생활과학석사학위논문

Composition of Triterpenoids in *Inonotus obliquus* and Their Anti-Proliferative Activity on Cancer Cell Lines

차가버섯(*Inonotus obliquus*)에 함유되어 있는
Triterpenoid의 조성과 암세포 증식 억제능

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ABSTRACT

Composition of Triterpenoids in *Inonotus obliquus* and Their Anti-Proliferative Activity on Cancer Cell Lines

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Inonotus obliquus has been known to have biological activities such as anti-cancer, anti-inflammatory, and anti-oxidant activities. Although triterpenoids in *I. obliquus* have been reported to have anti-proliferative activity, triterpenoids in outer part of *I. obliquus* have been little studied. The aim of the study was to determine composition of triterpenoids in the extract from inner and outer parts of *I. obliquus* and to evaluate their anti-proliferative activity against HT-29, AGS, MCF-7, and PC-3 cancer cells. Dried *I. obliquus* powder was extracted using 80% methanol. Triterpenoid fraction was obtained from the methanol extract using a Diaion HP-20

column. Betulin, betulinic acid, inotodiol, and trametenolic acid were identified in the triterpenoid fractions. Inotodiol and trametenolic acid were major triterpenoids in both of the inner and outer parts of *I. obliquus*. The amount of triterpenoids in the outer part of *I. obliquus* (369.9 ± 10.7 mg/g fraction, dry basis) was significantly higher than that of the inner part (324.3 ± 22.3 mg/g). The triterpenoid fractions had dose-dependent anti-proliferative activity against AGS, MCF-7, and PC-3 cells. Anti-proliferative activity of the triterpenoid fraction from the outer part against the cancer cells was also significantly higher than that of the inner part. The outer part of *I. obliquus*, a by-product of the mushroom from extraction process, may be used as a functional material for foods or drugs.

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INTRODUCTION

Inonotus obliquus, called chaga in Russia and kabanoanatake in Japan, is a white rot fungus that belongs to the family of Hymenochaetaceae (Handa et al., 2012). It has been used as a traditional medicine without toxicity in Russia (Reid, 1976) because it has been known to have biological activities such as anti-cancer (Kim et al., 2006), anti-inflammatory (Park et al., 2005), and anti-oxidant (Wang et al., 2001) activities. Especially, anti-cancer activity of *I. obliquus* has been studied since the sixteenth century. Previous studies have reported that triterpenoids in *I. obliquus* have a significant anti-cancer effect on various cancer cells (Kahlos et al., 1986; Shin et al., 2001). Ma et al. (2013) also reported that petroleum ether fraction from *I. obliquus,* which has triterpenoid compounds, was more active against cancer cells than other solvent extracts of *I. obliquus*. However, most of these studies were focused on crude extracts or organic solvent fractions of *I. obliquus*. It cannot be concluded that the anti-cancer activity is directly related to triterpenoids in *I. obliquus* because phenolic compounds, polysaccharides, and proteins are also present in crude extracts. Therefore, fractionation process may be needed to demonstrate anti-cancer effect of triterpenoids in *I. obliquus*. Solid phase extraction can be a more appropriate method than solvent fractionation to obtain triterpenoid fraction in the extracts. Oleszek et al. (2002) used a C18 micro-columns to obtain triterpenoid saponins from

Trifolium seeds. Li et al. (2007) also used a Diaion HP-20 column to remove phenolic compounds and obtain a triterpenoid fraction from *Acanthopanax senticosus*.

The outer part of *I. obliquus* is usually discarded before extraction or pulverization in the industry because it was reported to have no biological activities (Bazyleva et al., 1958). Nakajima et al. (2007) also reported that anti-oxidant activity of *I. obliquus* was higher in the extract of fruiting body (inner part) than that of sclerotium (outer part). Zhong et al. (2009), however, reported that triterpenoids, which have anti-virus activity, exist mainly in the outer surface of *I. obliquus*. Triterpenoid composition and biological activities of triterpenoids in the outer part of *I. obliquus* have been little studied.

The purpose of this study was to identify triterpenoids in the inner and outer parts of *I. obliquus*. In addition, anti-proliferative activities of methanol extracts and triterpenoid fractions from the inner and outer parts of *I. obliquus* were comparatively determined.

MATERIALS AND METHODS

1. Chemicals and reagents

Betulin, betulinic acid, ursolic acid, and vanillin were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Inotodiol was purchased from ALB Technology Ltd. (Henderson, NV, USA). Diaion HP-20, formic acid, glacial acetic acid (99.7%), and perchloric acid (70%) were purchased from Samchun Chemical Co. (Seoul, Korea). Acetonitrile, *n*-butanol, and methanol were from JT Baker (Phillipsburg, NJ, USA). Cancer cell lines (AGS, HT-29, MCF-7, and PC-3) and macrophage cell line (RAW 264.7) were obtained from Korea Cell Line Bank (Seoul, Korea). Roswell Park Memorial Institute (RPMI) 1640, Dulbecco's Modified Eagle Medium (DMEM), and phosphate buffered saline (PBS) were purchased from GIBCO Invitrogen (Grand Island, NY, USA). Fetal bovine serum (FBS) and penicillin/streptomycin were purchased from WelGENE Inc. (Daegu, Korea) and GE Healthcare Life Sciences (South Logan, UT, USA), respectively. N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS), dimethyl sulfoxide (DMSO), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT), and pyridine were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

2. Material

Inner and outer parts of *I. obliquus* collected in Tyumen, Russia and provided from DHF Co. (Seoul, Korea) were ground to fine powder and stored at 15 ºC with vacuum packing until used for extraction.

