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Ethnopharmacological communication

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

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ABSTRACT

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 $5C_{18}$-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 $5C_{18}$-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

penicillin, and 100 mg/mL streptomycin. Caco-2 cells were cultured in DMEM supplemented with 20% FBS. HT-29 cells were maintained in Roswell Park Memorial Institute (RPMI-1640) medium containing 10% FBS. LoVo cells were cultured in F12K cell culture medium supplemented with 20% FBS. All cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2.

2.4. Cytotoxicity assay

Cell viability was monitored as described before (Huang et al., 2013). Briefly, cancer cells were treated with 3–300 μM of pinicolol B (**1**) for 24 h. The range of concentrations of pinicolol B was determined based on preliminary trial experiments. The MTS dye (500 μg/mL) was added, and the solution was incubated for 4 h. Absorbance was measured at 490 nm using a spectrophotometer to determine the number of viable cells which produce a blue product. Ethanol served as a negative control for comparison.

2.5. Apoptosis assay

Apoptosis was monitored by flow cytometry using the Annexin V-FITC Apoptosis Detection Kit (BioVision) based on established procedure (Huang et al., 2011). Briefly, 5×10^5 cells grown to 60% confluence were treated with pinicolol B (**1**) at the indicated concentrations for 24 h. Adherent and floating cells were collected and washed with serum-containing medium. Washed cells were resuspended in 500 μl of 1× binding buffer, followed by addition of 5 μl of annexin V-FITC and 5 μl of propidium iodide. The mixture was then incubated for 5 min at room temperature in the dark and analyzed by flow cytometry based on the guidelines provided by the manufacturer.

2.6. Statistical analysis

The results shown correspond to mean values ± standard deviation (SD). Three independent experiments were performed. Samples were processed in triplicate. Statistical significance was assessed using Student's *t*-test. *P* values < 0.05 were considered statistically significant.

3. Results and discussion

3.1. Identification of compound *1*

We prepared an ethanol extract of *A. cinnamomea* mycelium and fractionated the extract by chromatographic separation. This process led to the isolation of a fraction which was submitted to chemical analysis. Compound **1** was obtained as a white, amorphous powder. Mass spectrometry data showed a protonated molecule at *m/z* 457.3703 $[M+H]^+$(see Supplementary Fig. 1). Its chemical formula was identified as $C_{30}H_{48}O_3$. Functional groups were identified by infrared spectroscopy (KBr, υ_{max} cm^{-1}) (Supplementary Fig. 2): 1091 cm^{-1} and 1050 cm^{-1} (C–O); 1380 cm^{-1} (CH_3); 1457 cm^{-1} (CH_2); 1651 cm^{-1} (C=C); 1686 cm^{-1} (C=C stretching, six-carbon ring); 2929 cm^{-1} (CH); 3,422 cm^{-1} (OH).

The ^{1}H-NMR spectrum of compound **1** showed signals corresponding to three olefinic protons (δ 5.28, 5.38 and 6.50), and two oxygenated-methine (δ 3.45, 4.78), one oxygenated-methylene (δ 3.61) and seven primary hydrogen signals corresponding to methyl groups (δ 1.08, 1.11, 1.13, 1.18, 1.43, 1.58, and 1.63) (Supplementary Fig. 3 and Table 1). The ^{13}C-NMR spectrum and DEPT135 spectrum showed 30 carbon signals, including six olefinic carbons (δ 116.10, 122.00, 124.64, 131.43, 141.74, 146.73), two secondary carbons bound to oxygen (δ 73.74, 77.66) and one primary carbon bound to oxygen (δ 70.44) (Supplementary Fig. 4 and Table 2). Based on this information, the compound was identified as having a lanosta-7,9(11),24-triene skeleton.

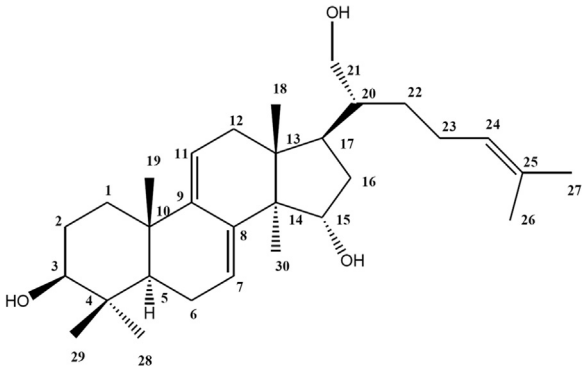

Fig. 1. Chemical structure of compound **1**.

The position of hydrogen and carbon atoms and functional groups was determined using DEPT135 spectroscopy (Supplementary Fig. 5) combined with HSQC (Supplementary Fig. 6). Based on HMBC spectroscopy analysis, the following information was obtained: 28-CH_3 (δ_H 1.11), 29-CH_3 (δ_H 1.18) and C-4 (δ_C 39.39), as well as C-3 (δ_C 77.66) and C-5 (δ_C 49.25) show $^{2,3}J$ long range coupling, implying that C-4 is bound to two methyl groups; C-3 is bound to OH; 19-CH_3 (δ_H 1.08) and C-10 (δ_C 37.69), as well as C-1 (δ_C 36.17) and C-9 (δ_C 146.73) show $^{2,3}J$ long range coupling, implying that 19-CH_3 is a substituent at C-10; 18-CH_3 (δ_H 1.13) and C-13 (δ_C 44.66), as well as C-12 (δ_C 36.59) and C-14 (δ_C 52.10) show $^{2,3}J$ long range coupling, implying that 18-CH_3 is a substituent at C-13; 30-CH_3 (δ_H 1.43) and C-14 (δ_C 52.10), as well as C-8 (δ_C 141.74), C-13 (δ_C 44.66) and C-15 (δ_C 73.74) show $^{2,3}J$ long range coupling, implying that 30-CH_3 is a substituent at C-14 and C-15 is bound to a OH group (Supplementary Figs. 7 and 8).

Compound **1** was identified as lanosta-7,9(11),24-trien-3β,15α,21-triol (**1**) (Fig. 1) and was isolated for the first time here from *A. cinnamomea* mycelium. While compound **1** (also called pinicolol B) was isolated previously from the fungus *Fomitopsis pinicola* (Rösecke and König, 1999), the effect of this compound on cancer cells had not been described until now.

