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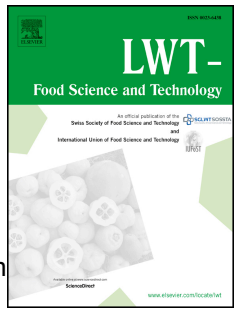

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Effects of Non-Traditional Extraction Methods on Extracting Bioactive Compounds from Chaga Mushroom (*Inonotus obliquus*) Compared with Hot Water Extraction

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Abbreviations: CMP, chaga mushroom powder; CME, chaga mushroom extract(s); CMEP, chaga mushroom extract powder; HWE, hot water extract; HE, high temperature and pressure extract; EE, enzyme extract; UE, ultrasound extract.

Abstract

Chaga mushroom (*Inonotus obliquus*) is mainly consumed as an extract. Hot water extraction, which is a traditional method preparing chaga mushroom extract, has limitations requiring a large amount of solvent and long extraction time. The objective of this study was to compare non-traditional methods for extracting bioactive compounds in chaga mushroom with traditional hot water extraction. β-Glucans and total phenolic compounds were highest in high temperature and pressure extract. Enzyme extract contained more β-glucans than hot water extract. Enzyme and ultrasound extracts contained more triterpenoids than the hot water extract.

Keywords

Chaga mushroom; *Inonotus obliquus*; High temperature and pressure extraction; Enzyme extraction; Ultrasound extraction

1. Introduction

Chaga mushroom (*Inonotus obliquus*) is a fungus belonging to the family Hymenochaetaceae. Chaga mushroom has bioactive compounds such as *β*-glucans, triterpenoids, and phenolic compounds to protect itself from environmental stresses (Zhong, Ren, Lu, Yang, & Sun, 2009). Chaga mushroom has traditionally been consumed as an extract due to its rigid cell walls, which consist of cross-linked chitin, *β*-glucans, and other components. Traditionally chaga mushroom extract (CME) has been prepared by heating crushed mushroom in water. However, this traditional extraction requires long extraction time, possibly leading to degradation and coagulation of extracted bioactive compounds in chaga mushroom (Rosello-Sato et al., 2016). Thus, different extraction methods could be screened to improve their extractability. The objective of this study was to determine bioactive compounds in CME prepared by non-traditional extraction methods such as high temperature and pressure, enzyme, and ultrasound, compared with hot water extraction.

2. Materials and methods

2.1. Chemicals and reagents

Viscozyme L was purchased from Novozymes (Bagsvaerd, Denmark). *β*-Glucan assay kit was purchased from Megazyme (Bray, Ireland). HCl (37% (v/v) and 1 N), KOH, NaOH, 99.7% (v/v) glacial acetic acid, sodium bicarbonate anhydrous, 70% (v/v) perchloric acid, and formic acid were purchased from Samchun Pure Chemicals (Pyeongtaek, Korea). Vanillin and 2,5-dihydroxyterephthalic acid were from Tokyo Chemical Industry Co. (Tokyo, Japan). Folin-Ciocalteu reagent, N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS), pyridine, gallic acid, caffeic acid, ferulic acid, syringic acid, and vanillic acid were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

Protocatechuic acid was from HWI Analytik (Rheinzabern, Germany). 3,4-Dihydroxybenzaldehyde was from Wako Chemical Co. (Osaka, Japan). Methanol and acetonitrile were from JT Baker (Phillipsburg, NJ, USA).

2.2. Sample preparation

Chaga mushroom (containing about 12% moisture) (Tyumen, Russia) was provided by DHF Company (Seoul, Korea). The outer part of chaga mushroom was discarded and the inner part was used for extraction. It was pulverized using a blender (Hanil Co., Bucheon, Korea). The chaga mushroom powder (CMP) was sealed using a vacuum packaging machine (M-6TM, Leepack Co., Incheon, Korea) and stored at 15 °C until extraction within 2 days.

2.3. Preparation of water extracts

Hot water extract (HWE): The CMP (10 g) and 400 mL water were refluxed for 2 h in a water bath (Daihan Scientific Co., Seoul, Korea) at 100 °C.

High temperature and pressure extract (HE): The CMP (10 g) and 400 mL water were heated for 2 h in an autoclave (AC-11, Jeiotech, Seoul, Korea) at 121 °C and 1.1 - 1.2 kg/cm^2.

Enzyme extract (EE): The CMP (10 g) was dispersed in 400 mL water. pH was adjusted to 4.5 (optimum pH of Viscozyme L) using 1 N HCl. Viscozyme L was added at 5% (v/w). The mixture was heated for 2 h, stirring at 200 rpm with an overhead stirrer (HS-30D, Daihan Scientific Co.) in the water bath at 50 °C. The enzyme was inactivated by heating the extract at 90 °C for 10 min.

Ultrasound extract (UE): The CMP (10 g) and 400 mL water were heated for 2 h in an ultrasonic bath (5510E-DTH, 139 W, 42 kHz, Bransonic, Danbury, CT, USA) at 50 °C.

Each of the extracts was filtered through a Whatman No. 4 filter paper (Whatman

International Ltd., Maidstone, England). The filtrate was concentrated using a rotary evaporator (A-10005, Eyela Co., Tokyo, Japan) at 50 °C under vacuum to make the final volume to be 45 mL. The concentrated extract was frozen for 12 h in a deep freezer (Revco Elite Plus, ThermoFisher Scientific, Asheville, NC, USA) at -72 °C. The frozen extract was lyophilized for 4 or 5 days in a freeze dryer (NB-504, Ilshin Co., Dongducheon, Korea) at -45 - -55 °C and below 5 mTorr. The freeze-dried extracts were stored at -20 °C in a 50 mL tube for less than 3 weeks without nitrogen gas.