3. Extraction and fractionation

The dried *I. obliquus* powder (20 g) was refluxed with 180 mL 80% methanol for 24 h in a water bath (Daihan Scientific Co., Seoul, Korea) at 80 ºC. The extract was filtered twice through a Whatman No. 4 filter paper (Whatman International Ltd., Maidstone, England). Some of the filtrate was dried and stored before used for anti-proliferative activity assay and designated as methanol extract. The rest of the filtrate was fractionated by method of Shin et al. (2001) with a slight modification. Briefly, the solvent in the filtrate was evaporated under reduced pressure at 40 ºC to be 100 mL and 100 mL water was added to the concentrated filtrate. The solution (200 mL) was extracted twice with water-saturated *n*-butanol (200 mL) in separatory funnel, followed by collecting *n*-butanol layer. Meanwhile, a glass column (inner diameter, 5 cm; length, 25 cm) was filled with Diaion HP-20 dispersed in methanol (200 mL) and left for 24 h in order to eliminate bubble and impurities. The column was washed with water and methanol (1 L, each) just before loading the sample, followed by subjecting the collected *n*-butanol layer into the column. The column was washed with

50% methanol (500 mL) and sequentially eluted with absolute methanol (500 mL) to obtain triterpenoid fraction. The triterpenoid fraction was lyophilized and stored at -20 ºC until further analysis. The dried triterpenoid fractions from the inner and outer parts of *I. obliquus* were designated as IPTF and OPTF, respectively.

4. Determination of total triterpenoids

Total triterpenoids were determined according to the colorimetric method described by Zhang and Qu (2013). The extract dissolved in methanol (10 mg/mL) was vigorously shaken and centrifuged at 5000 × g for 20 min. The supernatant (100 µL) was mixed with 150 µL 5% (w/v) vanillin-glacial acetic acid and 500 µL 70% perchloric acid. The solution was heated for 45 min at 60 ºC and cooled to room temperature. Glacial acetic acid (2.25 mL) was added to the solution and absorbance was measured at 548 nm using a UV-vis spectrometer (Spectramax 190, Molecular Devices, Sunnyvale, CA, USA). Total triterpenoids were expressed as ursolic acid equivalent (UAE)/g extract on dry basis.

5. Analysis of triterpenoid composition

The IPTF or OPTF dissolved in methanol (10 mg/mL) was agitated using a sonicator (5510E-DHT, Bransonic, Danbury, CT, USA). The solution was centrifuged at 8000 × g for 20 min at 4 ºC and the supernatant was filtered

with a 0.2 μm nylon syringe filter (Whatman International Ltd., Maidstone, England) and stored at -20 ºC until analyzed.

Triterpenoids were identified using an LTQ XL (Thermo Fisher Scientific, Waltham, MA, USA) connected to an Ultimate 3000 RS system via ESI interface. Stationary phase was a U-VDSpher PUR C18-E (1.8 μm, 100 × 2.0 mm, VDS Optilab, Berlin, Germany) and mobile phases were water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B). Flow rate was 0.3 mL/min with a gradient as follows: 0 min, 90% B; 0-10 min, 100% B; 10-22 min, 100% B; 22-23 min, 90% B; and 23-30 min, 90% B. Injection volume was 5 μL. Mass parameters were set as follows: detection ion mode, positive ([M+H, Na]$^+$); scan range, m/z 100-2000; capillary temperature, 300 ºC; source voltage, 3.5 kV; sheath gas flow, 42 arbitrary unit; and software, X-Calibur 2.0 (Thermo Fisher Scientific, Waltham, MA, USA).

Triterpenoids in *I. obliquus* were quantified using an HPLC (Waters Alliance 2695, Waters, Milford, MA, USA) equipped with a ZORBOX Eclipse Plus C18 (5 μm, 4.6 × 250 mm, Agilent, CA, USA). Mobile phases were water (solvent A) and acetonitrile (solvent B). Flow rate was 1.0 mL/min with a gradient as follows: 0 min, 90% B; 0-10 min, 97% B; 10-30 min, 97% B; 30-30.1 min 90% B; and 9.9 min reconditioning. Injection volume was 20 μL. Detection wavelength was set at 206 nm. Column oven was maintained at 30 ºC. Triterpenoids were quantified by comparing peaks

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of corresponding standards on HPLC chromatogram.

6. Analysis of phytosterols

The triterpenoid fraction was silylated to obtain trimethylsilyl (TMS) derivatives of phytosterols as follows: The freeze-dried triterpenoid fraction was mixed with hexane at 1 mg/mL and sonicated for 30 min. The mixed solution was filtered through a 0.2 μL nylon filter and transferred into a vial. The solvent of the filtrate in the vial was evaporated to dryness using nitrogen gas. The dried filtrate was mixed with 300 μL BSTFA containing 1% TMCS and 200 μL pyridine, followed by incubation at 60 ℃ for 30 min and stored at -20 ℃ until analyzed.

TMS-derivatized phytosterols were analyzed using a QP 2010 Plus gas chromatograph (Shimazdu Co., Kyoto, Japan) equipped with a DB-5 capillary column (30 m × 0.25 mm i.d., 0.25 μm, J&W Scientific, Folsom, CA, USA). Split ratio was 1:15 and injection volume was 1 μL. Flow rate was 1 mL/min and carrier gas was helium. Temperature of transfer line from GC to mass detector was 250 ℃. Injector and detector temperatures were 280 ℃. The column temperature was as follows: initially set at 100 ℃ and held for 3 min, from 100 ℃ to 275 ℃ at 15 ℃/min held for 25 min, and finally increased to 280 ℃ at 3 ℃/min held for 3 min. The mass spectrometer was run in electron impact ionization mode with 70 electron voltage. Data were scanned in *m/z* range of 50-650. TMS-derivatized phytosterols were identified by comparing

standard mass spectra and similarity indices in National Institute of Standards and Technology (NIST) 08 MS library.

