ANTI-INFLAMMATORY PRINCIPLES OF CULTIVATED *PYCNOPORUS SANGUINEUS*

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Pycnoporus sanguineus is a parasitic fungus found on dead broadleaf trees and used as a nutraceuticals and functional food. LPS-induced NO production in a mouse microglial cell line, BV-2 cells was used as inflammatory model to screen neuro-protective compounds from the cultivated mycelia of *P. sanguineus*. Bioassay-guided fractionation was led to the isolation of four ergostane skeleton type steroids, including ergosta-7,22-dien-3β-ol (**1**), 3β,5α-dihydroxyergosta-7,22-dien-6-one (**2**), 3β,5α,9α-trihydroxy-ergosta-7,22-dien-6-one (**3**), and 3β,5α,6α-trihydroxy-ergosta-7,22-diene (**4**) from mycelia of cultivated *P. sanguineus*. Among that, compounds **2**, **3**, and **4** were newly isolated in this species. Compound **1** showed significantly inhibition of lipopolysaccharide (LPS)-induced nitric oxide (NO) production in BV-2 cells, at a concentration of 15 μM in the value of 43.5%.

Key words: *Pycnoporus sanguineus*, mycelia, NO, microglia, BV-2 cells, anti-inflammation, ergostane steroids

Introduction

Adequate formation of NO contributes to vasodilator, neurotransmitter, and in the immunological system as a defense against the diseases. However, the over production of nitric oxide (NO) in response to lipopolysaccharide (LPS) also react with superoxide anion radicals to form peroxynitrite leading to high oxidative stress, as a pro-inflammatory stimuli.[1-3] Under pathological conditions, activated microglia have been implicated as the predominant cell governing inflammation-mediated neural damage. Activated microglia induce neuronal death has been shown to involve nitric oxide.[4,5] Therefore, the application of dietary components aside from anti-inflammatory drugs has become a focus of interest. Diets for preventing chronic diseases associated with inflammation were recently proposed as a therapeutic strategy.[6]

Pycnoporus sanguineus, Polyporaceae, is a

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parasitic fungus found on dead broadleaf trees. The fungus effectively adsorbs lead, copper, and cadmium. Recently, attempts have been made to apply its biosorption to treat wastewater containing heavy metals[7] and an oil-polluted habitat.[8] On the other hand, the fungus plays a very important ecological role in degrading woody forest litter.[9] The fruiting bodies of *P. sanguineus* has also long been used as nutraceuticals and functional foods, in the Americas and Africa for treating a number of illnesses[10] and exhibits antiviral activity.[11] Chemical compounds found in this fungus include phenol oxidase,[12,13] sterols, and phenoxazine.[14] It is worthwhile to elevate the production from culture systems with a scalable method for pharmaceutical applications. Therefore, this article describes several compounds isolated from the mycelia of cultivated *P. sanguineus* and its bioactivity.

Materials and Methods

General methods

P. sanguineus strains were isolated by the fungal specialist, Dr. Tun-Tschu Chang (Taiwan Forestry Research Institute, Taipei, Taiwan). BV-2 microglia (a murine microglial cell line), a generous gift of Dr. Rongtai Wei (National Health Research Institute, Zhunan, Miaoli, Taiwan). Bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), lipopolysaccharide (LPS), and other chemicals were obtained from Sigma (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) was obtained from Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS) and antibiotic-antimycotic were obtained from Biological Industries (Haemek, Israel). Optical rotations were recorded on a JASCO DIP-370 polarimeter. IR and UV spectra were recorded on a Nicolet Avatar 320 FT-IR spectrometer and a Hitachi U-3310 spectrophotometer, respectively. NMR spectra were measured on a Varian Unity INOVA-500 MHz using $CDCl_3$ or CD_3OD as solvent. EIMS were recorded on a Finnigan DSQ II mass spectrometer at 70 eV. Column chromatography was performed with silica gel 60 (230-400 mesh, Merck, Darmstadt, Germany), Sephadex LH-20 (Amersham Biosciences, Uppsala, Sweden), and semi-preparative reversed-phase silica gel (LiChrosorb Si-60, Merck, Darmstadt, Germany).