3.2. Cytotoxic effects of compound *1* in NPC cell lines

The anti-cancer effect of compound **1** was examined using the MTS cell viability assay. Compound **1** produced no cytotoxic effects in non-tumorigenic nasopharyngeal epithelial NP69 cells (Fig. 2A). On the other hand, compound **1** produced cytotoxic effects in NPC cell lines TW02 and TW04, compared with control ethanol used as a solvent (Fig. 2B and C). Half maximal inhibitory concentrations (IC_{50}) of compound **1** against TW02 and TW04 cells were calculated at 63.3 and 115.0 μM, respectively. No significant cytotoxic effects were observed in the human liver cancer and colon cancer cell lines tested (i.e., HepG2, Hep3B, PLC/PRF/5, SK-Hep-1, Caco-2, HT-29, LoVo; data not shown). These results indicate that compound **1** may preferentially affect the viability of tumorigenic NPC cancer cells.

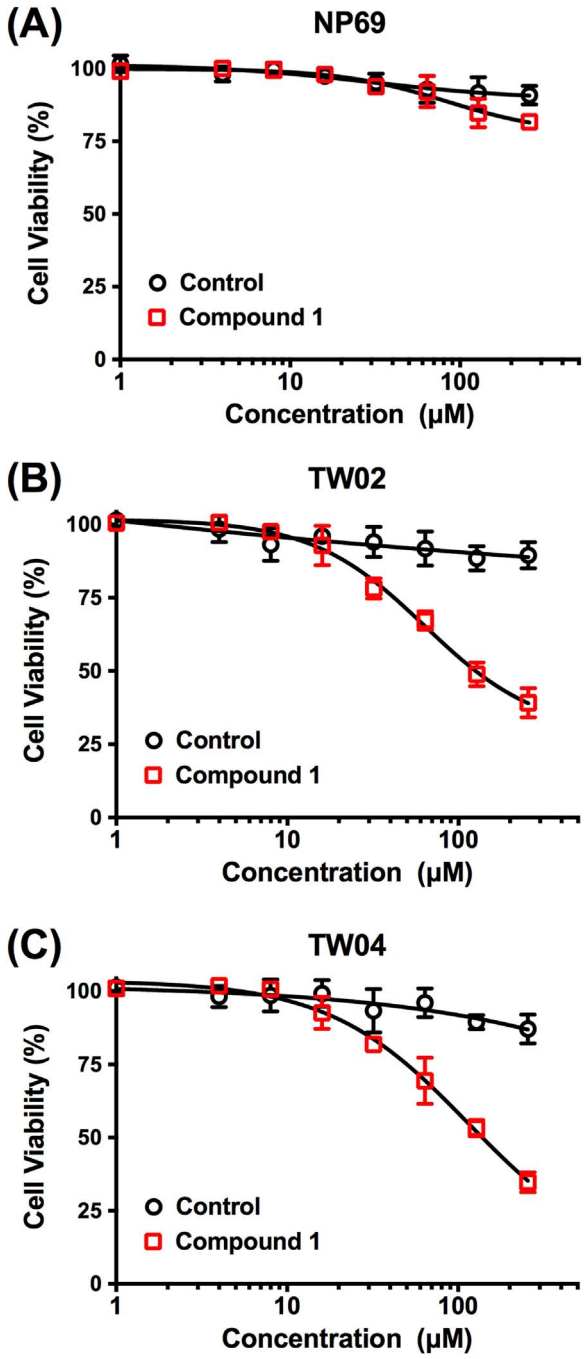

Fig. 2. Cytotoxic activity of compound **1** on NPC cancer cell lines. Cell viability was evaluated using the MTS assay. (A) Compound **1** did not affect the viability of control, non-tumorigenic NP69 cells. In contrast, compound **1** reduced the viability of NPC cancer cell lines TW02 (B) and TW04 (C).

3.3. Pro-apoptotic effects of compound *1* on NPC cells

We monitored the ability of compound **1** to induce apoptosis in NPC cells by monitoring the presence of phosphatidylserine on the cell surface using flow cytometry. We simultaneously analyzed the necrosis-inducing activity of compound **1** by using propidium iodide (PI), which stains cells with a ruptured membrane. Compound **1** induced the accumulation of apoptotic (annexin V-positive) and necrotic (PI-positive) cells in a dose-dependent manner in both TW02 and TW04 cell lines, whereas no significant effect was noted in non-tumorigenic NP69 cells (Fig. 3A). Quantification of the flow cytometry results confirmed the pro-apoptotic and necrosis-inducing activities of compound **1** in NPC cells and the absence of effect in NP69 cells (Fig. 3B).

Lanostanoids are a group of tetracyclic triterpenoids found in various fungi, including *A. cinnamomea* and *Ganoderma lucidum* (Rios et al., 2012). Lanostanoid compounds are derived from lanosterol, a molecule obtained from the cyclization of squalene. Yeh et al. isolated five lanostanoid compounds from *A. cinnamomea* fruiting bodies, and observed that two of these molecules are cytotoxic in human colon cancer cells (HT-29, HCT-116, and SW-480) and breast cancer cells (MDA-MB-231) (Yeh et al., 2009). Similarly, 24-methylenelanosta-7,9(11)-diene-3β,15α-diol-21-oic acid (also called MMH01) was isolated from *A. cinnamomea* and found to have anti-cancer effects against leukemic and pancreatic cancer cell lines via diverse cellular mechanisms (Chen et al., 2009). The compound lanosta-8,24-dien-3β,15α,21-triol (MMH02) was isolated from *A. cinnamomea* fruiting bodies, and experiments showed that this compound produces cytotoxic effects against human cervical cancer cells, esophageal cancer, hepatoma, leukemia, and pancreatic cancer, whereas no apparent cytotoxicity was observed in cultured monocytes (Chen et al., 2010).

