 96.3 and 82.5 µg /ml, respectively.*

Keywords: *Trichosporum* sp.; Diketopiperazine Alkaloid; Antileishmanial

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INTRODUCTION

The search for new and biologically active secondary metabolites is a contentious process. The endophytic fungi can be considered as a major source for chemically novel as well as biologically active compounds [1]. Endophytic fungi are the fungi which grow within their plant host (intercellular or intracellular) without causing apparent disease symptoms[2].

The endophytes have a great potential to protect their host plant from biotic stress factors such as herbivores and infections through the production of various secondary metabolites [3, 4], also it may protect the host plant from a biotic dangers by enhancing nutrient uptake[5], increasing host tolerance to heat [6, 7] and salinity [8].

The isolated secondary metabolites from endophytic fungi were found to be belong to several chemical classes including; alkaloids[9], steroids[10],flavonoids[11], α- pyrones[12], terpenoids[13], pyranones[14], quinones[15], isochromenes[16] and benzopyran derivatives[17].

Diketopiperazines are a widely spread class of bioactive secondary metabolites that has been isolated from different fungal sources such as; *Aspergillus fumigatus*[18], *Simplicillium* sp. [19]*Chromocleista* sp.[20] and *Paecilomyces variotii*[21]as well as from several marine organisms [22-24].

Diketopiperazine alkaloids may hold a great promise for the future due to its promising biological activities such as; antitumor[25, 26], antiviral[27], antifungal[28, 29], antibacterial[26] and antihyperglycemic [30].

We herein report on the isolation, structural elucidation and biological evaluation of two new diketopiperazine alkaloid isomers (**1, 2**) from the endophytic fungus *Trichosporum* sp. Absolute configuration for **1** and **2** couldn't identified due to lack of the isolated quantity.

MATERIALS AND METHODS

2.1. General Experimental Procedures: Optical rotations were measured using a Rudolph Research Analytical Autopol IV automatic polarimeter model 589-546. IR spectra were recorded on a Bruker Tensor 27 instrument. NMR spectra were recorded on a Varian Mercury 400 MHz spectrometer at 400 (^{1}H) and 100 MHz (^{13}C). The HR-ESI-MS spectra were measured using a Bruker Bioapex-FTMS with electrospray ionization (ESI). Column chromatographic separation was performed on silica gel 60 (0.04-0.063 mm) and sephadex LH-20 (0.25-0.1 mm, Merck). TLC was performed on precoated TLC plates with silica gel 60 F254 (0.2 mm, Merck). Semi preparative HPLC (Waters Delta Prep 4000) was performed using Luna® RP-18 (250,10mm x5 µm; flow rate 5 mL/min).

2.2. Fungal material: The endophytic fungus *Trichosporum* sp. was isolated from healthy seeds of *Trigonella foenum-graecum* (Fabaceae). The seeds were rinsed with water followed by surface sterilization in 70% EtOH for 1 min. The sterilized seeds were rinsed again with sterilized water, cut into small pieces and deposited on a petri dish containing Potato Dextrose Agar (PDA) medium (200 g potato, 20 g glucose, and 15 g agar in 1 L distilled water, supplemented with 100 mg/ L chloramphenicol) and cultivated at 28°C for 3 days. Hypha tips were observed and transferred on new PDA plates and subcultured until a pure culture was obtained. The fungus was identified at the Regional Center for Mycology and Biotechnology, Cairo, Egypt (RCMB). After purification the fungus was grown on PDA at 28 °C for 5 days. Ten pieces (0.5×0.5 cm^2) of mycelial agar plugs were inoculated into ten 1000 mL Erlenmeyer flasks containing 250 ml liquid fermentation media Potato Dextrose Broth (PDB) (200 g potato and 20 g glucose in 1 L distilled water). This was further fermented for the next 30 days at room temperature.

