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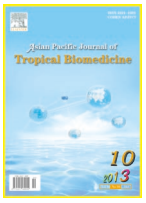

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Identification of an anticancer compound against HT-29 cells from *Phellinus linteus* grown on germinated brown rice

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PEER REVIEW

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Comments

This is a valuable research in which authors showed an anticancer compound isolated from PB which is an atractylenolide I, a eudesmane-type sesquiterpene lactone, not polysaccharides and proteoglycans.
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ABSTRACT

Objective: To isolate and identify the anticancer compound against proliferation of human colon cancer cells from ethyl acetate (EtOAc) extract of *Phellinus linteus* grown on germinated brown rice (PB).

Methods: EtOAc extract of PB was partitioned with *n*-hexane, EtOAc, and water-saturated *n*-butanol. Anticancer compound of *n*-hexane layer was isolated and identified by HPLC and NMR, respectively. Cytotoxicity against HT-29 cells was tested by SRB assay.

Results: The *n*-hexane layer obtained after solvent fractionation of PB EtOAc extracts showed a potent anticancer activity against the HT-29 cell line. Atractylenolide I, a eudesmane-type sesquiterpene lactone, a major anticancer substance of PB, was isolated from the *n*-hexane layer by silica gel column chromatography and preparative-HPLC. This structure was elucidated by one- and two-dimensional NMR spectroscopic data. Atractylenolide I has not been reported in mushrooms or rice as of yet. The isolated compound dose-dependently inhibited the growth of HT-29 human colon cancer cells.

Conclusions: Atractylenolide I might contribute to the anticancer effect of PB.

KEYWORDS
Atractylenolide I, Human colon cancer cells, NMR, *Phellinus linteus*, Germinated brown rice

1. Introduction

Colorectal cancer is one of the most commonly diagnosed cancers worldwide. During the last two decades colorectal cancer incidence rates have been rapidly increasing in Eastern Asia because of changes in dietary and lifestyle factors associated with westernization, including smoking and obesity[1,2]. Although various chemotherapeutic agents have been developed for the treatment of colorectal cancer, dietary interventions have recently attracted increasing attention from researchers and clinicians[2].

Phellinus linteus (*P. linteus*) belongs to the Hymenochaetaceae basidiomycetes and it is commonly called "Sanghwang" in Korean. *P. linteus* is a well-established medicinal mushroom that has been used to treat various human cancers in Asia[3,4]. We have previously produced *P. linteus* grown on germinated brown rice (PB) to enhance its biological effects, such as antitumor, immunomodulatory, and antioxidant activities. Indeed, PB has been shown to have a stronger immunomodulating

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activity compared to *P. linteus*, through modulation of the balance of Th1/Th2 cytokines in lymphocytes[5,6]. Also, we have recently shown that PB has antitumor activities which inhibit tumor metastasis in mice and induce apoptosis associated with cell cycle arrest at the G_0/G_1 phase in human colon cancer cells[7,8]. However, the anticancer activity ascribed to PB is limited to crude extracts and fractions and it is not known yet which compounds are responsible for the anticancer effect.

P. linteus contains various bioactive substances, including polysaccharides and proteoglycans, which have been shown to exert anticancer and immune-regulatory properties[9]. Although most studies have focused on polysaccharides as active ingredients responsible for the antitumor effects of *P. linteus*[10-12], our previous studies, interestingly, indicated that the anticancer activity of PB was observed in ethyl acetate extract, but not in boiled-water[8]. These observations suggested that the major anticancer constituent of PB might be retained in the nonpolar organic solvent fraction rather than in the polysaccharide-rich aqueous fraction.

In the current study, we isolated and identified atractylenolide I as the anticancer compound against the HT-29 human colon cancer cell line from ethanol (EtOH) and ethyl acetate (EtOAc) extracts of PB. Atractylenolide I is a eudesmane-type sesquiterpene lactone and one of the major bioactive components from *Atractylodes macrocephala*[13]. This is the first report on the isolation of anticancer compound from the PB.

2. Materials and methods

2.1. Chemicals

RPMI-1640 and fetal bovine serum were purchased from JRH bioscience (Lenexa, KS). Dimethyl sulfoxide, trichloroacetic acid and sulforhodamine B were purchased from Sigma Chemical Co. (St. Louis, MO).