In the present study, we examined the effects of lanostanoid compound **1** on several cancer cell lines, including NPC cells, which to our knowledge have not been thoroughly used to study the effects of *A. cinnamomea* compounds. Liver cancer cells were also chosen for testing since *A. cinnamomea* has been used in the past to treat human liver cancer (Lu et al., 2013). In addition, colon cancer cells were chosen based on previous studies performed to test the effects of triterpenoid compounds (Yeh et al., 2009). We observed that compound **1** possesses cytotoxic activity on NPC cells (Fig. 2), whereas it produces no cytotoxic effect on liver and colon cancer cells (data not shown). Of note, compound **1** shows no cytotoxic effects on non-tumorigenic NPC cells, allowing the possibility to specifically target cancer cells and reduce unwanted adverse effects in non-cancerous cells and tissues. Further studies are needed to examine the effects of compound **1** in laboratory animals.

We show that compound **1** induces apoptosis in NPC cells as shown by increased staining with annexin V (Fig. 3). Annexin V recognizes phosphatidylserine on the surface of apoptotic cells and is widely used to assess apoptosis (Schlegel and Williamson, 2001). A recent study reported that translocation of phosphatidylserine onto the cell surface of apoptotic cells relies on activation of caspase-3 and caspase-7 (Marino and Kroemer, 2013), suggesting that a similar phenomenon may occur in NPC cells treated with compound **1**. The mechanism of apoptosis occurring in cancer cells in response to triterpenoid compounds isolated from *A. cinnamomea* has been studied in previous studies and involves activation of the caspase cascade and the mitochondria-dependent pathway (Tsai et al., 2010; Lee et al., 2012). Further studies are needed to determine whether these pro-apoptotic responses occur in NPC cells as well as to explore in more detail the mechanism of apoptosis in this context.

Herbal remedies used as traditional Chinese medicines may consist of whole extracts of herbal or fungal material as well as complex mixtures of natural products obtained from various sources. The

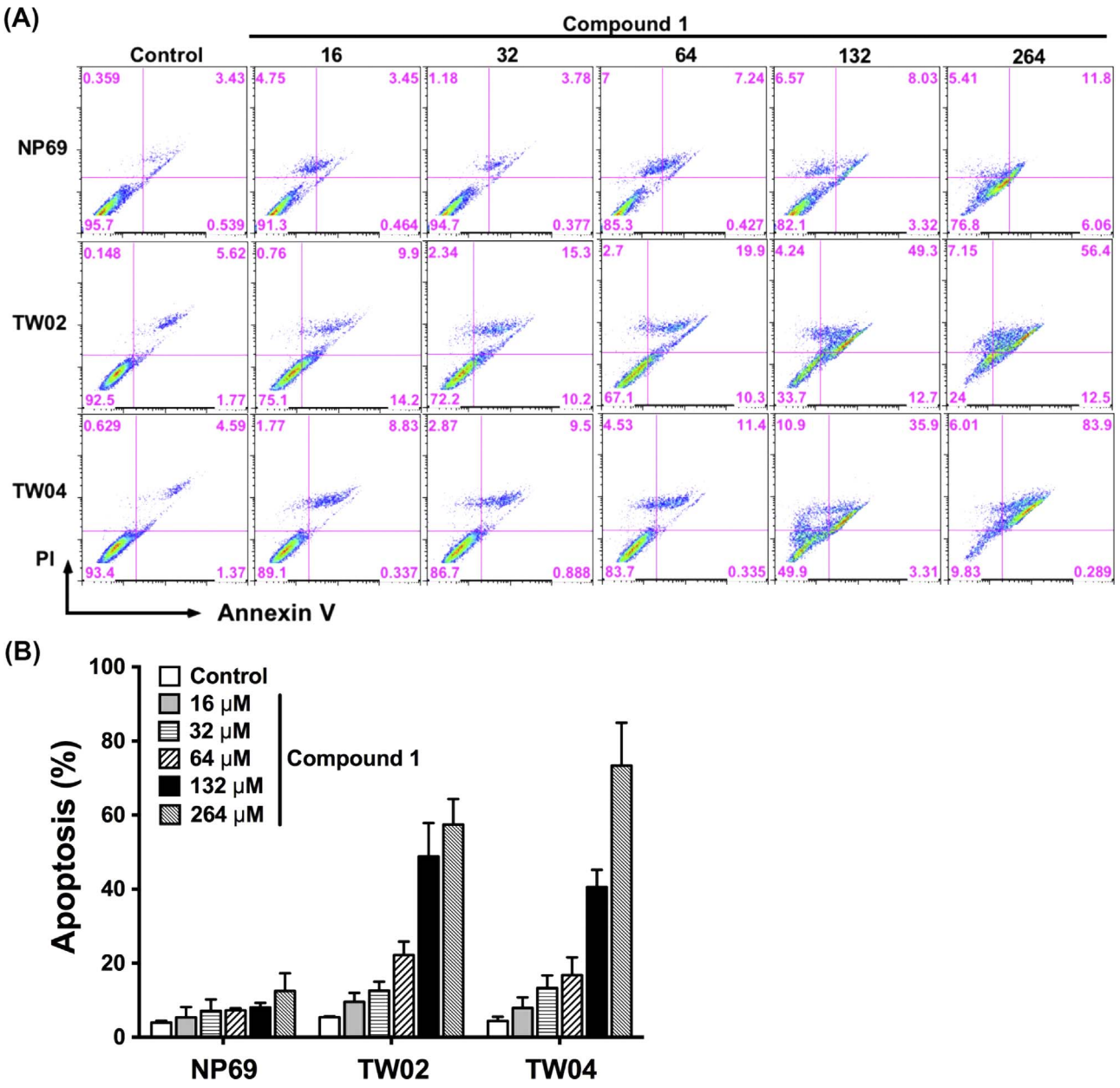

Fig. 3. Pro-apoptotic and necrotic activity of compound **1** on NPC cancer cell lines. Apoptosis was assessed by flow cytometry using fluorescent annexin V-fluorescein isothiocyanate (FITC). Propidium iodide (PI) was used to evaluate necrotic cell death. (A) Representative flow cytometry plots showing annexin V and propidium iodide staining. Compound **1** did not induce significant levels of apoptosis in control, non-tumorigenic NP69 cells. However, compound **1** induced dose-dependent accumulation of annexin-V-positive and PI-positive cells in NPC cancer cell lines (TW02 and TW04). (B) Average quantification of top right quadrant values of flow cytometry plots based on three independent experiments.

composition and quality of these remedies is variable and depends on the culture conditions of the source material. A determination of the quality of herbal remedies is of prime importance for the adequate use of such products (Martel et al., 2016). Given that pinicolol B (**1**) induces cell death of NPC cancer cells, we propose that this compound may be used alone or in combination with other active molecules to monitor the quality of *A. cinnamomea* products sold on the market.

