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Precursor-feeding strategy on the triterpenoid production and anti-inflammatory activity of *Antrodia cinnamomea*

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ABSTRACT

Antrodia cinnamomea has commercially been used in the formulation of nutraceuticals and functional foods. *A. cinnamomea* was fed exogenous sterols including squalene, cholesterol, and stigmasterol to enhance its triterpenoid content. Four triterpenoids were identified in *A. cinnamomea*, namely dehydrosulphurenic acid (De-sul), zhankuic acid A (ZaA), 15α-acetyl-dehydrosulphurenic acid (15α) and dehydroeburicoic acid (De-eb), and one polyphenyl compound, 4,7-dimethoxy-5-methyl-1,3-benzodioxole (4,7-D). Maximum ZaA and 15α contents of 2.84 and 48.07 μg/mg dry weight, respectively were achieved by 100 μM squalene-feeding, and maximum De-sul and De-eb contents of 69.08 and 47.91 μg/mg dry weight were achieved by 10 and 100 μM stigmasterol-feeding, respectively. To study the anti-inflammatory potential of *A. cinnamomea*, lipopolysaccharide-induced nitric oxide (NO) production, NADPH oxidase (NOX), inducible NO synthetase (iNOS) and cyclooxygenase-2 (COX-2) expression in murine microglial cells, BV-2, were evaluated. ZaA and 50 μM squalene-feeding of *A. cinnamomea* inhibited LPS-induced NO, iNOS and COX-2 expression in BV-2 cells.

1. Introduction

Antrodia cinnamomea (Polyporaceae) is an edible mushroom. It has also been used in the formulation of neutraceuticals and functional foods. *A. cinnamomea* is a slow-growing species with a limited distribution in Taiwan, so it is not possible to collect sufficient quantities for extensive use as drug remedies [1]. Mycelial culture of this species is an alternative strategy for the mass production of secondary metabolites to overcome its insufficient presence in local ecosystems. Optimization of cultural conditions could improve the triterpenoids content in *A. cinnamomea*. Monoterpenes added to liquid medium showed increased production of crude triterpenoids [2]. Physical parameters of temperature changes during fermentation was reported to enhance triterpenoids in this fungus [3]. The chemical compositions of *A. cinnamomea* include terpenoids [4,5], benzoids [6], lignans [8], maleic acid and succinic acid derivatives [9]. In this study, triterpenoids were the targets for manipulation in the *A. cinnamomea* mycelial culture system (Scheme 1). The targeted compounds of ergostane- and lanostane-type triterpenoids are characteristic of this species and have been described as anticancer. There is growing interest in their use to treat diseases [6]. They may also play important roles in the fungal efforts to protect itself against stress and microbial pathogens, and be responsible for maintain membrane permeability [7]. Using *in vitro* systems to improve the production of these compounds is of value and importance.

This study is the first report to use precursor-feeding strategy to enhance the biosynthesis of triterpenoids of *A. cinnamomea*. Squalene, cholesterol, and stigmasterol were used as precursors in this study (Scheme 2). According to the documented pathway [10], ergostane-type triterpenoids were mainly produced by squalene synthesis. Triterpenoids of *A. cinnamomea* are characterized by the 29 carbon skeleton, and contained a structure of a double bond between C-8 and C-9, and a 24-exo-methylene-26-oic acid chain. Lanostane-type triterpenes are important intermediate products of the biosynthesis of triterpenes, which have the 31 carbon skeleton, double bonds in between C-7, C-8, C-9 and C-11 and a 24-exo-methylene-21-oic acid chain structure (Scheme 1). Squalene, cholesterol, and stigmasterol (24*S*-ethylcholesta-5,22-dien-3β-ol) are the intermediates and the major plant sterols [11]. This study used squalene, cholesterol, and stig masterol as precur-

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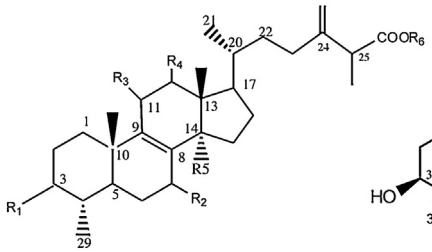
1359-5113/