7. Cell culture

AGS human gastric adenocarcinoma cells, HT-29 colorectal adenocarcinoma cells, MCF-7 breast adenocarcinoma cells, PC-3 prostatic adenocarcinoma cells were used for anti-proliferative activity assay. These cell lines were cultured in RPMI 1640 containing 10% FBS and 1% penicillin/streptomycin. RAW 264.7 murine macrophage cells were used as a normal cell line. The cells were cultured in DMEM containing 10% FBS and 1% penicillin/streptomycin. All the cell lines were incubated in 5% CO_2 at 37 ℃ and the cells with passage numbers between 2 and 10 were used for assay.

8. Anti-proliferative activity

MTT assay was conducted to confirm anti-proliferative activity of the IPTF and OPTF. For comparison, 80% methanol extract of *I. obliquus* was also used for the assay. Briefly, 2×10^4 cells/well were seeded in a 96 well plate and incubated for 24 h in 5% CO_2 at 37 ℃. Methanol extracts and triterpenoid fractions were dissolved with DMSO (never excess 0.1%) and diluted with non-serum medium. Various concentrations (100-300 μg/mL) of the samples were treated on the cells and incubated for 24 h. The control

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was treated with non-serum medium containing 0.1% DMSO. After the exposure period, the medium was discarded and MTT solution (100 μL) was added to each well and incubated for 4 h. The MTT solution was replaced by 100 μL DMSO to solubilize formazan, followed by incubation for 20 min at room temperature. Absorbance was measured at 540 nm. Cell viability was calculated as percentage viability: (absorbance of treated cells/ absorbance of control cells) × 100.

9. Statistical analysis

All experiments were performed in triplicate. The results were expressed as means ± standard deviations. Data were subjected to one-way analysis of variance (ANOVA), Duncan's new multiple range test ($p<0.05$) and Student's t-test ($p<0.05$ and $p<0.01$) using a SPSS 23.0 software (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Extraction yields of the IPTF and OPTF were 0.51 ± 0.04, and $0.78 \pm 0.14\%$ on a dry basis, respectively (Table 1). Extraction yield of the OPTF in this study was quite similar to that of an ethyl acetate extract $(0.72 \pm 0.03\%$, dry basis) in a previous study (Wang et al., 2014). The previous study also reported that the extraction yield of bark from *I. obliquus* was higher than that of its inside.

1. Total triterpenoids in *I. obliquus*

Total triterpenoids in the OPTF (554.5 ± 13.9 mg UAE/g extract) were significantly higher ($p<0.05$) than those in the IPTF (469.2 ± 10.2 mg UAE/g extract), even when calculated on the basis of the raw materials (Table. 1). Wang et al. (2014) also reported a similar result that total triterpenoids in methanol extracts of birch core and bark of *I. obliquus* were 2.36 and 2.43 mg/g raw material, respectively.

However, the method for determining total triterpenoids in the present study may not exclusively detect triterpenoids only because the reagents (perchloric acid and vanillin) used in the method can react with other compounds which have unsaturated bonds (Zhang and Qu, 2013). To more accurately determine triterpenoids, LC-MS was performed.

Table 1. Extraction yields and total triterpenoid contents in *I. obliquus*

Fraction	Yield (%, w/w)	Triterpenoid content (mg UAE/g)	
	Dry basis of *I. obliquus*	Dry basis of fraction	Dry basis of *I. obliquus*
IPTF	0.51±0.04	469.2±10.2	2.51±0.04
OPTF	0.78±0.14[*]	554.5±13.9[*]	4.32±0.11[*]

Values are expressed as means ± S.D. (*n*=3).

[*] Significant difference (p<0.05).

IPTF: triterpenoid fraction from inner part of *I. obliquus*; OPTF: triterpenoid fraction from outer part of *I. obliquus*; and UAE, ursolic acid equivalent