4. Conclusions

Our results show for the first time that pinicolol B (**1**) is found in *A. cinnamomea* mycelium cultured in vitro. This compound (**1**) induces apoptosis and necrosis of NPC cell lines and may thus be used as a candidate molecule for the development of pharmacological agent to prevent the development of NPC, either alone or in combination with existing anticancer treatments. Pinicolol B (**1**) may also be used as the starting basis for design of chemical analogs that might induce cell death of nasopharyngeal tumor cells at a lower IC_{50} than the natural compound. Finally, pinicolol B (**1**) may be used as a molecular marker to monitor the quality of *A. cinnamomea* products available on the market.

Potential conflict of interest

YFK is President of Chang Gung Biotechnology. JCL, WTJ, and ITC are employees of Chang Gung Biotechnology. JDY is Chairman of the Board of Chang Gung Biotechnology. The authors have filed patent applications related to the isolation and use of the compound described

in the present study. Part of the data and information presented here has been included in Taiwan patent I583671 and a U.S. patent application currently in preparation.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jep.2017.02.008.

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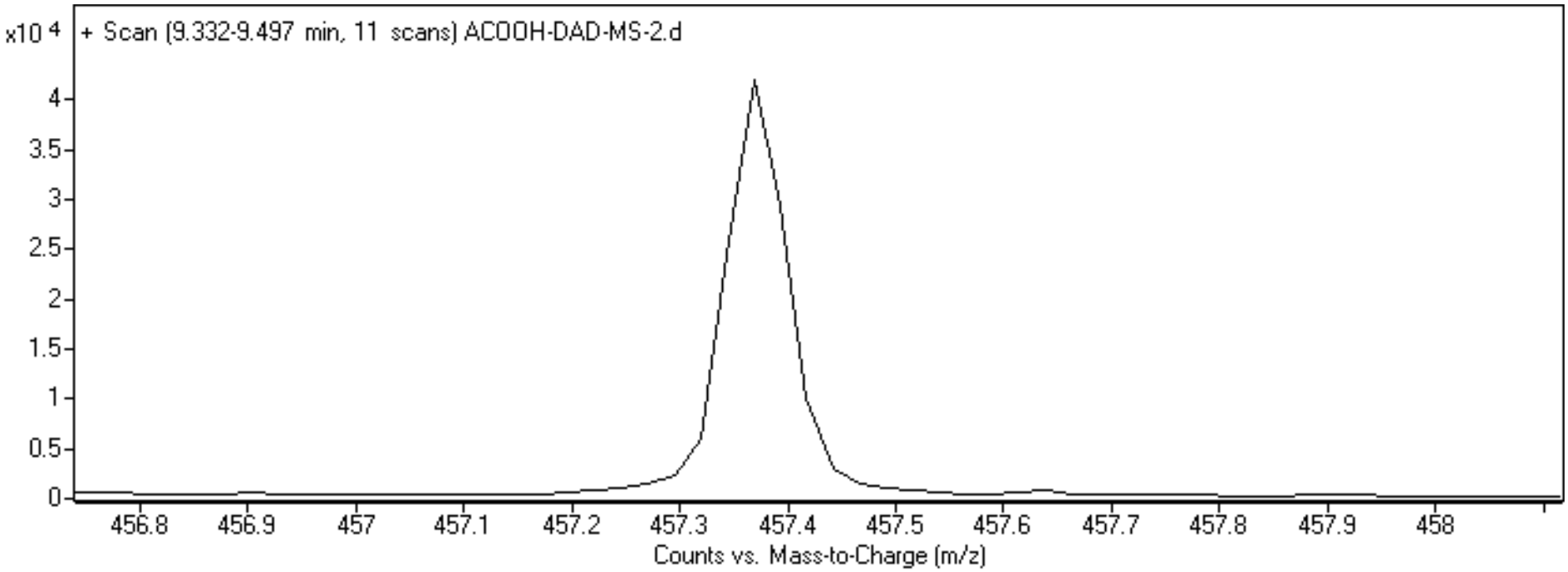
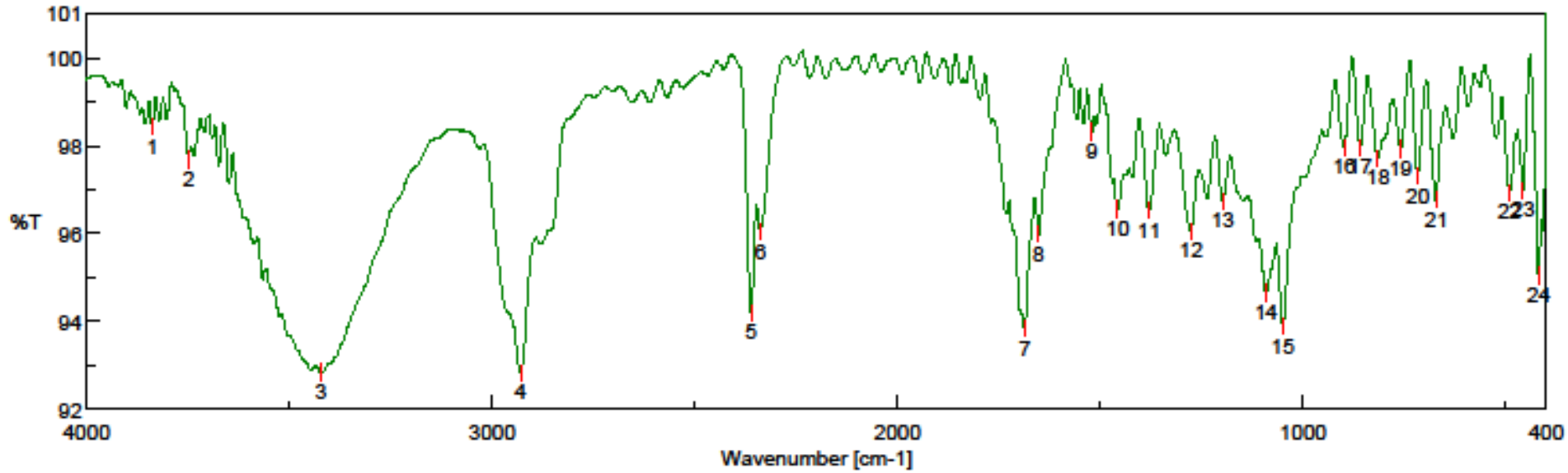
Supplementary material