2.3. Extraction and Isolation:Supernatant was separated from biomass via filtration through sieving paper and then extracted by adding 1 L EtOAc to each flask twice and separated using a separating funnel. The EtOAc extracts were collected, filtrated, concentrated under vacuum and partitioned with distilled water. The ethyl acetate portion was evaporated to dryness and partitioned with hexane and 90% MeOH to afford the hexane fraction (10.1 g) and MeOH fraction (11.3 g). The water portion was fractionated against n-butanol to afford the water fraction (18.6 g) and the butanol fraction (7.2 g). The *Trichosporum* sp. butanol fraction (7.2 g) was subjected to Si gel VLC eluted with chloroform and MeOH in a manner of increasing polarity. Six fractions were collected (500 mL each). Fraction 5 (255 mg) was chromatographed with a sephadex LH-20 column eluted with MeOH to yield three groups (1-3). Group 2 was chromatographed on a semi-preparative HPLC with linear gradient elution 20–85% aqueous methanol to obtain compounds **1** (2 mg) and **2** (1.8 mg).

2.4. 3-(1,3-dihydroxypropyl)-6-(2-methylpropyl)piperazine-2,5-dione (**1**): white solid powder; $[\alpha]^{20}_{D}$ -17.9 (*c* 0.1, in MeOH); IR ν_{max} 1665, 1680, 3220, 3310, 3360, 3400 cm^{-1}; ^{1}H NMR (400 MHz, D_2O) and ^{13}C NMR (100 MHz, D_2O) see Table 1.

2.5. 3-(1,3-dihydroxypropyl)-6-(2-methylpropyl)piperazine-2,5-dione (**2**): white solid powder; $[\alpha]^{20}_{D}$ +37.6 (*c* 0.1, in MeOH); IR ν_{max} 1668, 1675, 3225, 3315, 3360, 3400 cm^{-1}; ^{1}H NMR (400 MHz, D_2O) and ^{13}C NMR (100 MHz, D_2O) see Table 1.

2.6. Antimalarial assay: Crude extracts, fractions and isolated compounds were tested on chloroquine sensitive (D6, Sierraleon) and resistant (W2, Indo-china) strains of *Plasmodium falciparum* using previously reported method; Artemisinin and chloroquine have been used as positive controls[31].

2.7. Antileishmanial assay: The anti-leishmanial screen tests samples for their ability to inhibit *Leishmania donovani*, a fly-borne protozoan that causes visceral leishmaniasis.

2.7.1. Primary Screen; at 80µg/mL in duplicate and percent inhibitions (% inh.) are calculated relative to controls. Extracts showing ≥50% inhibition proceed to the secondary assay.

2.7.2. Secondary Assay; in the secondary LEM assay, all samples (2 and 20mg/mL) are tested at 40, 8.0 and 1.6µg/mL and IC_{50}s as well as IC_{90}s (test concentration that affords 90% inhibition of the protozoan relative to controls) are reported. Samples that have an IC_{50} of <1.6 µg/mL in the secondary LEM assay proceed to the tertiary assay.

2.7.3. Tertiary Assay; where the sample is tested at 40, 8, 1.6, 0.32, 0.064, 0.0128µg/mL and IC_{50}s and IC_{90}s are reported. All IC_{50}s and IC_{90}s are calculated using the XLFit fit curve fitting software. The drug controls pentamidine and amphotericin B are used as positive controls.[32].

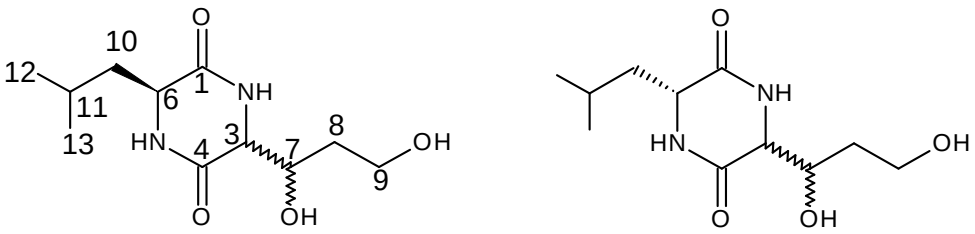
2.8. Antimicrobial assay: The antimicrobial screen tests samples for their ability to inhibit a panel of 5 bacteria and 5 fungi those are pathogenic to humans including; *Staphylococcus aureus,* Methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli, Pseudomonas aeruginosa, Mycobacterium intracellulare, Candida albicans, Candida glabrata, Candida krusei, Aspergillus fumigates and Cryptococcus neoformans.*

The antimicrobial assay has been proceeded using previously reported method [31, 33]

RESULTS AND DISCUSSION

In this study, chemical and biological investigations of the endophytic fungus *Trichosporum* sp. were conducted. The fungus was cultivated on liquid fermentation media (PDB). The crude ethyl acetate extract of the culture was subjected to different chromatographic techniques, which led to the isolation and structural elucidation of two new (**1**, **2**) diketopiperazine alkaloids. We herein report on the isolation, structural elucidation and biological evaluation of those compounds.

