Anti-inflammatory and anti-diabetic naphthoquinones from an endophytic fungus
Dendryphion nanum (Nees) S. Hughes

Piramal Enterprises Limited, 1-Nirlon Complex, Goregaon (E), Mumbai 400 063, India
E-mail: sunil.deshmukh@piramal.com

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Chemical investigation of the culture filtrate of an endophytic fungus Dendryphion nanum (Nees) S. Hughes, isolated from the leaves of Ficus religiosa collected from Goregaon, Mumbai, has resulted in the isolation of two naphthoquinones. These compounds have been evaluated for anti-inflammatory and anti-diabetic activities. Compound 1 showed very good anti-inflammatory and anti-diabetic activities, but compound 2 did not show the above said activities. This is the first report of the naphthoquinone compounds isolated from endophytic fungus D. nanum, evaluated for anti-inflammatory and anti-diabetic activities.

Keywords: Dendryphion nanum, Ficus religiosa, Endophyte, naphthoquinones, anti-diabetic, anti-inflammatory

Fungal biodiversity exploration for novel bioactive molecules has been a topic of intense research in recent years. Several success stories have been scripted by the discovery of valuable therapeutically active compounds from relatively less investigated fungal groups, which have shown great promise as anti-fungal agents like caspofungin, micafungin and fungal groups, which have shown great promise as active compounds from relatively less investigated scripted by the discovery of valuable therapeutically active compounds from relatively less investigated.

Results and Discussion

Bioactivity guided isolation of active compound from crude extract yielded two compounds (Figure 1a). Compound 1 was obtained as yellow crystals with molecular weight 327.0842 [M+Na]+ and molecular formula C_{16}H_{13}O_{6} from HRMS. By 1H and 13C NMR this compound was identified as naphthoquinone antibiotic herbarin, which was further confirmed with literature values8.

Compound 2 was obtained as white crystals with molecular formula C_{16}H_{13}O_{6} from HRMS corresponding to mass 329.0997 [M+Na]+. The 1H and 13C NMR of this compound showed close similarities to compound 1.

Characteristic signals were obtained for naphthoquinone skeleton with two-methoxy group at δ_H 3.8 and 3.9 attached to aromatic ring. One methyl group at δ_H 1.6 attached to quaternary carbon (C-3) showed downfield shift at δ_C 95.3. 1H-1H COSY correlations supported the presence of meta coupling between signals at δ_H 6.9 (H-8) and δ_H 7.2 (H-6). Compound 2 showed close similarities to herbarin with exception of signals of two ABX spin system connected to each other CH₂ (H-1), CH (H-10a), CH (H-4a) and CH₂ (H-4). First ABX spin system showed three signals at δ_H 4.2 (1H, d, H-1a), 4.1 (1H, d, H-1b) and 2.9 (1H, ddd, J = 5.7, 9.5, 13.7 Hz, H-10a).

Second ABX system showed signal at δ_H 1.7 (1H, dt, J = 4.5, 12.6 Hz, H-4a), 2.4 (1H, dd, J = 3.9, 13.0 Hz, H-4b) and 3.4 (1H, ddd, J = 3.6, 10.7, 14.2 Hz, H-4a).

13C NMR of compound 2 showed sixteen carbons, DEPT experiment sorted seven quaternary carbons attributed to four aromatic carbons at δ_C 138.8 (C-5a), 118.2 (C-9a), 161.2 (C-9) and 164.4 (C-7), two carbons from carbonyl group at δ_C 197.1 and 193.8 and one at δ_C 95.3 (C-3). Four tertiary carbons were assigned to δ_C 102.0 (C-6), 104.8 (C-8), δ_C 45.1 (C-4a) and 48.7 (C-10a). Two secondary carbons showed
peaks at δC 60.1 (C-1) and 34.6 (C-4) and remaining three primary carbons showed peaks similar to compound 1. Key HMBC correlations are depicted in Figure 1b. After extensive 1D and 2D NMR analysis and literature search, compound 2 was identified as a herbaridine A9-12.

Compound 1 showed potent inhibition of cytokine production in the LPS induced human mononuclear cell line (THP-1). The production of both cytokines TNF-α and IL-6 was blocked with an IC50 of 0.60±0.100 µM and 0.06±0.009 µM respectively. Compound 2 was found to be inactive against TNF-α and IL-6 inhibitory activity. Dexamethasone, a widely used anti-inflammatory agent with multi-targeted effects, was used as a standard in the assay. Dexamethasone blocked cytokine production with IC50 of 0.06±0.007 µM and 0.01±0.001 µM for TNF-α and IL-6, respectively, indicating that the assay conditions were appropriate.

Compound 1 induced glucose uptake in rat skeletal muscles in the presence of insulin with an EC50 of 0.80±0.090 µM. Rosiglitazone, a known glucose uptake activator (EC50 = 3.0±0.040 µM) was used as a standard in the assay. Compound 2 was found to be inactive. The ability of compound 1 to induce glucose uptake at such low concentration indicated that it could therefore, be a good scaffold as a starting point for drug development.

** Experimental Section **

TLC was performed on Si-gel 60 F254 (0.20 mm thickness) plates (Merck, Germany). Naphthoquinones were separated using Waters HPLC system (Waters 600, USA) employing normal phase preparative column (Eurospher 100-10 Si, 250×20 mm), with the detection on PDA detector (Waters 2996, USA). 1H and 13C NMR spectra were recorded in CDCl3 on Bruker Avance 75 MHz FT-NMR spectrometer with TMS as an internal standard. Melting points were determined by using visual melting range apparatus (Labindia MR-IVS). High resolution MS of the compounds were determined by using Micro TOFQ (Bruker Daltonics).