Liquid culture

Fungi were maintained on potato dextrose agar (PDA) slants and transferred to fresh medium at 3-week intervals. In each pasteurized Petri dish, 25 ml of PDA medium (39 g/l) was used and incubated at 28 °C for 14 days. The fine mycelia on the media surface were transferred to 800-ml culture flasks containing 100 ml of 24 g/l potato-dextrose broth (PDB) (Difco™, BD Diagnostic Systems, Sparks, MD, USA), glucose at the concentration 20 g/l at pH 5.6. Mycelia were harvested from 49-day-old cultures. Following incubation, mycelia were rapidly washed with 1 L of 250 mM NaCl during aspiration to remove contaminating of culture media. Samples were then lyophilized and stored at 4 ºC, and the dry weight of the mycelia was measured.

Extraction and isolation

Dry mycelia (1.1 kg) were extracted with ethanol (3 x 6 L) at 80 ℃ refluxing (6h each). The combined ethanol extracts was evaporated under reduced

pressure to yield a brown residue (EtOH extract). The EtOH extract (158.3 g) was suspended in H_2O and then successively partitioned with ethyl acetate (EtOAc) and *n*-butanol (BuOH) to give EtOAc-, BuOH- and H_2O-soluble fractions. The bioactivity of each fraction was tested in the system described later. The bioactive fractions, EtOAc and BuOH fractions (Fig. 2), were subjected to investigating the bioactive components. EtOAc extract was fractionated by gradient Si gel (230–400 mesh) column chromatography (CC) eluting with a gradient of EtOAc in hexane (Hex) to give six fractions (EA-1, EA-2, EA-3, EA-4, EA-5 and EA-6). Subfractions EA-2 and EA-5 stowed bioactivity. Subfraction EA-2 (eluate of 30% EA/Hex) was further purified by Si gel CC to afford ergosta-7,22-dien-3β-ol (**1**, 3.4 mg). Fraction EA-5 (eluate of 75% EA/Hex) was subjected to a semi-preparative HPLC (LiChrosorb Si-60) to give 3β,5α-dihydroxy-ergosta-7,22-dien-6-one (**2**, 1.8 mg) and 3β,5α,9α-trihydroxyergosta-7,22-dien-6-one (**3**, 2.6 mg). BuOH-soluble fraction was further separated by Si gel CC ($CHCl_3$/MeOH gradient). Subfraction eluting with 5%MeOH $CHCl_3$ was collected and purified by Sephadex LH-20 (MeOH) and RP-18 columns (Cosmosil 75C_{18}-OPN) to yield 3β,5α,6α-trihydroxyergosta-7,22-diene (**4**, 2.4 mg).

Ergosta-7,22-dien-3 β -ol (1)[15,16]

Colorless amorphous powder; mp 168-170℃; [α]25 *D* +11.1° (*c* 0.27, $CHCl_3$); IR ν_{max} 3418, 1457, 1382, 1370, 1042 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): δ 0.52 (3H, s, H-18), 0.79, 0.81 and 0.89 (3H each, d, *J*=7.0 Hz, H-26, H-27 and H-28), 1.00 (3H, s, H-19), 1.00 (3H, d, *J*=7.0 Hz, H-21), 1.40 (1H, m, H-5), 3.57 (1H, m, H-3), 5.17 (1H, dd, *J*=15.2, 7.8 Hz, H-22), 5.18 (1H, dd, *J*=15.2, 6.5 Hz, H-23), 5.14 (1H, m, H-7); EIMS *m/z* (rel. int.): 398 [M^+ (25)], 300 (18), 271 (100), 255 (55), 147 (40), 107 (50).

3β,5α-dihydroxyergosta-7,22-dien-6-one (2)[17]

Colorless amorphous powder; 252-253 ℃ ; [α]25 *D* +5° (*c* 0.2, $CHCl_3$); IR ν_{max} 3454, 3322, 1668, 1619, 1461, 1375, 1364, 1166, 1073 cm^{-1}; UV (MeOH)(log ε) λ_{max} 248 nm; ^1H NMR ($CDCl_3$, 500 MHz): δ 0.59 (3H, s, H-18), 0.80, 0.81 and 0.90 (3H each, d, *J*=7.5 Hz, H-26, H-27 and H-28), 0.93 (3H, s, H-19), 1.01 (3H, d, *J*=7.0 Hz, H-21), 4.01 (1H, m, H-3), 5.12 (1H, dd, *J*=15.2, 7.6 Hz, H-22), 5.20 (1H, dd, *J*=15.2, 7.1 Hz, H-23), 5.63 (1H, s, H-7); EIMS *m/z* (rel. int.): 428 [M^+ (5)], 410 (45), 392 (60), 301 (25), 267 (30), 213 (35), 173 (45), 109 (75), 105 (80), 81 (100), 69 (95).

