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# Pinicolol B from *Antrodia cinnamomea* induces apoptosis of nasopharyngeal carcinoma cells

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A R T I C L E I N F O

*Keywords:*
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A B S T R A C T

*Ethnopharmacological relevance:* The medicinal mushroom *Antrodia cinnamomea* possesses anticancer properties but the active compounds responsible for these effects are mostly unknown.
*Aim of the study:* We aimed to identify novel *A. cinnamomea* compounds that produce cytotoxic effects on cancer cells.
*Materials and methods:* Using ethanol extraction and chromatography, we isolated the lanostanoid compound lanosta-7,9(11),24-trien-3β,15α,21-triol (**1**) from cultured *A. cinnamomea* mycelium. Cytotoxicity and pro-apoptotic effects of compound **1** were evaluated using the MTS assay and flow cytometry analysis, respectively.
*Results:* Compound **1** produced cytotoxic effects on the nasopharyngeal carcinoma cell lines TW02 and TW04, with $IC_{50}$ values of 63.3 and 115.0 μM, respectively. On the other hand, no cytotoxic effects were observed on non-tumorigenic nasopharyngeal epithelial cells (NP69). In addition, compound **1** induced apoptosis in TW02 and TW04 cells as revealed by flow cytometry analysis.
*Conclusions:* Our results demonstrate for the first time the presence of pinicolol B in *A. cinnamomea* mycelium and suggest that this compound may contribute to the anticancer effects of *A. cinnamomea*.

## 1. Introduction

Traditional Chinese medicine has a long history of use in Asian countries to improve health and longevity (Normile, 2003; Stone, 2008; Tang et al., 2008). While historical and anecdotal accounts suggest that traditional herbal remedies may produce beneficial effects against human chronic diseases, the efficacy and safety of these remedies remain to be established. Notably, various compounds used as phar-

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maceutical drugs in Western medicine have been isolated from traditional Chinese remedies, including the anti-malaria compound artemisinin—for which the 2015 Nobel Prize of Physiology or Medicine was awarded (Kong and Tan, 2015)—and the immuno-suppressive compound fingolimod, which has been approved in the U.S. to treat multiple sclerosis (Adachi and Chiba, 2007). We recently reviewed the anti-obesogenic and antidiabetic compounds found in traditional Chinese medicines and natural health products (Martel et al., 2016). The possibility that traditional herbal remedies may lead to the development of other active compounds continues to be investigated intensively.

*Antrodia cinnamomea* Chang and Chou (Fomitopsidaceae) is a medicinal mushroom initially used by aboriginal tribes in Taiwan to treat abdominal pain, food poisoning, hypertension, and liver cancer (Lu et al., 2013). Known in Chinese as "niu zhang zhi" or "niu zhang ku," this Basidiomycota fungus grows slowly in the inner trunk cavities of the tree *Cinnamomum kanehirai*, producing orange-to-red fruiting bodies. The slow growth and rarity of *A. cinnamomea* fruiting bodies in nature has led to the development of mycelium culture as an alternative for the preparation of this natural health product. Recent studies have shown that *A. cinnamomea* mycelium and fruiting bodies produce similar biological effects on cultured cells and laboratory animals with regards to anti-fatigue, anti-inflammatory, anti-viral, and liver-protecting activities (Ao et al., 2009; Huang et al., 2014a; Lee et al., 2002; Lu et al., 2013). Our previous work showed that an ethanol extract of *A. cinnamomea* mycelium inhibits the NLRP3 inflammasome and secretion of pro-inflammatory cytokines (interleukin-1β, interleukin-18 and tumor necrosis factor-α) by human macrophages (Huang et al., 2014a), suggesting that this medicinal mushroom may be used to treat chronic inflammation.

Extracts of *A. cinnamomea* mycelium and fruiting bodies are also known to produce potent anti-cancer effects. Song et al. observed that a methanol extract of *A. cinnamomea* mycelium induces apoptosis in hepatocellular carcinoma HepG2 cells (Song et al., 2005a, 2005b). Peng and colleagues showed that an *A. cinnamomea* extract inhibits the proliferation and migration of urinary cancer cell lines in vitro (Peng et al., 2007). Similarly, a fermented broth of *A. cinnamomea* mycelium was shown to induce apoptosis in breast cancer cell lines (Hseu et al., 2008; Yang et al., 2006). Another study showed that *A. cinnamomea* mycelium delays breast tumor formation and induces tumor regression in a xenograft mouse model (Hseu et al., 2008).