Ergostane type



Antcin C, R_1= =O , R_2= β -OH,
R_3= =O , R_4=H_2, R_5=H, R_6=H

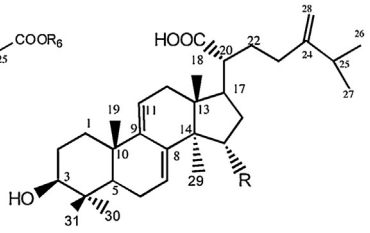
Antcin K, R_1= α -OH, R_2= β -OH,
R_3= =O , R_4=**H**, R_5=H, R_6=H

Zhankuic acid C, R_1= α -OH, R_2= =O ,
R_3= =O , R_4= α -OH, R_5=H, R_6=H

Zhankuic acid B, R_1= α -OH, R_2= =O ,
R_3= =O , R_4=H_2, R_5=H, R_6=H

Zhankuic acid A, R_1= =O , R_2= =O ,
R_3= =O , R_4=H_2, R_5=H, R_6=H

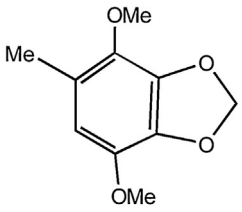
Lanostane type



Dehydroeburicoic acid, R=H

Dehydrosulphurenic acid, R=OH

15α-Acetyl-dehydrosulphurenic acid, R=OA



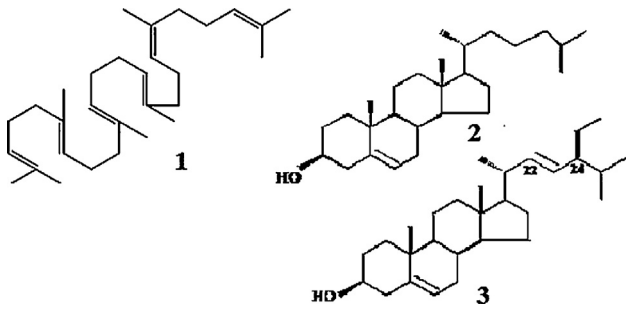
4,7-Dimethoxy-5-methyl-1,3-benzodioxole

Scheme 1. Structures of triterpenoids evaluated in this study.

sors to induce the production of triterpenoids in mycelial cultures of *A. cinnamomea*.

There are several molecular mechanisms of anti-inflammation, including inhibition of reactive oxygen species (ROS), inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) production. Recent studies on methanolic extracts of *Antrodia* mycelium have observed inhibition of ROS production in peripheral human neutrophils (PMN) or mononuclear cells (MNC) [12]. The methanolic extracts of *A. cinnamomea* inhibited mouse liver catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx), leading to increase in malondialdehyde (MDA) and serum levels of NO and TNF-α in the mouse foot [13]. Fermentation of *A. cinnamomea* inhibited LPS-induced nitric oxide (NO), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and prostaglandin E2 (PGE2) production. It also inhibited nuclear factor-κB (NF-κB) pathway of iNOS and COX-2 expression in macrophages [14]. Also, it was reported the inhibition of IκB degradation by methanolic extract of the fruiting body and that of LPS-induced iNOS and COX-2 expression in macrophages [15].

This study investigated the mechanisms behind the inhibitory effect of the triterpenoid-enriched fraction of *A. cinnamomea* on LPS-induced NO production in BV-2, the murine microglial cell. Microglia exist in brain parenchyma and participate in host defense and tissue repair of the central nervous system. Activated microglia had been reported as the predominant cells governing inflammation-mediated neural damage [16]. Microglia could induce neuronal death and are involved in NO production [17]. This study showed that zhankuic acid A was involved in the inhibitory effect of NO production as well as iNOS and COX-2 expression in BV-2 cells.



Scheme 2. Structures of precursors used in this study: (1) squalene; (2) cholesterol; and (3) stigmasterol.

2. Materials and methods

2.1. *Precursor-feeding of* A. cinnamomea

A. cinnamomea isolate (strain WFB 33) obtained from Ilan province in Taiwan, was a generous gift from Dr. Cheng-Jen Chou (National Research Institute of Chinese Medicine, Taipei, Taiwan). For liquid culture, *A. cinnamomea* was inoculated and incubated with 100 ml of medium 24 g/l potato-dextrose-broth (PDB), with 20 g/l glucose (pH 5.6), with or without (control) the addition of 10, 50, and 100 μM squalene, cholesterol, and stigmasterol at 28 °C for 49 day. Samples were then lyophilized, and stored at 4 °C, and the dry weight of mycelia was measured. Squalene, cholesterol,