2.2 Materials

P. linteus, grown on germinated brown rice (PB), was provided by Cell Activation Research Institute (CARI, Korea). This commercial preparation was made according to an original method, as described previously[14].

2.3. Extraction and solvent fractionation

PB (3 kg) was extracted with EtOH (36 L) for 1 d at room temperature. After filtration with No. 2 paper filter (GE healthcare, PA), the residue was extracted with EtOAc (24 L and 30 L, respectively) for 2 d at room temperature and was then filtered again. The EtOH and EtOAc extracts were combined and concentrated by a vacuum rotary evaporator (EYELA, Tokyo, Japan) at 40 °C. The mixture (55 g) of PB extracts obtained by EtOH and EtOAc extraction was suspended with 3 L of distilled water and was then successively partitioned with *n*-hexane (3 L, 3 times), EtOAc (3 L, 3 times), and water-saturated *n*-butanol (BuOH, 3 L, 3 times). Each layer was concentrated by a vacuum rotary evaporator (EYELA, Tokyo, Japan) at 40 °C.

2.4. Isolation and purification

The portion (14.8 g) of the *n*-hexane layer was chromatographed on a silica gel (70-230 mesh, Merck, Germany) column (7.7×67.0 cm) and was then eluted in a step-wise manner using *n*-hexane/EtOAc=10:0, 8:2, 6:4, 4:6, 2:8, and 0:10 (v/v, each 6 L). Fraction G (3 g) was subjected to HPLC equipped with a Pak SIL (10×250 mm, 5 μm, YMC, Kyoto, Japan). The solvent mixture of *n*-hexane/isopropyl alcohol (IPA)/EtOH=1 000:0.7:0.07 (v/v) was used as a mobile phase. The flow rate was 4 mL/min and the eluates were monitored at 210, 254, 280, and 365 nm. Fraction G5 (t_R 19.5 min, 197 mg) was further purified by silica gel column-HPLC with the same conditions as described above. Fraction G5-B (t_R 25.9 min, 17 mg) was finally purified by HPLC (Prevail Amino, 250×4.6 mm, *n*-hexane/IPA/EtOH=10:0.3:0.05, v/v/v, Alltech, Deerfield, IL). The flow rate was 1 mL/min and the eluates were monitored at 210, 254, 280, and 365 nm (Dionex, München, Germany).

2.5. NMR analysis

The ^{1}H- and ^{13}C-NMR spectra were measured in C_6D_6 and $CDCl_3$ with an INOVA 500 spectrometer (500 MHz, Varian, Walnut creek, CA, USA), using *tetra*-methylsilane (TMS, δ=0) as an internal standard.

2.6. Cytotoxicity assay

Human colon carcinoma (HT-29) and human fibroblast normal (CCD-986sk) cell lines were obtained from Korean Cell Line Bank (KCLB, Korea). The cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum at 37 °C in 5% CO_2 incubator. The cells (5×10^4 cell/mL) were seeded in 96-well culture plates and were incubated overnight. Samples were dissolved in dimethyl sulfoxide and were diluted at the appropriate concentrations with cultivation medium added to triple culture wells. After cultivation for 48 h, the cells were fixed by 50% trichloroacetic acid and were incubated at 4 °C for 1 h. The cells were then stained with 0.1% SRB solution containing 1% acetic acid. After washing with 1% acetic acid solution, the bound protein stain was solubilized with 10 mmol/L unbuffered Tris base. The absorbance was measured at 492 nm with an ELISA reader (Tecan, Austria). IC_{50} values were calculated with Prism 5.0 (GraphPad, CA) software using the sigmoidal dose-response function.

2.7. Statistics

The data are presented as mean±SEM. Significance of differences between means was tested using the Student's *t*-test and was assumed for $P<0.05$. The statistical software package Prism 5.0 (GraphPad Software) was used for these

analyses. All the experiments were performed in triplicate.