2. Composition of triterpenoids in *I. obliquus*

Total ion chromatogram and MS spectra of the IPTF and OPTF are shown in Fig. 1. Triterpenoids were identified from their protonated molecular ions $[M+H]^+$, by comparing to literature information. Four peaks were observed to be major components in the IPTF and OPTF. Mass values of the peak 1 and 2 in Fig. 1 were 457 $[M+H]^+$ and 439 $[M+H-H2O]^+$, respectively. These mass values were also the same as those of betulinic and trametenolic acids (Leliebre-Lara et al., 2016). Retention time (RT) of the peak 1 on the HPLC was the same as that of the betulinic acid standard (Fig. 2), suggesting the peak 1 is betulinic acid. The peak 2 was determined as trametenolic acid referring to the previous study (Leliebre-Lala et al., 2016). The elution order of betulinic and trametenolic acids on the HPLC was similar to the result of a previous study (Xu X et al., 2016). The peak 3 might be lupenone or inotodiol due to its mass value which was detected at *m/z* 425. As molecular weights of lupenone and inotodiol are 424 and 442, respectively, they could have *m/z* 425 ($[M+H]^+$ and $[M+H-H2O]^+$ ion fragments, respectively). To exactly confirm the peak 3, lupenone and inotodiol standards were analyzed by the HPLC. The peak 3 was identified as inotodiol because its RT and UV-spectrum on the HPLC corresponded to those of inotodiol standard. The peak 4 could not be identified.
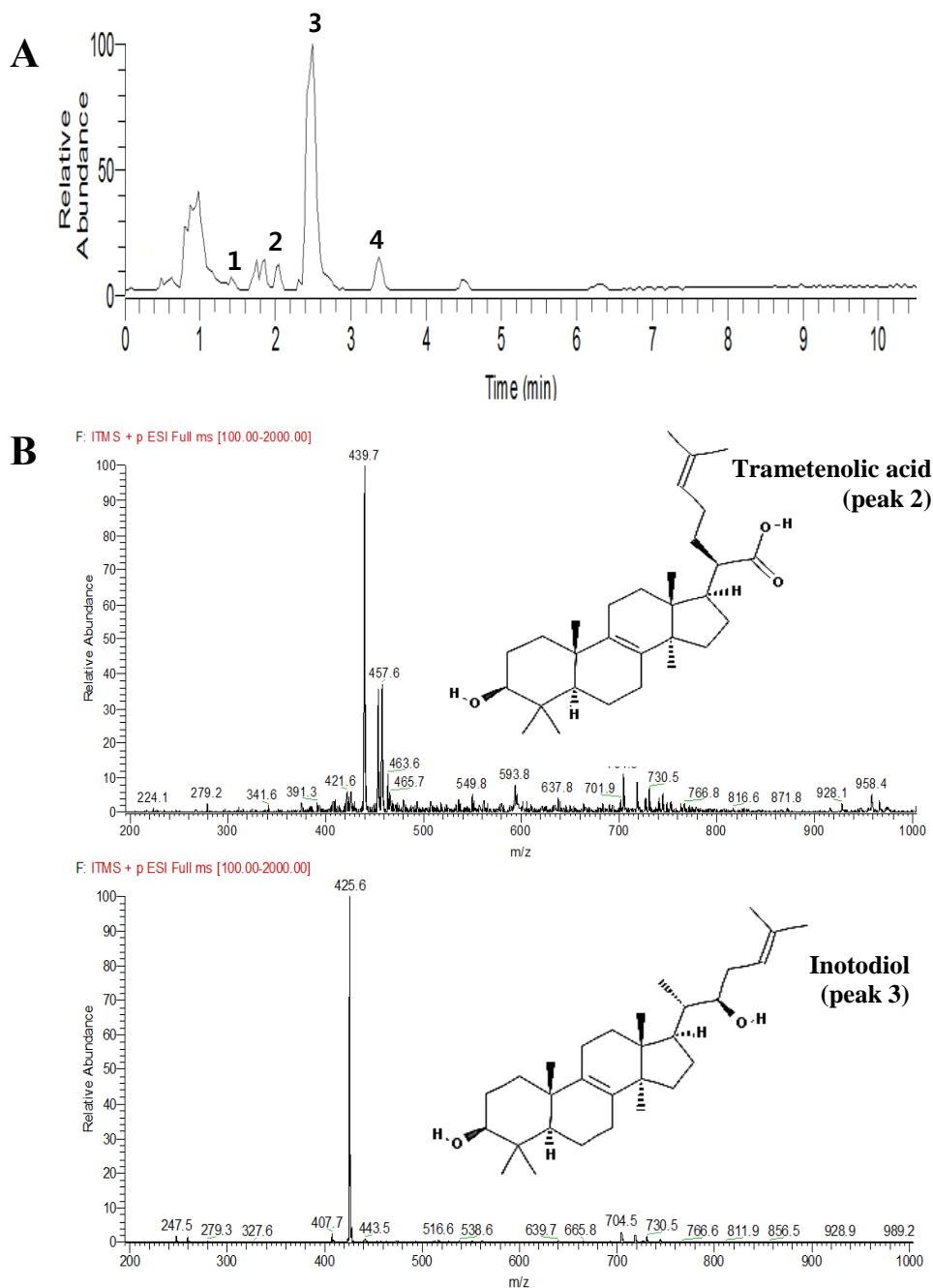
Figure 1. LC-ESI-MS total ion chromatogram (TIC) (A) of triterpenoid fraction from *I. obliquus* and mass spectra (B) of the peak 2 (trametenolic acid) and 3 (inotodiol) (B) shown in the TIC.

HPLC chromatograms of triterpenoids in the IPTF and OPTF are shown in Fig. 2. RT of the peaks on the HPLC chromatograms (Fig. 2) were not the same as those of the peaks on LC-MS chromatograms (Fig. 1) because the used instruments and columns for the HPLC and LC-MS analyses were different. The peak 1, 3, 4, and 5 detected by the HPLC (Fig. 2) were betulinic acid, trametenolic acid, inotodiol, and an unknown compound, respectively. The peak 2 was determined as betulin by matching RT and UV-spectrum of the peak with betulin standard. The peaks were quantified using betulin, betulinic acid, and inotodiol as standards. Contents of the peak 3 and 5 were calculated as betulinic acid equivalent. There was no big difference in triterpenoid constituents between the IPTF and OPTF. However, the content of triterpenoids in the OPTF was significantly higher than in the IPTF ($p<0.05$). This might be because contents of betulinic acid and inotodiol were significantly higher in the OPTF ($p<0.05$). Major triterpenoids in *I. obliquus* were trametenolic acid, inotodiol, and an unknown compound. The three compounds in the IPTF and OPTF accounted for 94.8 and 94.0%, respectively, of the total triterpenoids quantified by the HPLC. This is similar to the report of Xu et al. (2016), in which inotodiol and trametenolic acid were major components in *I. obliquus*. Du et al. (2011) also reported that contents of inotodiol and trametenolic acid in *I. obliquus* were 130 and 70 mg/g extract, respectively.
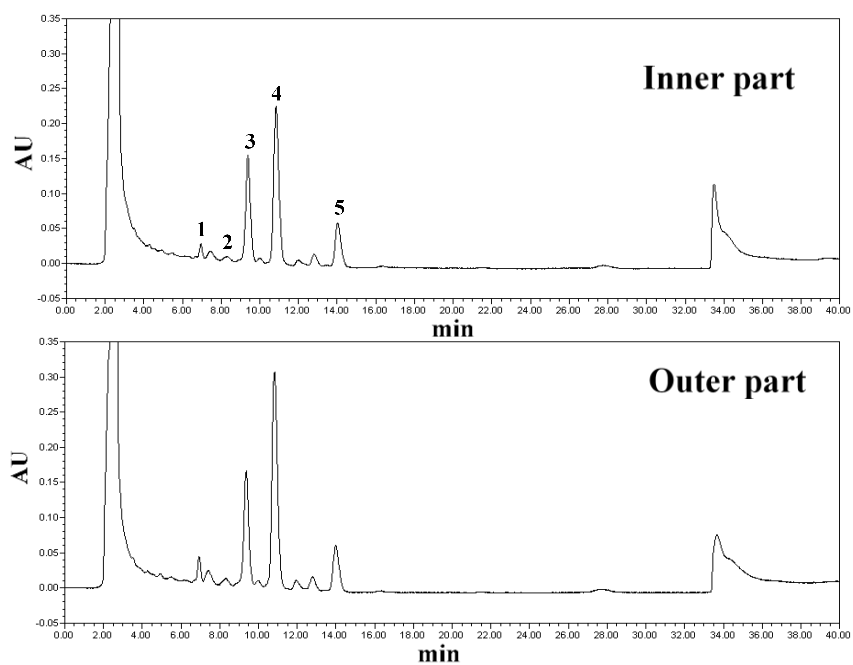
Figure 2. HPLC chromatograms of triterpenoid fractions from inner and outer parts of *I. obliquus*. Peak 1, betulinic acid; 2, betulin; 3, trametenolic acid; 4, inotodiol; and 5, unknown.