Supplementary Fig. 1. Mass spectrum of compound **1**.



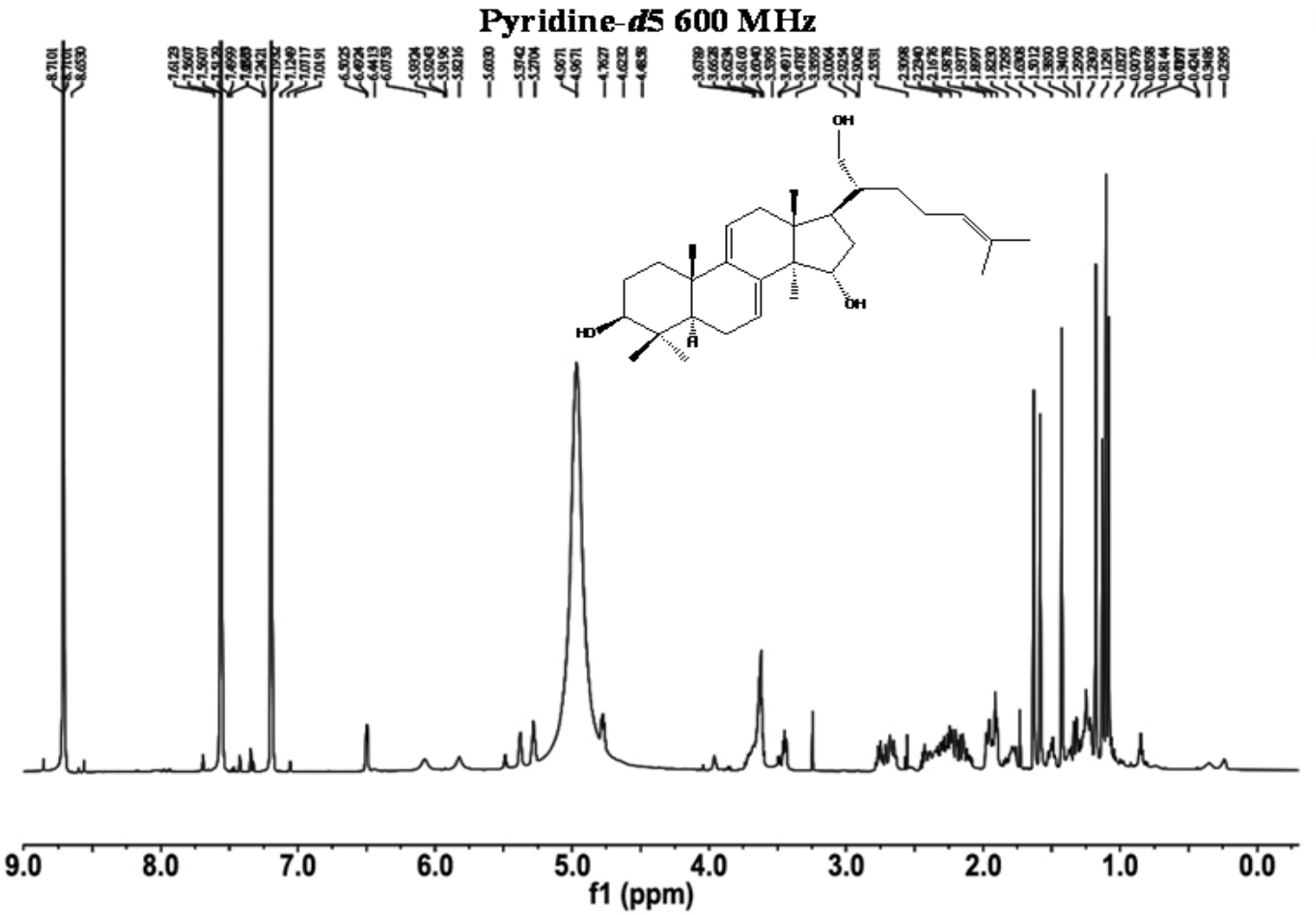
Supplementary Fig. 2. Infrared spectrum of compound **1**. T, transmission.