3. Results

3.1. Anticancer activity of PB extracts and its solvent-fractionated layers

The mixture (55 g) of PB extracts obtained by EtOH and EtOAc extraction was suspended with H_2O and successively partitioned with *n*-hexane, EtOAc, and BuOH to give their soluble extracts: *n*-hexane (15.1 g), EtOAc (6.5 g), BuOH (5.9 g), and H_2O (19 g) layers. The anticancer activities of PB extracts and its solvent-fractionated layers were evaluated using the human colon cancer cell line, HT-29, and normal cell line, CCD-986sk. The PB extracts reduced the percentage of viable HT-29 cells in a dose-dependent manner and its IC_{50} value was 149.9 μg/mL. Interestingly, the *n*-hexane layer among the layers obtained after the solvent fractionation of the PB extracts showed the highest anticancer activity and its IC_{50} value was 69.8 μg/mL (Table 1). However, no toxicities were observed for normal cells treated with concentrations of the PB extracts, and its solvent-fractionated layers up to 1 000 μg/mL. The *n*-hexane layer may contain a potent anticancer compound of PB extracts against HT-29. Therefore, the purification and isolation of anticancer compounds in the *n*-hexane layer was performed.

Table 1
Cytotoxicity of PB against HT-29 cell line.

Cell line	IC_{50} (μg/mL)				
	PB	*n*-hexane	EtOAc	BuOH	water
HT-29 (human colon carcinoma)	149.9	69.8	77.8	>1 000	>1 000
CCD-986sk (normal cell)	>1 000	>1 000	>1 000	>1 000	>1 000

Cells were incubated with various concentrations of extract and fractions for 48 h and were subjected to SRB assay. The data were obtained from three independent experiments. IC_{50} is the concentration at which 50% of cells are no longer viable.

3.2. Isolation of anticancer active compound

The *n*-hexane layer (14.8 g) was chromatographed on a silica gel column chromatography eluting with a step-wise system of *n*-hexane/EtOAc/MeOH to give 16 fractions (A-P). Fraction G (3.0 g, *n*-hexane/EtOAc=8:2, v/v) was injected into the silica gel column-HPLC (*n*-hexane/IPA/EtOH=10:0.3:0.05, v/v) to obtain 8 fractions (G1-G8). Fraction G5 (t_R 19.5 min, 197 mg) was further purified by silica gel column-HPLC under the same conditions, to give 2 subfractions (G5-A and G5-B). Fraction G5-B (t_R 25.9 min, 17 mg) was finally purified by prevail amino column-HPLC (*n*-hexane/IPA/EtOH=10:0.3:0.05, v/v) to obtain G5-B4 (t_R 4.3 min, 3.0 mg).

3.3. Structural elucidation of the active compound

The structure of the isolated compound (G5-B4) was elucidated by ^{1}H- and ^{13}C-NMR, ^{1}H-^{1}H COSY, HSQC, HMBC, and NOESY experiments.

The ^{13}C-NMR (125 MHz, C_6D_6) and HSQC spectra of G5-B4 exhibited the presence of 15 carbons, including a carbonyl carbon (δ 171.0), six sp^2 carbons (δ 148.9-107.6), two methyl carbons (δ 18.8 and 8.7), four sp^3 methylene carbons (δ 39.4, 23.6, 36.8, and 22.8), a methine carbon (δ 48.6), and a quaternary carbon (δ 38.2). These results were supported by the observations of proton signals of a typical sesquiterpene in the ^{1}H-NMR (500 MHz, C_6D_6) spectrum. In particular, the proton signals of sesquiterpene, including germinal sp^3 methylene proton signals at δ 4.75 (1H, dd, *J*=1.5, 3.0 Hz, H-14a) and 4.40 (1H, dd, *J*=1.5, 3.0 Hz, H-14b), a sp^2 methine proton signal at δ 5.20 (1H, s, H-9), a sp^3 methine proton [δ 1.81 (1H, m, H-5)], and two methyl proton signals at δ 1.56 (3H, d, *J*=2.0 Hz, H-13) and δ 0.65 (3H, s, H-15) were observed. From the 1D-NMR and HSQC spectra, G5-B4 was suggested to be a tricyclic eudesmanolide-type sesquiterpene. A partial structure of three continuous methylene protonated carbons (C-1~C-3) was assigned from the cross peaks between δ 1.36 (2H, m, H-2) and δ 1.18 (2H, m, H-1), 2.11 (1H, H-3a), and 1.75 (1H, H-3b) detected in the ^{1}H-^{1}H COSY spectrum. In addition, the cross peaks of a methine proton [δ 1.81 (1H, H-5)] and methylene proton [δ 2.08 (1H, H-6a) and 1.97 (1H, H-6b)] were observed. The long-range correlations between protons and carbons were determined by the HMBC experiment. The important correlations (Figure 1, arrows) between protons and carbons are shown in Figure 1. In particular, the position of two methyl groups was assigned by the presence of the cross peaks from H-15 (δ 0.65) to C-9 (δ 118.1), C-5 (δ 48.6) and C-1 (δ 39.4) and from H-13 (δ 1.56) to C-12 (δ 171.0) and C-7 (δ 147.8). In addition, the C-14 position of the olefinic methylene group was decided by the correlations between H-14a (δ 4.75) to C-3 (δ 36.8) and C-5 (δ 48.6). The *S* configurations (Figure 1) of the C-5 methine proton and that of the C-10 methyl group were deduced from the correlations between the methyl group (δ 0.65, H-14) and δ 1.36 (H-2), 2.11 (H-3a), and 2.08 (H-6a), as well as between the methine proton (δ 1.81, H-5) and δ 1.75 (H-3b), as observed in the NOESY spectrum. The structure of G5-B4 was determined to be 8-eudesmatrienolide (atractylenolide I). The ^{13}C-NMR ($CDCl_3$) data of G5-B4 was also consistent with that of atractylenolide I which was reported previously (Table 2)[15,16]. Therefore, G5-B4 was unambiguously identified as (4a*S*,8a*S*)-4a,5,6,7,8,8a-hexahydro-3,8a-dimethyl-5-methylene-naphtho[2,3-b]furan-2(4H)-one (atractylenolide I).