Table 2. Triterpenoids in triterpenoid fractions from *I. obliquus*.

Peak No.[a]	Compound	Fraction (mg/g dry basis of fraction)	
		IPTF	OPTF
1	Betulinic acid	8.69±1.71	13.6±2.29*
2	Betulin	8.24±0.65	8.79±0.40
3	Trametenolic acid[b]	94.5±9.15	106.3±8.23
4	Inotodiol	153.9±15.4	194.1±11.5*
5	Unknown[b]	59.0±4.04	47.1±15.7
Total		324.3±22.3	369.9±10.7*

Values are means and standard deviations (*n*=3).

* Significant difference ($p < 0.05$).

[a] Peak numbers of triterpenoids in HPLC chromatogram in Fig. 2.

[b] Trametenolic acid and unknown compound were calculated as betulinic acid equivalent.

IPTF: triterpenoid fraction from inner part of *I. obliquus*; and OPTF: triterpenoid fraction from outer part of *I. obliquus*

3. Phytosterols in *I. obliquus*

TMS-derivatized ergosterol and lanosterol detected on GC were identified by matching with mass spectra of NIST library (Fig. 3). The molecular weights of ergosterol and its TMS derivative are 396 and 468, repectively. In the MS spectrum of the TMS-derivatized ergosterol, principal ions with *m/z* 468, 363, and 337 were detected. Axelsson et al. (1995) suggested that *m/z* 363 $[M-105]^+$ is produced by loss of trimethylsilanol and a methyl group from the ergosterol derivative, and *m/z* 337 $[M-131]^+$ is produced by loss of trimethylsilanol and C_1-C_3 fragment. Molecular weights of lanosterol and its TMS derivative are 426 and 498, respectively. In the MS spectrum of the TMS-derivatized lanosterol, principal ions with *m/z* 498, 483, and 393 were detected. Cuesta-Rubio et al. (2017) reported that *m/z* 483 $[M-15]^+$ is produced by loss of a methyl group from the lanosterol derivative and *m/z* 393 $[M-105]^+$ is produced by loss of trimethylsilanol and a methyl group. Since the IPTF and OPTF have very low amounts of ergosterol and lanosterol, quantitative analysis was not performed. A previous study reported that contents of ergosterol and lanosterol in fruiting bodies of *I. obliquus* were 0.33 and 1.38 mg/g dry sample, respectively. They also determined stigmasterol and sitosterol, but their amounts were only about half that of ergosterol (Gao et al., 2009).
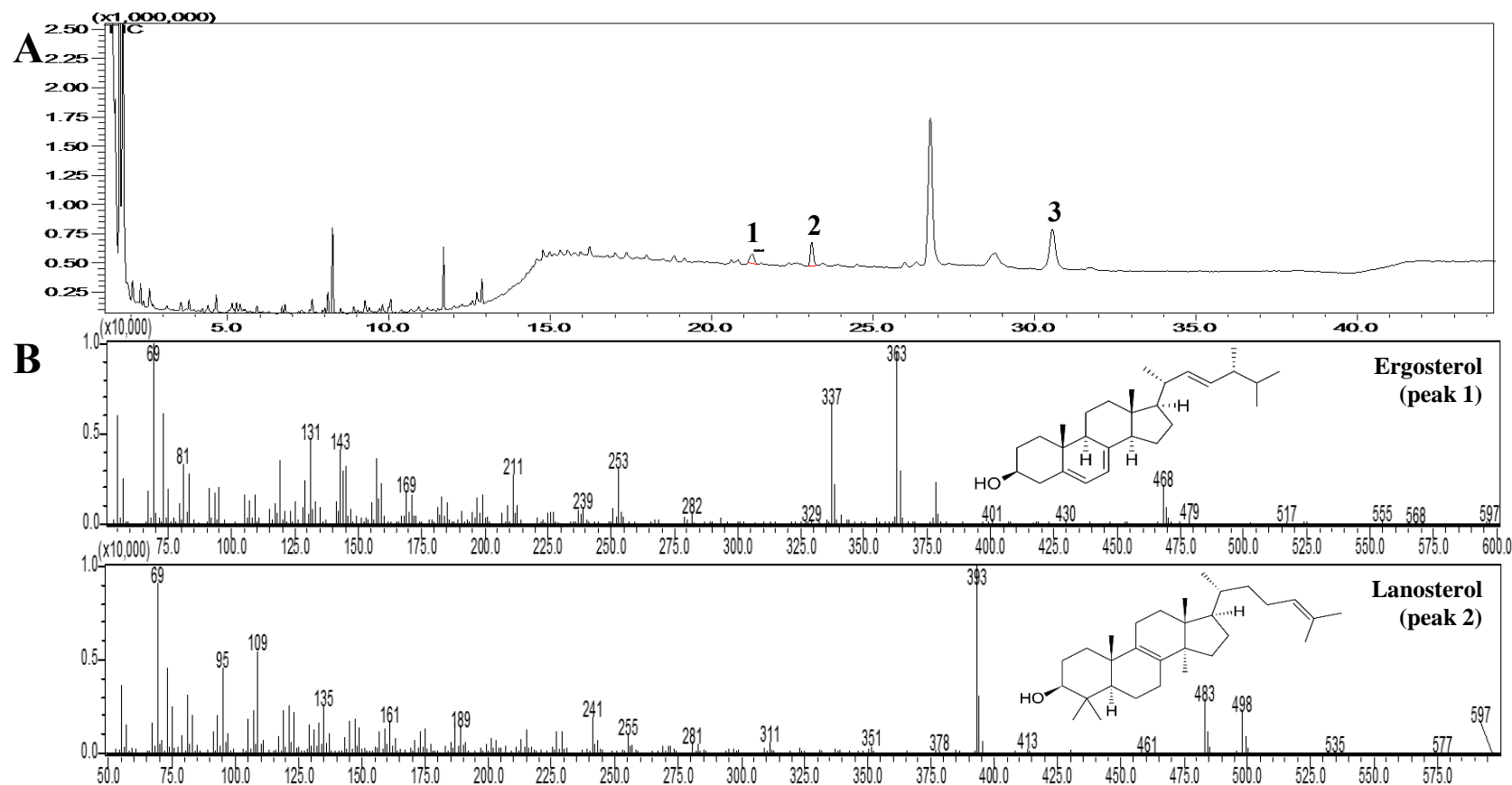
Figure 3. GC-MS total ion chromatogram (TIC) (A) of triterpenoid fraction from outer part of *I. obliquus* and mass spectra (B) of phytosterols (peak 1 and 2) and inotodiol (peak3) detected in the TIC.