3β,5α,9α−trihydroxyergosta-7,22-dien-6-one (3)[18,19]

Colorless amorphous powder; 225-228 ℃; [α]25 *D* -40° (*c* 0.2, $CHCl_3$); IR ν_{max} 3422, 3315, 3220, 1679, 1620, 1449, 1378, 1366, 1160, 1150, 1105 cm^{-1}; UV (MeOH) (log ε) λ_{max} 239 nm; ^1H NMR ($CDCl_3$, 500 MHz): δ 0.60 (3H, s, H-18), 0.80, 0.81 and 0.90 (3H each, d, *J*=7.0 Hz, H-26, H-27 and H-28), 1.01 (3H, d, *J*=7.0 Hz, H-21), 1.02 (3H, s, H-19), 4.04 (1H, m, H-3), 5.17 (1H, dd, *J*=15.0, 8.1 Hz, H-22), 5.22 (1H, dd, *J*=15.0, 7.1 Hz, H-23), 5.64 (1H, s, H-7); EIMS *m/z* (rel. int.): 426 [$M-H_2O$] $^+$, 369 (25), 300 (8),139 (100).

3β,5α,6α−trihydroxyergosta-7,22-diene (4)[17]

Colorless amorphous powder; 223-226 ℃; [α]25 *D* -15° (*c* 0.2, $CHCl_3$); IR ν_{max} 3465, 3303, 1461, 1384, 1374, 1152, 1034 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz):

δ 0.58 (3H, s, H-18), 0.83, 0.85 and 0.90 (3H each, d, *J*=7.0 Hz, H-26, H-27 and H-28), 1.02 (3H, d, *J*=6.6 Hz, H-21), 1.53 (3H, s, H-19), 3.61 (1H, d, *J*=5.3 Hz, H-6), 4.07 (1H, m, H-3), 5.14 (1H, dd, *J*=15.2, 7.8 Hz, H-22), 5.19 (1H, dd, *J*=15.2, 6.5 Hz, H-23), 5.33 (1H, d, *J*=5.3 Hz, H-7); EIMS *m/z* (rel. int.): 412 [(M-H_2O)$^+$, 60], 394 (35), 379 (50), 269 (30), 251 (40), 69 (100).

BV-2 cell culture

BV-2 cells was cultured in DMEM with 10% heat-inactivated FBS 100 U/ml penicillin-streptomycin, and 2% glutamine in a 5% CO_2 incubator at 37 °C. Confluent cultures were passaged by trypsinization.

Assay of cell viability

Cell viability of BV-2 cells was performed by MTT assay as common method. The cell viability from various test groups was determined by (absorbance of the test group/absorbance of the control) × 100. All test samples mentioned above were dissolved in DMSO, and the final concentration of DMSO was < 0.1%; using 0.1% DMSO as a control group.

Assay of NO release

NO release assay was carried out according to the method described by Mastuda *et al*.[20] Briefly, BV-2 cells were seeded at 1x10^4 cells/well in 96-well plate for overnight. After washing with PBS, cells were pretreated with test sample at the indicated concentrations in serum-free medium for 2 h and then added 500 ng/ml LPS for 24 h. Supernatant (100 μl) was used to investigate NO release by NO quantitative kit (Active Motif). The absorbance of the water soluble purplish red product was read at 550 nm on an ELISA reader. The amount of NO were calculated by calibration curve established with 0.15-50 μM $NaNO_2$.

Statistical analysis

Data are presented as the mean ± SE, and *n* represents the number of experiments. In bar graphs, S.E. values are indicated by error bars. Statistical analyses were carried out using Student's unpaired *t*-tests when applicable. *p* values of < 0.05 were considered to be statistically significant.

Results and Discussion

Time-course study of growth

To maximize the production of biomass of *P. sanguineus*, a time-course study was performed on the dry mass accumulation (Table 1). At 49 days, the culture achieved the maximal dry mass accumulation at a value of 16.13 ± 2.10 g/l. Therefore, based on the mass accumulation, the 49-day culture was chosen to mass produce this species for compound isolation.