Figure 1: *Trichosporum* sp.



1, 2

Figure 2: chemical structure for 1 and 2

Compound **1** was isolated as a white amorphous powder. The molecular formula was determined to be $C_{11}H_{20}N_2O_4$ by HR-ESI-MS (*-ve* mode) showing molecular ion peak $[M-H]^-$ at *m/z* 243.1339 (calcd. for $C_{11}H_{19}N_2O_4$, 243.1350) indicating three degrees of unsaturation. The IR spectrum of **1** showed absorption bands at 1665, 1680, 3220, 3310, 3360 and 3400 cm^{-1} indicating the presence of two carbonyl groups, two amides and two alcoholic hydroxyls. The ^{13}C NMR spectrum (Table 1) of compound **1** displayed 11 carbon resonances, while the DEPT 135 experiment differentiated these signals into two methyl groups at (δC 20.7 and 21.1), three methylenes at (δC 35.9, 53.6 and 37.3), four methines, two of them attached to nitrogen atoms and resonating at (δC 57.1 and 53.2), with a signal of an oxygenated methine at (δC 67.7), while the last methine resonating at (δC 23.8). The ^{13}C NMR spectrum showed also two quaternary ester carbonyl signals resonating at (δC 168.4 and 171.2). By analysis of ^{1}H NMR (Table 1) and HMQC spectral data, compound **1** exhibited two signals for methyl groups [δ 0.85 (6H, m, H-12 and H-13)], a signal of oxygenated methie at δ 4.30 (m, H-7), three methylene group signals, the first one resonating at [δ 2.04 (m, 1H), and 2.20 (m, 1H), H-8)], the second one resonating at [δ 3.37 (m, 1H), and 3.59 (m, 1H), H-9)], while the third one resonating at [δ 1.37 (m, 1H), and 1.69 (m, 1H), H-10)]. The diketopiperazine structure was confirmed by HMBC correlations from H-3 to C-1 and from H-6 to C-4. HMBC spectrum confirmed the presence of a 1, 3 dihydroxy propyl side chain attached to C-3 by showing several correlations between H-7 to C-4 and H-9 to C-7. The attachment of propyl side chain at C-3 was confirmed from HMBC correlations of H-8 to C-3 and C-9. HMBC spectrum confirmed the presence of a 2-isobutyl side chain attached to C-6 by showing several

correlations between H-12 to C-10 and C-13. The attachment of the 2-isobutyl side chain attached to C-6 was confirmed by HMBC correlations between H-10 to C-1 and H-6 to C-11. The structure of **1** was authenticated by ^{2}D NMR experiments, giving pertinent COSY and HMBC correlations Fig. 3. Compound **1** was identified to be 3-(1,3-dihydroxypropyl)-6-(2-methylpropyl)piperazine-2,5-dione.

There are three chiral centers at C-3, C-6 and C-7 couldn't be identified due to shortage of the isolated amounts.