4. Anti-proliferative activity of triterpenoid fractions from *I. obliquus*

In this study, concentrations up to 300 µg/mL of the triterpenoid fractions exert little or no toxic effect on RAW 264.7 cells (data not shown). To compare with methanol extracts which have high amount of phenolic compounds and low amount of triterpenoids, the anti-proliferative activities of the methanol extracts from the inner and outer parts of *I. obliquus* were also evaluated. The control cells were treated with up to 0.1% DMSO. The concentration is known to have no toxic effect on the cells in another study (Attiga et al., 2000).

Anti-proliferative activities of the methanol extracts and the triterpenoid fractions were determined with various cancer cell lines (Fig. 4). All the extracts and fractions were not able to significantly inhibit proliferation of HT-29 cells. The methanol extracts had no anti-proliferative activity, while PC-3 cells were significantly inhibited at the highest concentration tested. However, the triterpenoid fractions had dose-dependent anti-proliferative activity on AGS, MCF-7, and PC-3 cells. The highest concentration of the triterpenoid fractions and even 250 µg/mL of the OPTF significantly inhibited proliferation of AGS cells. Proliferation of MCF-7 cells was significantly inhibited at 300 µg/mL of the OPTF ($p<0.05$). The highest concentration of the methanol extract and triterpenoid fraction of the inner part of *I. obliquus* significantly inhibited proliferation of PC-3 cells ($p<0.01$ and $p<0.05$, respectively). The OPTF were able to significantly inhibit