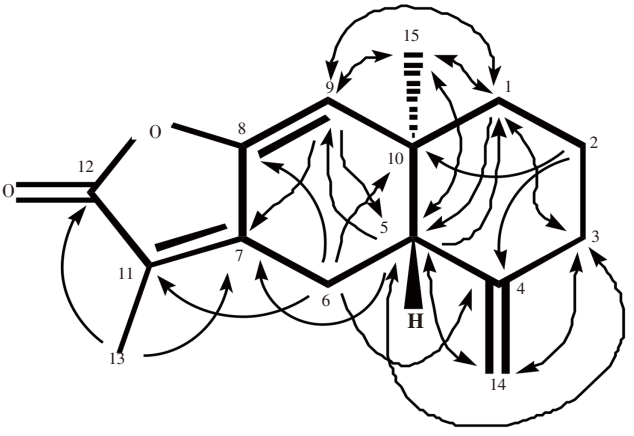

Figure 1. Important HMBC correlations (arrows) and structure of G5-B4.

Table 2.
^{1}H- (500 MHz) and ^{13}C-NMR (125 MHz) data for G5-B4 and reference compound

Position	δH (int, mult, J in Hz)[a]	δ_c[a]	δ_c[b]	Reference compound (δ_c)
1	1.18 (2H, m)	39.4	39.1	39.1
2	1.36 (2H, m)	23.6	23.0	23.0
3a	2.11 (1H, m)	36.8	36.2	36.2
3b	1.75 (1H, m)			
4		148.6	148.0	148.0
5	1.81 (1H, m)	48.6	48.4	48.4
6a	2.08 (1H, d, 3.5)	22.8	22.7	22.7
6b	1.97 (1H, m)			
7		147.8	148.3	148.3
8		148.9	148.1	148.1
9	5.20 (1H, s)	118.1	119.2	119.1
10		38.2	38.1	38.1
11		121.2	120.4	120.5
12		171.0	171.4	171.2
13	1.56 (3H, d, 2.0)	8.7	8.5	8.5
14a	4.75 (1H, dd, 1.5, 3.0)	107.6	107.5	107.5
14b	4.40 (1H, dd, 1.5, 3.0)			
15	0.65 (3H, s)	18.8	18.6	18.6

[a]The ^{1}H- and ^{13}C-NMR spectra of G5-B4 were measured in C_6D_6.
[b]The ^{13}C-NMR spectrum of G5-B4 was measured in $CDCl_3$.

3.4. The cytotoxicity of atractylenolide I against HT-29

To investigate the effect of atractylenolide I on cytotoxicity against HT-29, the cells were treated with various concentrations of atractylenolide I for 48 h. As shown in Figure 2, treatment of atractylenolide I significantly reduced the percentage of viable cells in a dose-dependent manner.