Table 1. Yield of dry mass from cultured mycelia of *P. sanguineus*

Days	Dry weight (g/l)
7	7.0 ± 0.1
14	8.6 ± 0.2
21	11.3 ± 0.2
28	10.5 ± 0.3
35	11.2 ± 0.2
42	12.5 ± 0.3
49	16.1 ± 2.1
56	12.7 ± 0.1

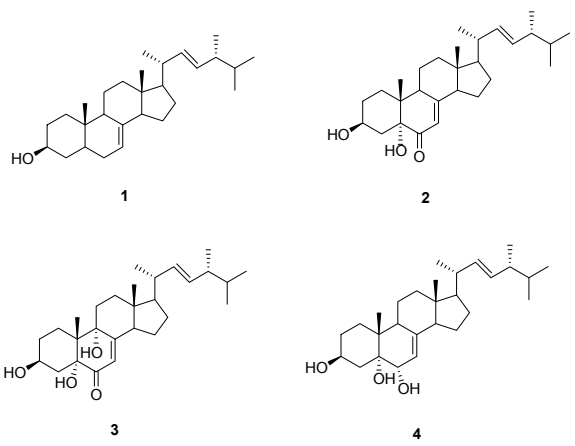

Fig. 1. Structures of isolated compounds (1-4).

to yield EtOAc-, *n*-BuOH- and H_2O-soluble fraction, respectively. The bioactive fractions, EtOAc- and BuOH-soluble fractions, were subjected to CC over Si gel, Sephadex LH-20 and RP-18 to yield 4 pure compounds. Their structures were elucidated as ergosta-7,22-dien-3β-ol (**1**), 3β,5α-dihydroxyergosta-7,22-dien-6-one (**2**), 3β,5α, 9α-trihydroxyergosta-7,22-dien-6-one (**3**), and 3β,5α,6α-trihydroxyergosta-7,22-diene (**4**) (Fig. 1). The structure identification of **1-4** were based on spectroscopic data including NMR and MS and comparison with authentic samples or with published data.[15-19]

Identification of isolated compounds from *P. sanguineus*

The ethanol extract from mycelia of *P. sanguineus* was successively partitioned with EtOAc and *n*-BuOH

Effects of NO release in BV-2 cells

Multiple studies suggested that activated microglia have been implicated as the predominant cell governing inflammation-mediated neural damage

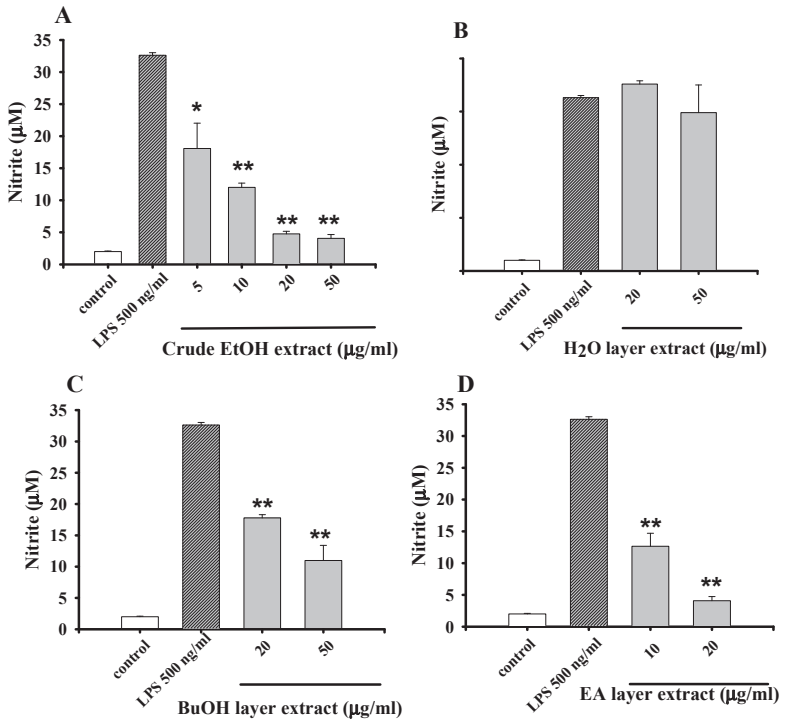

Fig. 2. Effects of ethanol extract and EtOAc-, BuOH-, and H_2O-soluble fractions from mycelia of *P. sanguineus* on LPS-induced NO production in BV-2 cells. Data are presented as mean ± SE (n=3). *p<0.05 in comparison with LPS-treated BV-2 cells by *t*-test. **p <0.01 in comparison with LPS-treated BV-2 cells by *t*-test.