proliferation of PC-3 cells at 250 and 300 µg/mL ($p<0.01$ and $p<0.001$, respectively). The highest concentration of the methanol extract from the outer part of *I. obliquus* also significantly inhibited proliferation of PC-3 cells ($p<0.05$). Thus, the triterpenoid fractions might have higher anti-proliferative activity on cancer cells than the methanol extracts and the effects of the OPTF were better than those of the IPTF. This is in accordance with the result of Ma et al. (2013), reporting that petroleum ether extract of *I. obliquus*, which had large amount of triterpenoids, was the most active against cancer cell lines among butanol, ethanol, ethyl acetate, petroleum ether, and water extracts. Moon and Lee (2009) also reported that extracts containing a higher amount of hydrophobic substances had a stronger anti-cancer activity on various cancer cell lines. Cha et al. (2007) reported that viability of AGS and MCF-7 cells fell below 80% when treated with water extract of *I. obliquus* at a concentration of 1 mg/mL. Triterpenoid fraction from *I. obliquus* seemed to have less anti-proliferative activity than a single compound such as inotodiol. Zhao et al. (2014) reported that inhibitory rate of inotodiol against human cervical cancer cells (HeLa) was 24.8% at a concentration of 50 µg/mL.
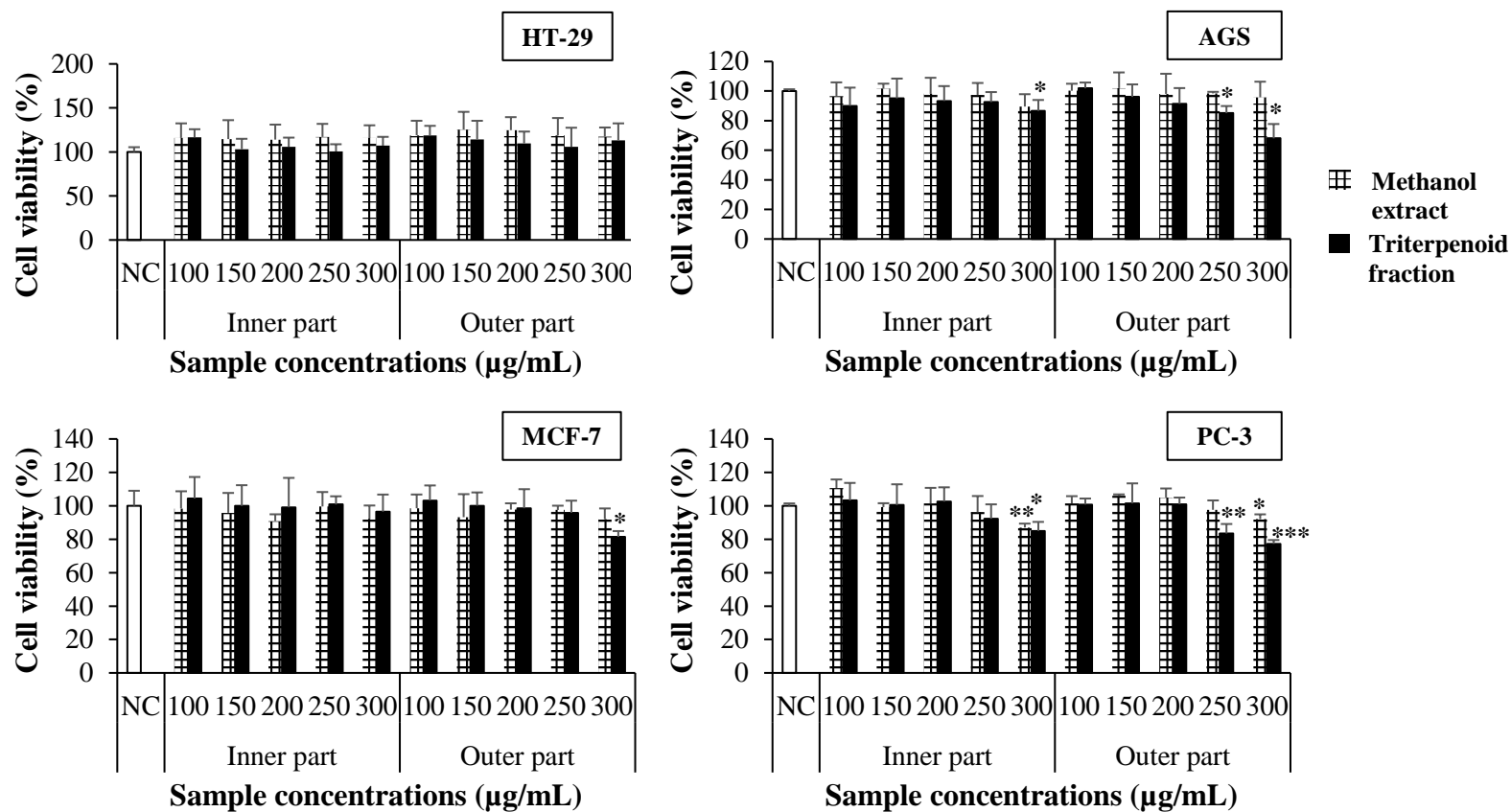
Figure 4. Anti-proliferative activity of triterpenoid fractions and methanol extracts of *I. obliquus* on cancer cell lines.

CONCLUSION

Triterpenoids from inner and outer parts of *I. obliquus* were identified and their anti-proliferative activity was evaluated. Betulin, betulinic acid, inotodiol, and trametenolic acid were identified in both of the inner and outer parts of *I. obliquus*. Inotodiol and trametenolic acid were major triterpenoids in both of the parts. Anti-proliferative activity of the triterpenoid fraction from the outer part was higher than that of the inner part. This might be because the amount of triterpenoids was higher in the outer part. The results of the study suggest the outer part of *I. obliquus* may be used as functional foods or drugs.

REFERENCES

Attiga, F. A., Fernandez, P. M., Weeraratna, A. T., Manyak, M. J., & Patierno, S. R. (2000). Inhibitors of prostaglandin synthesis inhibit human prostate tumor cell invasiveness and reduce the release of matrix metalloproteinases. *Cancer Research*, 60, 4629-4637.

Axelsson, B. O., Saraf, A., & Larsson, L. (1995). Determination of ergosterol in organic dust by gas chromatography-mass spectrometry. *Journal of Chromatography B: Biomedical Sciences and Applications*, 666, 77-84.

Bazyleva, N. N., Bolotkina, L. I., Laskina, E. M., Osvetimskaia, N. P., & Smirnova, L. I. (1958). Pharmacological and biological characteristics of *Inonotus obliquus*. *Farmakologiia I Toksikologiia*, 21, 89.

Cha, J. Y., Jun, B. S., Lee, C. H., Yooi, K. S., Moon, J. C., & Cho, Y. S. (2005). Hypoglycemic and antioxidative effects of fermented chaga mushroom (*Inonotus obliquus*) on streptozotocin-induced diabetic rats. *Journal of Life Science*, 15, 809-818.

Cuesta-Rubio, O., Fernández, M. C., Hernández, I. M., Jaramillo, C. G. J., González, V. H., Porto, R. M. D. O., Delange D. M., Fidalgo L. M., Piccinelli A. L., Campone L., & Rastrelli, L. (2017). Chemical profile and anti-leishmanial activity of three Ecuadorian propolis samples from Quito, Guayaquil and Cotacachi regions. *Fitoterapia*, 120, 177-183.

Du, D., Zhu, F., Chen, X., Ju, X., Feng, Y., Qi, L. W., & Jiang, J. (2011).
Rapid isolation and purification of inotodiol and trametenolic acid from
Inonotus obliquus by high-speed counter-current chromatography with
evaporative light scattering detection. *Phytochemical Analysis*, 22, 419-
423.

Gao, Y., Xu, H., Lu, Z., & Xu, Z. (2009). Quantitative determination of
steroids in the fruiting bodies and submerged-cultured mycelia of *Inonotus
obliquus*. *Se pu= Chinese Journal of Chromatography*, 27, 745-749.

Handa, N., Yamada, T., & Tanaka, R. (2012). Four new lanostane-type
triterpenoids from *Inonotus obliquus*. *Phytochemistry Letters*, 5, 480-485.

Kahlos, K., Kahlos, L., & Hiltunen, R. (1986). Anti-tumor tests of inotodiol
from the fungus. *Acta Pharmaceutica Fennica*, 95, 173-177.