**Isolation and Identification of the Fungus**

Culture PM0752236 was isolated from the leaves of the plant *F. religiosa*, (family Moraceae) which was collected from Nirlon Complex, Goregaon, Mumbai, (Maharashtra), India in the month of February 2007, by the method as described13 using Potato Dextrose Agar (PDA) medium and maintained for identification and fermentation. The fungus was identified based on partial sequence analysis of the internal transcribed spacer region (ITS) of r-DNA using ITS-1 and ITS-4 primers14. A nucleotide to nucleotide BLAST15 query of the gene bank database (http://www.ncbi.nlm.nih.gov/BLAST) recovered AY387657, D. nanum as the closest match to the ITS r-DNA of PM0752236 (93%).

**Large-Scale Production of the Fungus**

A loop full of the well grown culture from slant maintained on potato dextrose agar (PDA) was transferred to a 500 mL conical flask with 100 mL liquid medium containing 1.5 g soluble starch, 1.5 g soya bean meal, 0.2 g yeast extract, 0.1 g corn steep liquor, 0.5 g glucose, 0.2 g CaCO3, 0.5 g NaCl and 1.0 g glycerol in demineralized water at pH 5.5. This was grown on rotary shaker at 220 rpm for 72 hr at 28°C and was used as seed medium. Potato dextrose broth medium (Hi Media) was used for production. The pH of the medium was adjusted to 6.5 prior to sterilization. Fifty (1 L) flasks containing 200 mL of the above medium were inoculated with 1% of the seed culture and incubated on rotary shaker at 220 rpm for 72 hr at 28°C.

**Extraction and Isolation**

The culture filtrate and mycelia were separated by centrifugation of fermented broth (9 L). The filtrate (3×3 L) was extracted three times with equal volumes of EtOAc to give 1 g of brown coloured semi solid crude extract. The crude extract was subjected to silica gel column chromatography (230-400 mesh) and eluted gradient wise using petroleum ether and
EtOAc. Column eluted with 20% EtOAc gave 100 mg enriched fraction. Compounds 1 (15 mg) and 2 (40 mg) were isolated from enriched fraction by normal phase preparative HPLC using isocratic solvent system of CHCl₃ and petroleum ether (9:1). Rₜ of the compound 1 and 2 in solvent system petroleum ether and EtOAc (2:3) were 0.50 and 0.44, respectively.

Compound 1: Yellow colored, crystalline solid, m.p. 191.1-93.3°C. (Lit 190-92°C). ¹H NMR (300 MHz, CDCl₃): δ_H 1.6 (3H, s, CH₃), 2.5 (1H, tt, J = 3.9, 11.1 Hz, H-4β), 2.8 (1H, dd, J = 1.2, 10.2 Hz, H-4α), 3.9 (3H, s, OCH₃), 3.8 (3H, s, OCH₃), 4.7 (1H, d, H-1α), 4.7 (1H, d, H-1β), 6.6 (1H, d, J = 2.4 Hz, H-8), 7.2 (1H, d, J = 2.4 Hz, H-6); ¹³C NMR (75 MHz, CDCl₃): δ_C 58.5 (C-1), 94.6 (C-3), 31.8 (C-4), 181.7 (C-5), 203.6 (C-6), 104.1 (C-8), 162.0 (C-7), 103.6 (C-6), 164.7 (C-7), 104.8 (C-8), 183.9 (C-10), 136.0 (C-9a), 136.8 (C-4a), 143.0 (C-5a), 114.0 (C-9a), 56.6 (OCH₃), 56.0 (OCH₃), 29.4 (CH₃-3); MS: m/z 327.1 [M+Na]+; HRFABMS: m/z 327.0842 [M+Na]+ (calculated for C₁₀H₁₅O₆Na)²⁹.

Compound 2: White, crystalline solid, m.p. 120.8-21.5°C (Lit 120-21°C). ¹H NMR (300 MHz, CDCl₃): δ_H 1.52 (3H, s, CH₃), 1.7 (1H, dt, J = 4.5, 12.6 Hz, H-4α), 2.4 (1H, dd, J = 3.9, 13.0 Hz, H-4β), 2.9 (1H, ddd, J = 5.7, 9.5, 13.7 Hz, H-10a), 3.4 (1H, ddd, J = 3.6, 10.7, 14.2 Hz, H-4a), 3.8 (3H, s, OCH₃), 3.9 (3H, s, OCH₃), 4.1 (1H, d, H-1β), 4.2 (1H, d, H-1α), 7.2 (1H, d, J = 2.4 Hz, H-6), 6.9 (1H, d, J = 2.4 Hz, H-8); ¹³C NMR (75 MHz, CDCl₃): δ_C 30.3 (CH₃), 34.6 (C-4), 45.1 (C-4a), 48.7 (C-10a), 55.9 (OCH₃), 56.4 (OCH₃), 60.1 (C-1), 95.3 (C-3), 102.0 (C-6), 104.8 (C-8), 118.2 (C-9a), 138.8 (C-5a), 164.2 (C-9), 164.4 (C-7), 193.8 (C-5), 197.1 (C-10); MS: m/z 329.1 [M+Na]+; HRFABMS: m/z 329.0997 [M+Na]+ (calculated for C₁₀H₁₄O₆Na)²⁹.

Biological Activity of Isolated Compounds

The anti-inflammatory and anti-diabetic activities of these compounds were evaluated by the method as described by Kulkarni-Almeida, et al⁶ and Somwar, et al⁷.

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References