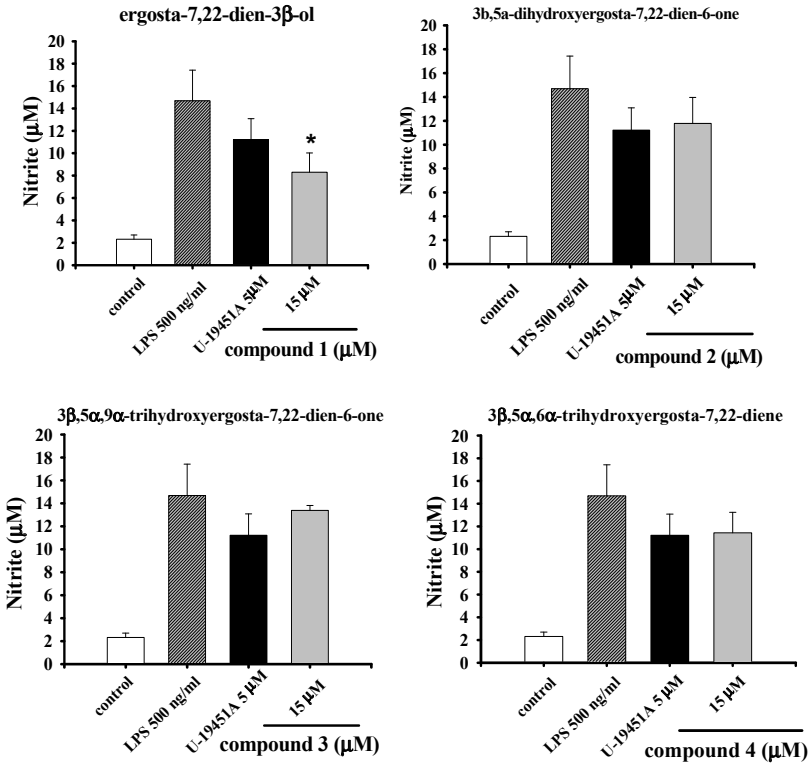

Fig. 3. Effects of isolated compounds from EtOAc- and BuOH-soluble fractions of mycelia of *P. sanguineus* on LPS-induced NO production in BV-2 cells. U-19451A was used as a positive control. Data are presented as means ± SE (n =3). * $p<0.05$ is in comparison with LPS- treated BV-2 cells by *t*-test. ** $p<0.01$ in comparison with LPS-treated BV-2 cells by *t*-test.

and associated with excess nitric oxide expression.[4,5] Our study showed that the suppressive effect of ethanol extract, EtOAc-, BuOH- and H_2O-soluble fractions on NO production was evaluated by LPS-induced BV-2 cells. As shown in Fig. 2, LPS significantly increased the NO production as compared with unstimulated control, and EtOH crude extract, EtOAc- and BuOH-soluble subfraction presented significantly inhibitory effect on NO formation at 20 μg/ml with 87.5% and 45.5%, respectively and in a concentration dependent manner. The isolated pure compound **1** showed a significant inhibition of NO production at the concentration of 15 μM even more potent than U-19451A, but not the other compounds (Fig. 3). U-19451A, a selective inducible nitric oxide synthase (iNOS) inhibitor[21] at 5 μM was used as a positive control with similar inhibitory level.

This is the first report to investigate the inhibition of NO production in BV-2 cells and the chemical study on the cultured mycelia of *P. sanguineus*. Our results suggest that *P. sanguineus* significantly inhibit NO release, and its ergostane components may be involved in its anti-inflammatory effect.

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血紅密孔菌菌絲體之抗發炎活性成分研究

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血紅密孔菌 (*Pycnoporus sanguineus* (Fr.) Murr) 為一種木材腐朽菌，被用於保健及機能性食品。本研究以脂多醣 (lipopolysaccharide, LPS) 誘導小鼠微膠小細胞株, BV-2細胞產生一氧化氮 (nitric oxide, NO) 的抗發炎模式，篩選人工培養之血紅密孔菌之菌絲體的活性成分。依此生物活性導向，進行成分分離純化，共得到四個麥角甾醇衍生物，分別為：ergosta-7,22-dien-3β-ol (**1**), 3β,5α-dihydroxyergosta-7,22-dien-6-one (**2**), 3β,5α,9α-trihydroxyergosta-7,22-dien-6-one (**3**) 及 3β,5α,6α-trihydroxyergosta-7,22-diene (**4**)。其中化合物**2**，**3**和**4**三個化合物是首次自血紅密孔菌中分離得到之已知化合物。化合物**1**對LPS誘導之微神經膠細胞株有顯著的抑制，在15 μM濃度有43.5 % 的抑制率。

關鍵字：血紅密孔菌、菌絲體、一氧化氮、微膠小細胞、BV-2細胞、抗發炎、麥角甾醇

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