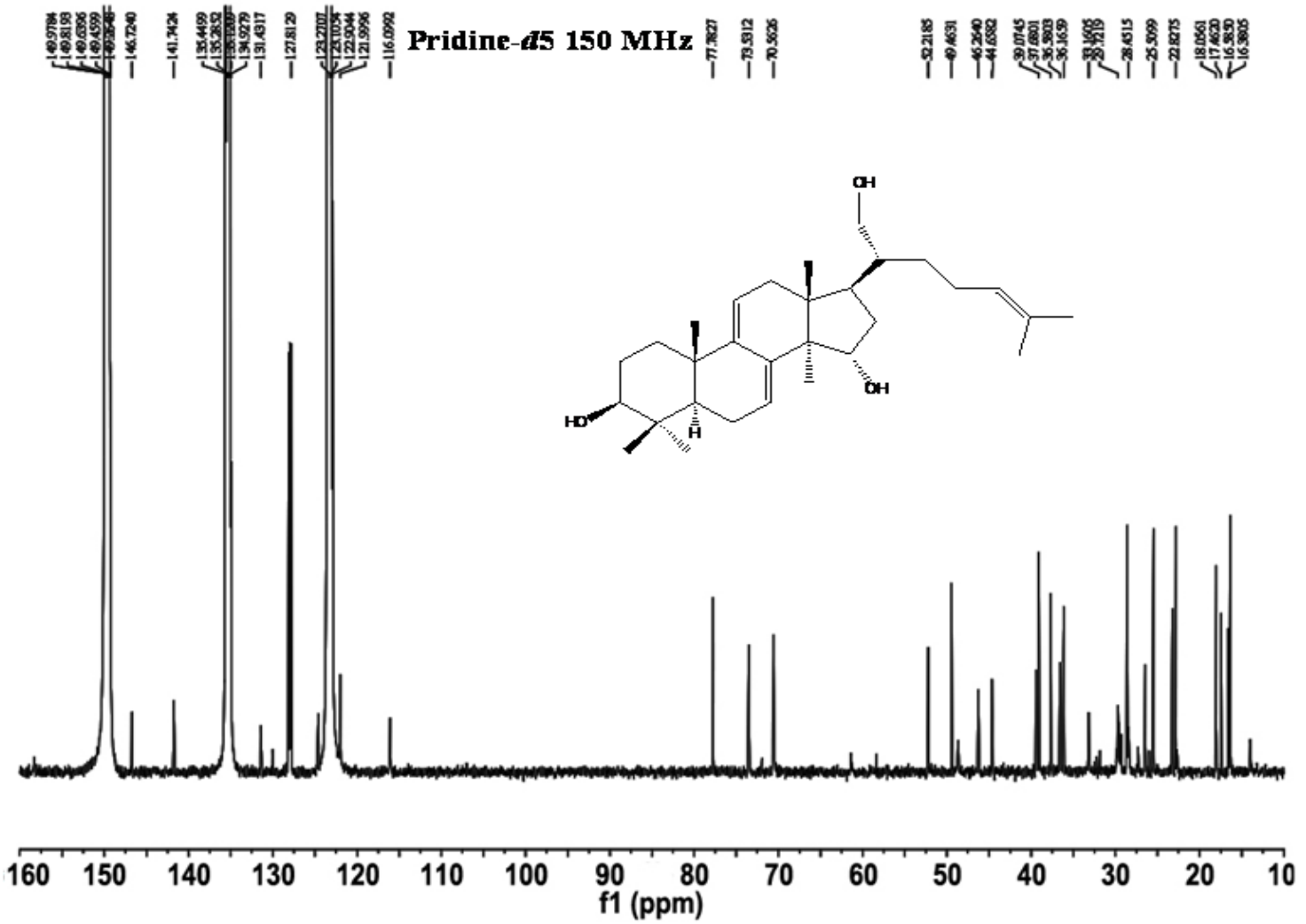
[Result of Peak Picking]

No.	Position	Intensity	No.	Position	Intensity	No.	Position	Intensity	No.	Position	Intensity
1	3838.61	98.4293	2	3748.94	97.6835	3	3422.06	92.841	4	2929.34	92.8196
5	2361.41	94.1904	6	2338.27	96.0409	7	1686.44	93.8285	8	1651.73	95.9824
9	1520.6	98.3144	10	1456.96	96.5502	11	1379.82	96.5177	12	1276.65	96.0518
13	1198.54	96.7388	14	1091.51	94.6498	15	1050.05	93.8881	16	898.666	97.9617
17	859.132	97.9555	18	815.742	97.7136	19	759.816	97.9213	20	717.39	97.3119
21	672.071	96.7579	22	491.759	96.9333	23	458.011	96.9775	24	419.442	95.0406

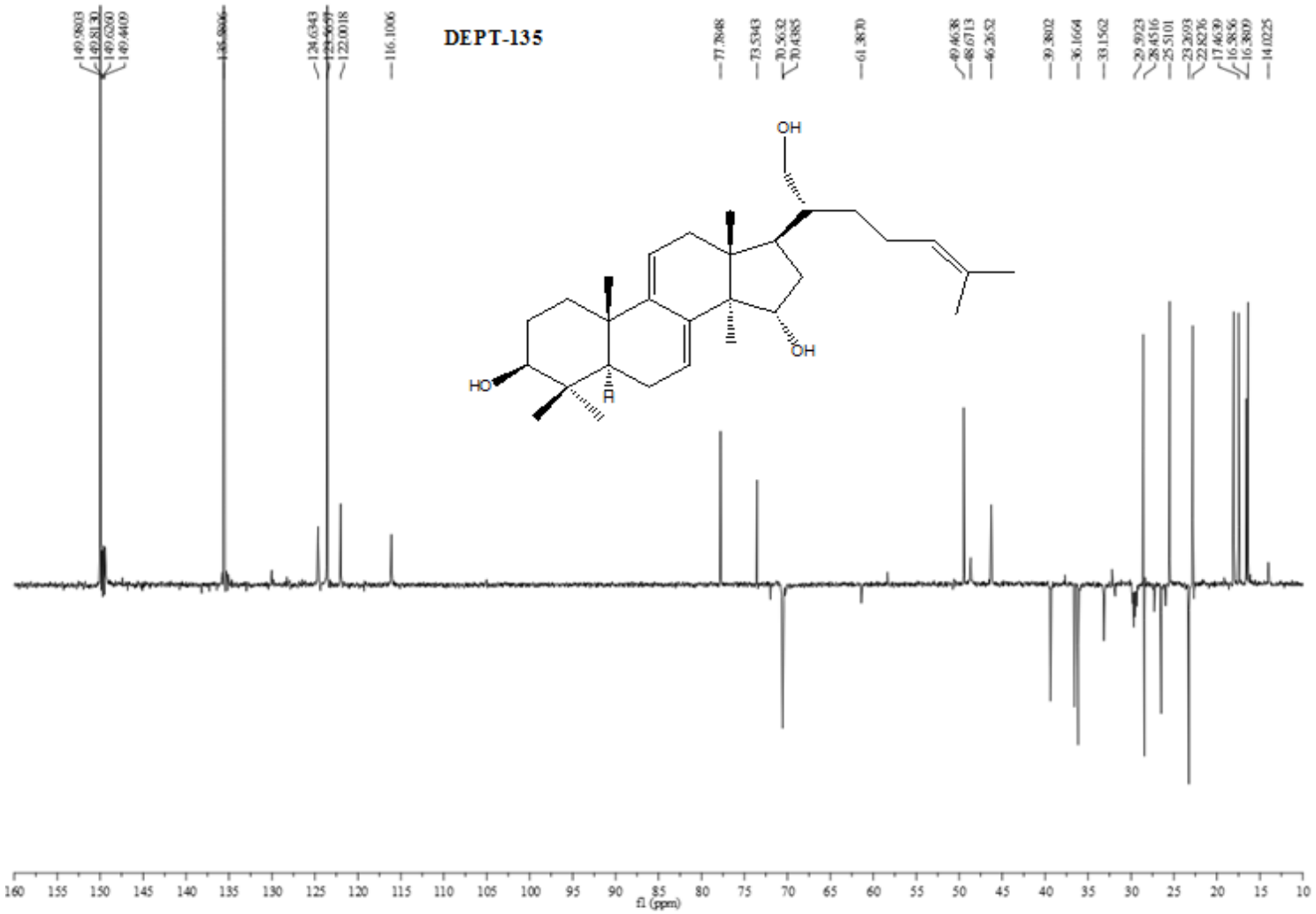
Supplementary Fig. 3. ^{1}H-NMR (pyridine-d_5, 600 MHz) spectrum of compound **1**.