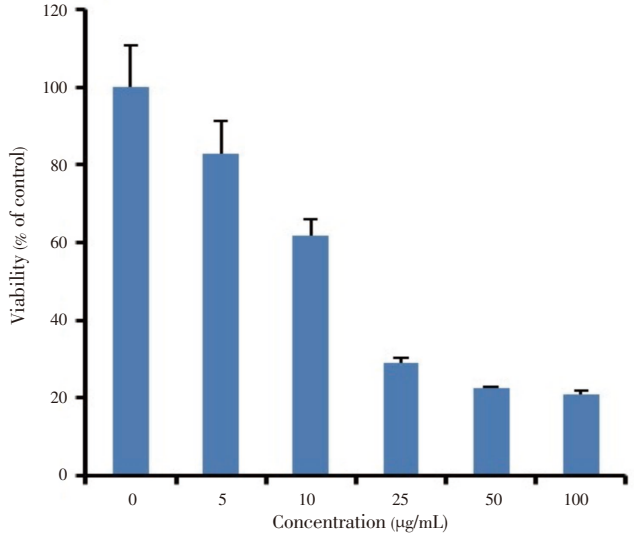

Figure 2. Effects of atractylenolide I on the viability of HT-29 cells.
The cells were incubated with different concentrations of atractylenolide I for 48 h, and cell viability was then determined using the SRB assay by measuring the absorbance at 492 nm. Values are expressed as means±SEM of three independent experiments.

4. Discussion

Various bioactive properties of PB have been explored and attracted considerable attention[5-8,14]. Because PB was grown under new cultivation condition, we expected a possibility for formation of new antitumor active compound through specific biosynthesis pathway. Therefore, present study was undertaken to isolate an active compounds related to antitumor activity using cytotoxicity-leaded purification method from PB.

We initially investigated cytotoxic activity of PB extracts and its solvent-fractionated layers against HT-29 and purified the *n*-hexane layer by silica gel column chromatography and preparative-HPLC. The structure of the isolated active compound was elucidated to be atractylenolide I, a eudesmane-type sesquiterpene lactone, by one- and two-dimensional NMR spectroscopic data. Atractylenolide I is well known to be one of the main compounds in *Atractylodes* spp. and has also been found in *Solarum lyratum*[15,16]. However, this compound was identified from PB for the first time, and the occurrence of atractylenolide I in mushrooms including *P. linteus* and rice has not been reported until now.

Anti-inflammation and anti-cancer properties of atractylenolide I have been well-documented[17,18]. Moreover, in clinical studies, atractylenolide I can be beneficial for treating gastric cancer cachexia by modulating cytokine production and proteolysis[19]. These observations have suggested that atractylenolide I may act as a promising anti-cancer agent.

In conclusion, we isolated and identified atractylenolide I as an anticancer active compound from PB and it may contribute to the anti-inflammatory and chemopreventive effects of PB.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

This study was supported by a grant from the Korea Food Research Institute (Grant number: E0131601).

Comments

Background

P. linteus belongs to the family of Hymenochaetaceae basidiomycests, and it is a well-established medicinal mushroom that has been used to treat various human cancers and has been shown to have an immune-modulating activity through various bioactive substances, including polysaccharides and proteoglycans.

Research frontiers

The research is being performed in order to investigate the significant anti-cancering substances from PB. Atractylenolide I, a eudesmane-type sesquiterpene lactone and a major anticancer substance, was isolated from the *n*-hexane layer by silica gel column chromatography and preparative-HPLC.

Related reports

P. linteus contains various bioactive substances, including polysaccharides and proteoglycans, which have been shown to exert anti-cancer and immune-regulatory properties Therefore, almost physiological researches of *P. linteus* showed the various functional effects due to just polysaccharides and proteoglycans.

Innovations and breakthroughs

This study suggested that the major anticancer constituent of PB might be retained in the non-polar organic solvent fraction rather than in the polysaccharide-rich aqueous fraction.

Applications

P. linteus is commonly called 'Sanghwang' in Korea, and a well-established medicinal mushroom in Asia. This scientific research suggests the use of this material as functional food resources.

Peer review

This is a valuable research in which authors showed an anticancer compound isolated from PB which is an atractylenolide I, a eudesmane-type sesquiterpene lactone, not polysaccharides and proteoglycans.

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