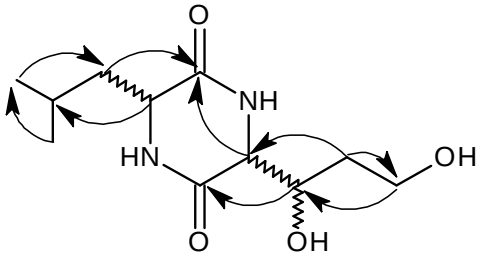


Figure 3: Key HMBC correlations for 1

Compound **2** was isolated as a white amorphous powder. The molecular formula was determined to be $C_{11}H_{20}N_2O_4$ by HR-ESI-MS (*-ve* mode) showing molecular ion peak $[M-H]^-$ at *m/z* 243.1342 (calcd. for $C_{11}H_{19}N_2O_4$, 243.1350) indicating three degrees of unsaturation. The IR spectrum of **2** showed absorption bands at 1668, 1675, 3225, 3315, 3360 and 3400 cm^{-1} indicating the presence of two carbonyl groups, two amides and two alcoholic hydroxyls. The ^{13}C NMR spectrum (Table 1) of compound **2** displayed 11 carbon resonances, while the DEPT 135 experiment differentiated these signals into two methyl groups at (δC 20.4 and 22.1), three methylenes at (δC 35.2, 52.4 and 41.1), four methines, two of them attached to nitrogen atoms and resonating at (δC 56.0 and 55.3), with a signal of an oxygenated methine at (δC 67.4), while the last methine resonating at (δC 23.9). The ^{13}C NMR spectrum showed also two quaternary ester carbonyl signals resonating at (δC 168.0 and 170.0). The ^{1}H, ^{13}C NMR (Table 1) , HMQC and HMBC spectral data of compound **2**and those for **1** were found to be similar, with a slight difference observed inthe chemical shift of carbons C-6 and C-10 it found to be resonating at (δC 55.3 and 41.1) for **2** instead of (δC 53.2 and 37.3) for **1**, respectively. The same slight difference was noticed in both of H-6 and H-10, it was resonating at *δ* 3.94.30 (m, H-6), [*δ* 1.53 (m, 1H), and 1.62 (m, 1H), H-10)] for 2instead of *δ* 4.15 (m, H-6), [*δ* 1.37 (m, 1H), and 1.69 (m, 1H), H-10)] for **1**, respectively.

These differences suggest that **1**and **2** are isomers to each other and they differ only in one chiral center at C-6.

The structure of **2** was authenticated by ^{2}D NMR experiments, giving pertinent COSY and HMBC correlations. Compound **2** was identified to be 3-(1,3-dihydroxypropyl)-6-(2-methylpropyl)piperazine-2,5-dione.

There are three chiral centers at C-3, C-6 and C-7 couldn't be identified due to shortage of the isolated amounts.

Compounds (**1** and **2**) were examined for *in vitro* antileishmanial, antifungal, antibacterial and antimalarial activities. Compound **1** and **2** showed antileishmanial activities with IC_{50} values of 96.3 and 82.5 µg /ml, respectively.

Table 1: ^{1}H NMR and ^{13}C spectroscopic data (400 MHz in D_2O) for (1and2)

Position	1			2		
	δ_C	δ_H (J in Hz)	HMBC	δ_C	δ_H (J in Hz)	HMBC
1	168.4	-		168.7	-	
3	57.1	4.43, m	168.4, 171.2	56.5	4.40, m	
4	171.2	-		170.4	-	
6	53.2	4.15, m	23.8, 37.3, 171.2	55.3	3.94, m	
7	67.7	4.30, m	171.2	67.4	4.49, m	
8	35.9	8a;2.04, m 8b;2.20, m	53.6, 57.1, 67.7	35.2	8a; 2.12, m 8a; 2.14, m	67.4
9	53.6	9a; 3.37, m 9b; 3.59, m	35.9, 67.7	52.4	9a; 3.44, m 9b; 3.61, m	67.4
10	37.3	10a; 1.37, m 10b; 1.69, m	168.4	41.1	10a; 1.53, m 10b; 1.62, m	20.4, 23.9, 41.1
11	23.8	1.69, m	168.4	23.9	1.64, m	
12	20.7	0.85, m	21.1, 37.3	20.4	0.86, m	22.1, 41.4
13	21.1	0.85, m	20.7	22.1	0.86, m	20.4

CONCLUSION

Two new diketopiperazine alkaloid isomers have been isolated from The endophytic fungus *Trichosporum* sp. and chemically identified chemically as; (6-*S*)-3-(1,3-dihydroxypropyl)-6-(2-methylpropyl)piperazine-2,5-dione and (6-*R*)-3-(1,3-dihydroxypropyl)-6-(2-methylpropyl)piperazine-2,5-dione (**1** and **2**). The isolated compounds were identified using different 1D and 2D NMR as well as IR and HR-MS techniques. The isolated compounds were examined for *in vitro* antileishmanial, antifungal, antibacterial and antimalarial activities. Compound **1** and **2** showed antileishmanial activities against *Leishmania donovani*, a fly-borne protozoan that causes visceral leishmaniasis with IC_{50} values of 96.3 and 82.5 µg /ml, respectively

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