Kim, Y. O., Park, H. W., Kim, J. H., Lee, J. Y., Moon, S. H., & Shin, C. S.
(2006). Anti-cancer effect and structural characterization of endo-
polysaccharide from cultivated mycelia of *Inonotus obliquus*. *Life
Sciences*, 79, 72-80.

Leliebre-Lara, V., Monzote Fidalgo, L., Pferschy-Wenzig, E. M., Kunert, O.,
Nogueiras Lima, C., & Bauer, R. (2016). In vitro anti-leishmanial activity
of sterols from *Trametes versicolor* (Bres. Rivarden). *Molecules*, 21, 1045.

Li, F., Li, W., Fu, H., Zhang, Q., & Koike, K. (2007). Pancreatic lipase-

inhibiting triterpenoid saponins from fruits of *Acanthopanax senticosus*.
Chemical and Pharmaceutical Bulletin, 55, 1087-1089.

Ma, L., Chen, H., Dong, P., & Lu, X. (2013). Anti-inflammatory and anti-cancer activities of extracts and compounds from the mushroom *Inonotus obliquus*. *Food Chemistry*, 139, 503-508.

Moon, B. H., & Lee, W. C. (2009). Studies on the anti-cancer activity of chaga mushroom extract. *Journal of Korean Oriental Medicine*, 30, 1-12.

Nakajima, Y., Sato, Y., & Konishi, T. (2007). Antioxidant small phenolic ingredients in *Inonotus obliquus* (persoon) pilat (chaga). *Chemical and Pharmaceutical Bulletin*, 55, 1222-1226.

Oleszek, W., & Stochmal, A. (2002). Triterpene saponins and flavonoids in the seeds of *Trifolium* species. *Phytochemistry*, 61, 165-170.

Park, Y. M., Won, J. H., Kim, Y. H., Choi, J. W., Park, H. J., & Lee, K. T. (2005). In vivo and in vitro anti-inflammatory and anti-nociceptive effects of the methanol extract of *Inonotus obliquus*. *Journal of Ethnopharmacology*, 101, 120-128.

Reid, D. A. (1976). *Inonotus obliquus* (pers. Ex Fr.) pilat in Britain. *Transactions of the British Mycological Society*, 67, 329-332.

Shin, J. Y., Choi, E. H., & Wee, J. J. (2001). New methods for separation of crude ginseng saponins. *Korean Journal of Food Science and Technology*,

33, 166-172.

Shin, Y., Tamai, Y., & Terazawa, M. (2001). Chemical constituents of *Inonotus obliquus* II: a new triterpene, 21, 24-cyclopentalanosta-3*β*, 21, 25-triol-8-ene from sclerotium. *Journal of Wood Science*, 47, 313-316.

Wang, L. X., Lu, Z. M., Geng, Y., Zhang, X. M., Xu, G. H., Shi, J. S., & Xu, Z. H. (2014). Stimulated production of steroids in *Inonotus obliquus* by host factors from birch. *Journal of Bioscience and Bioengineering*, 118, 728-731.

Xu, X., Zhang, X., & Chen, C. (2016). Stimulated production of triterpenoids of *Inonotus obliquus* using methyl jasmonate and fatty acids. *Industrial Crops and Products*, 85, 49-57.

Zhang, J., & Qu, F. (2013). Methods for analysis of triterpenoid saponins. *Natural Products*, 3311-3323. Springer Berlin Heidelberg.

Zhao, L. W., Zhong, X. H., Yang, S. Y., Zhang, Y. Z., & Yang, N. J. (2014). Inotodiol inhibits proliferation and induces apoptosis through modulating expression of cyclinE, p27, bcl-2, and bax in human cervical cancer HeLa cells. *Asian Pacific Journal of Cancer Prevention*, 15, 3195-3199.

Zhong, X. H., Ren, K., Lu, S. J., Yang, S. Y., & Sun, D. Z. (2009). Progress of research on *Inonotus obliquus*. *Chinese Journal of Integrative Medicine*, 15, 156-160.

국문초록

차가버섯(*Inonotus obliquus*)에 함유되어 있는 Triterpenoid의 조성과 암세포 증식 억제능

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차가버섯은 항암, 항산화, 항염증 등의 생리활성을 지닌 약용버섯으로 사용되어 왔다. 차가버섯에 함유되어 있는 triterpenoid의 암세포 증식 억제능은 보고되었으나 차가버섯의 껍질 부분에 존재하는 triterpenoid에 대한 연구는 거의 이루어지지 않았다. 따라서 본 연구의 목적은 차가버섯 내부와 껍질의 triterpenoid 조성을 확인하고 여러 암세포 (HT-29, AGS, MCF-7, PC-3)에 대한 증식 억제능을 확인하는 것이다.

본 연구에서는 차가버섯을 80% 메탄올로 추출한 뒤 Diaion HP-20 resin을 이용하여 triterpenoid fraction을 얻었다. Betulin, betulinic acid, inotodiol trametenolic acid, unknown 성분이 차가버섯의 내부와 껍질 분획물에서 동일하게 검출되었으며, 주요 triterpenoid는 inotodiol과 trametenolic acid였다. 그러나 차가버섯 내부

분획물에서보다 껍질분획물에 betulinic acid와 inotodiol이 유의적으로 많았으며, 전체적인 triterpenoid의 함량도 껍질 분획물에 유의적으로 많았다. 차가버섯의 triterpenoid 분획물을 암세포에 처리하였을 때 농도 의존적으로 암세포의 증식이 억제되었다. 껍질 분획물의 암세포 증식 억제 효과도 내부 분획물보다 유의적으로 높았다. 따라서, 차가버섯의 껍질 부분은 추출 과정의 부산물이지만 triterpenoid의 함량이 많고 암세포 증식 억제능이 내부보다 뛰어나기 때문에 식품이나 의약품에 기능성 소재로 활용될 수 있을 것으로 판단한다.