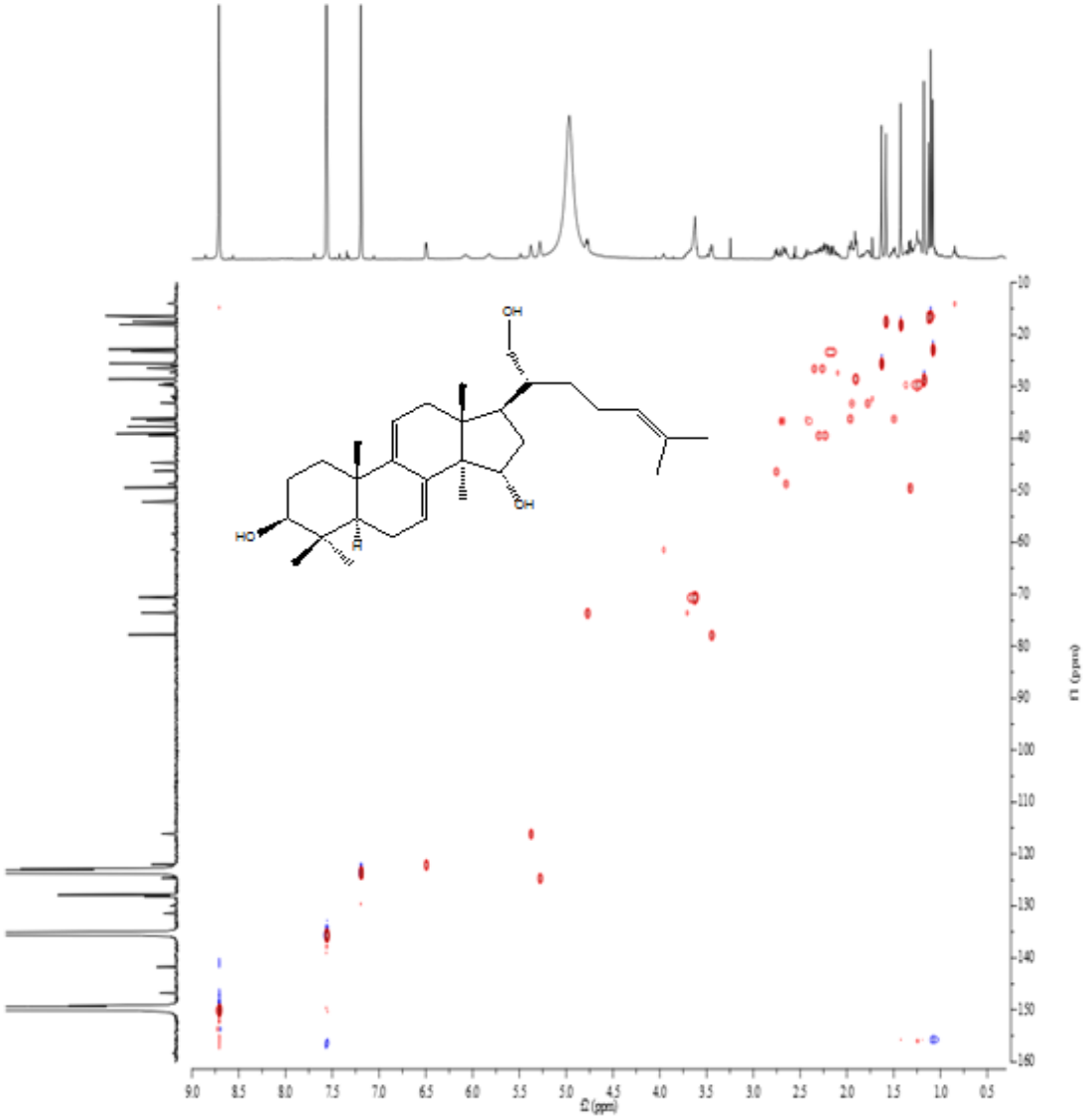
Supplementary Fig. 4. ^{13}C-NMR (pyridine-d_5, 150 MHz) spectrum of compound **1**.