Several anti-cancer compounds have been isolated from *A. cinnamomea* mycelium and fruiting bodies. Nakamura et al. identified five maleic and succinic acid derivatives from *A. cinnamomea* mycelium, and demonstrated the cytotoxic properties of these compounds in cancer cell lines (Nakamura et al., 2004). The ubiquinone compound antroquinonol isolated from *A. cinnamomea* mycelium possesses cytotoxic activity against various cancer cell lines cultured in vitro (Lee et al., 2007). Huang et al. purified 12 ergostanoid triterpenoids from *A. cinnamomea* and showed that these compounds produce cytotoxic activities against MDA-MB-231 breast cancer cells and A549 lung carcinoma cells, while no significant toxic effects were observed in non-cancerous cells (Huang et al., 2014b).

The incidence of nasopharyngeal carcinoma (NPC) is especially high in Southeast Asia, including the Chinese province of Guangdong, Hong Kong, and Taiwan (Cao et al., 2011). Identification of compounds that could selectively kill NPC cells is highly needed. We report here the isolation of pinicolol B from cultured *A. cinnamomea* mycelium and show that this compound produces cytotoxic and pro-apoptotic effects in cultured NPC cells.

## 2. Materials and methods

### 2.1. Fungal strain

The *A. cinnamomea* strain initially selected and characterized by Chang Gung Biotechnology was validated by comparison of DNA sequences corresponding to 5.8S rDNA and internal transcribed spacers (ITS-1 and ITS-2) with the sequences of type strain deposited in the GenBank database (99.68% homology with AJ496398 sequence).

### 2.2. Extraction and isolation

*A. cinnamomea* was inoculated onto potato dextrose agar plates (20 g/L dextrose, 4 g/L potato extract, 2% agar, pH 5.6) and cultured at 28 °C for seven days. A 1-$cm^2$ piece of agar containing *A. cinnamomea* mycelium was transferred to 100 mL of liquid potato dextrose medium and incubated as above for seven days with gentle agitation. A 15-mL aliquot was transferred to solid-state fermentation culture medium (200 g wheat, 2 g soy peptone, 0.01 g $MgSO_4 \cdot H_2O$, 100 mL distilled water) and the medium was incubated at 24 °C for 120 days in the dark. *A. cinnamomea* mycelium obtained from solid-state fermentation was dried by lyophilization (1000 g) and mixed with 10 L of 95% ethanol (v/v), followed by incubation with gentle shaking at 78 °C for 3 h. The extract was filtered to remove undissolved material, and the solvent of the liquid phase was evaporated using a vacuum concentrator to obtain a dried precipitate (~125 g). The precipitate (100 g) was resuspended in 1 L of double distilled water at 80 °C and n-hexanes were added at a 1:1 volume ratio to obtain organic and aqueous fractions. After stirring for 1 h, the aqueous fraction was collected and ethyl acetate was added at a 1:1 volume ratio to obtain ethyl acetate and aqueous fractions. After stirring for 1 h, the ethyl acetate fraction was collected (~12.4 g).

The ethyl acetate fraction obtained above was subjected to silica gel column chromatography using n-hexanes and ethyl acetate as the mobile phase. HPLC was performed using a Cosmosil 5$C_{18}$-MS-II column (4.6×250 mm, 5 µm) with a mobile phase of acetonitrile/water/phosphoric acid. A gradient of n-hexanes and ethyl acetate was used as eluent. Four fractions were collected and the fourth fraction was subdivided into eight sub-fractions of equal volume. The fifth sub-fraction (~0.6 g) was subjected to preparative HPLC using a Cosmosil 5$C_{18}$-MS-II column (20×250 mm, 5 µm), a mobile phase of acetonitrile/methanol/water/acetic acid, and a photodiode array detector (PDA). The isolated compound (**1**) was obtained as a white, amorphous powder.

### 2.3. Cell culture

The NPC cell lines (keratinizing squamous TW02 and undifferentiated TW04 cells) characterized previously (Lin et al., 1993) were kindly provided by Dr. C.-T. Lin (Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan). NPC cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, 100 µg/mL streptomycin and 25 µg/mL amphotericin B. The human, non-tumorigenic, nasopharyngeal epithelial NP69 cell line characterized earlier (Tsao et al., 2002) was kindly provided by Dr. Yu-Sun Chang (Molecular Medicine Research Center, Chang Gung University; these cells have been used as control, non-tumorigenic cells in previous studies (Chan et al., 2008; Chen et al., 2015; Zhang et al., 2008). NP69 cells were cultured in keratinocyte serum-free medium (K-SFM) under cell culture conditions as described above. Human liver cancer cells (HepG2, Hep3B, PLC/PRF/5, SK-Hep-1) and human colon cancer cell lines (Caco-2, HT-29, LoVo) were obtained from collaborators or the Bioresource Collection and Research Center (Hsinchu, Taiwan). Human liver cancer cell lines were cultured in DMEM supplemented with 10% FBS, 100 IU/mL

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