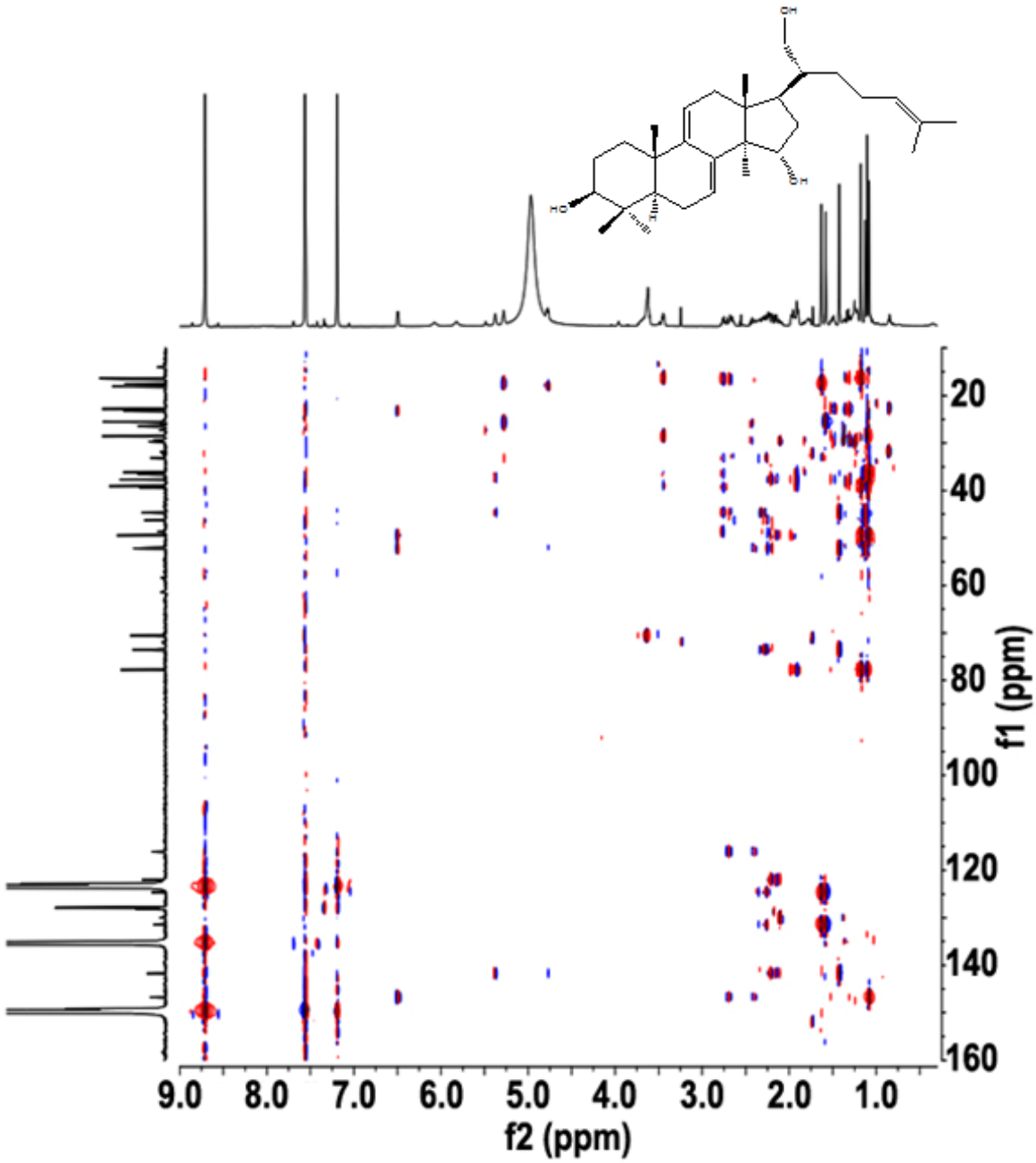
Supplementary Fig. 5. Distortionless enhancement by polarization transfer (DEPT135) spectrum of compound **1**.



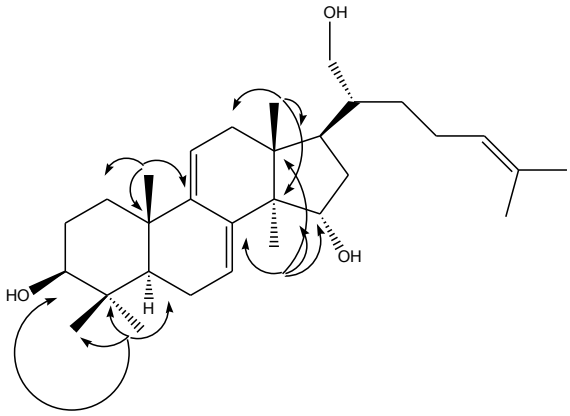
Supplementary Fig. 6. Heteronuclear single quantum correlation (HSQC) spectrum of compound **1**.



Supplementary Fig. 7. Heteronuclear multiple bond correlation (HMBC) spectrum of compound **1**.



Supplementary Fig. 8. Heteronuclear multiple bond correlations (HMBC) for compound **1**.



Supplementary Table 1. ^{1}H-NMR data of compound 1 (600 MHz in pyridine-d_5)

Position	δppm, [Multiplicity, *J* (Hz)]	
3α	3.45 (dd, *J*=10.8, 4.8 Hz)	1H
7	6.50 (d, *J*=6.0 Hz)	1H
11	5.38 (br.s, *J*=5.6 Hz)	1H
15β	4.78 (dd, *J*=10.6, 6.4 Hz)	1H
18	1.13 (s)	3H
19	1.08 (s)	3H
21α	3.61 (m)	2H
21β	3.61 (m)	2H
24	5.28 (t, *J*=6.0 Hz)	1H
26	1.58 (s)	3H
27	1.63 (s)	3H
28	1.11 (s)	3H
29	1.18 (s)	3H
30	1.43 (s)	3H

Supplementary Table 2. ^{13}C-NMR data of compound 1 (150 MHz in pyridine-d_5)

Position	δ ppm	Position	δ ppm
1	36.17 (t)	16	39.08 (t)
2	28.46 (t)	17	46.27 (d)
3	77.66 (d)	18	16.59 (q)
4	39.39 (s)	19	22.83 (q)
5	49.25 (d)	20	49.00 (d)
6	23.27 (t)	21	70.44 (t)
7	122.00 (d)	22	33.17 (t)
8	141.74 (s)	23	26.50 (t)
9	146.73 (s)	24	124.64 (d)
10	37.69 (s)	25	131.43 (s)
11	116.10 (d)	26	17.47 (q)
12	36.59 (t)	27	25.52 (q)
13	44.66 (s)	28	16.39 (q)
14	52.10 (s)	29	28.58 (q)
15	73.80 (d)	30	18.06 (q)