2.4. Determination of glucans

Content of *β*-glucans was determined according to protocol of *β*-glucan assay kit. Contents of total glucans and *α*-glucans were calculated by comparing with D-glucose standard. *β*-Glucan content was calculated by subtracting the *α*-glucan content from the total glucan content.

2.5. Determination of total triterpenoids

Total triterpenoids were determined as described by Chen, Xie, and Gong (2007) with some modification. The chaga mushroom extract powder (CMEP) was dispersed in 100% methanol at 5 mg/mL, followed by filtering through 0.2 μm nylon syringe filter (Whatman International Ltd.). One hundred μL of the filtrate, 0.15 mL 5% (w/v) vanillin-acetic acid solution, and 0.5 mL 70% perchloric acid were mixed and heated at 60 °C for 45 min. It was left at room temperature for 3 min and mixed with 2.25 mL acetic acid. Absorbance was measured at 548 nm. Total triterpenoids were expressed as ursolic acid equivalent (UAE).

2.6. Determination of total phenolic compounds

Total phenolic compounds were determined by a modified method of Singleton, Orthofer, and Lamuela-Raventos (1999). The CMEP (2 mg) was dispersed in 1 mL 50% methanol. The methanol dispersion (20 μL) was mixed with water (1.58 mL) and Folin-Ciocalteu reagent (100 μL). The mixture was vortexed, left for 3 min at room temperature, and mixed with 300 μL 20% (w/v) sodium bicarbonate solution, followed by incubation at 40 °C for 30 min. Absorbance was measured at 765 nm. Content of total phenolic compounds was expressed as gallic acid equivalent (GAE).

2.7. Identification of phenolic compounds

The CMEP (100 mg) dispersed in 1 mL methanol was sonicated for 20 min, followed by filtering with 0.2 μm nylon syringe filter. The filtrate (5 μL) was injected into an UltiMate 3000 RS HPLC system coupled with an LTQ XL mass spectrometer (Thermo Fisher Scientific) with ESI. Stationary phase was a U-VDSpher PUR C18-E (100 × 2.0 mm i.d., 1.8 μm, VDS Optilab, Berlin, Germany). Mobile phases were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) with a gradient as follows: 0-6 min, 8-12% B; 6-15 min, 12-17.5% B; 15-25 min, 17.5-27% B; 25-26 min, 27-100% B; 26-27 min, 100% B; 27-27.5 min, 8% B; and 27.5-35 min, 8% B. Flow rate was 0.3 mL/min. The MS was operated in both positive and negative modes with source voltages of 3.5 and 2.7 kV, respectively. Capillary temperature was 300 °C. MS data were obtained in a range of 100-1000 *m/z*.

GC-MS analysis was performed by method of Ju et al. (2010). The CMEP was dispersed in methanol at 100 mg/mL and sonicated for 20 min. The methanol dispersion was filtered through 0.2 μm nylon syringe filter. After the solvent in the filtrate was evaporated under nitrogen stream, BSTFA containing 1% TMCS (300 μL) and pyridine (200 μL) were added, followed by incubation at 60 °C for 30 min to silylate. The silylated sample (1 μL) was

injected into a GCMS-QP2010 Plus system (Shimadzu Co., Kyoto, Japan) with a DB-5 capillary column (30 m × 0.25 mm i.d., 0.25 μm, J&W Scientific, Folsom, CA, USA). Column temperature was initially held at 100 °C for 2 min, then raised to 270 °C at 5 °C /min, and held for 6 min. Carrier gas was helium. Flow rate was 1.2 mL/min. Injector, interface, and ion source temperature were 250 °C, 230 °C, and 230 °C, respectively. Electron energy was 70 eV. Mass spectra were scanned from 100 to 1000 *m/z* and matched with National Institute of Standards and Technology (NIST) library.

2.8. Quantification of phenolic compounds

The CMEP was dispersed in 50% methanol (20 mg/mL), followed by filtering with 0.2 μm nylon syringe filter. The filtrate (20 μL) was loaded on a Waters 2695 HPLC system equipped with a Zorbax Eclipse Plus C18 column (250 × 4.6 mm i.d., 5 μm , Agilent) and a diode array detector. Mobile phases were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) with a gradient as follows: 0-6 min, 8-12% B; 6-15 min, 12-17.4% B; 15-27 min, 17.4-27% B; 27-27.1 min, 27-8% B; and 27.1-35 min, 8% B. Column oven was 30 °C and flow rate was 1 mL/min. Absorbance was measured at 280 nm. Phenolic compounds in the CME were quantified by their corresponding standards (gallic acid, protocatechuic acid, 3,4-dihydroxybenzaldehyde, caffeic acid, and syringic acid).

2.9. Statistical analysis

All experiments were conducted in triplicate except the MS analysis, which was conducted once. One-way ANOVA and Duncan's multiple range test ($p<0.05$) were conducted using a SPSS program (SPSS, Chicago, IL, USA).

3. Results and discussion

3.1. Contents of glucans, triterpenoids, and phenolic compounds

β-Glucan content was the highest in the HE (Table 1). The *β*-glucan content was significantly ($p<0.05$) higher in the EE than in the HWE. Rosello-Sato et al. (2016) reported that enzymes with chitinase or glucanase activity could intensify extraction of compounds in mushrooms by breaking bonds between cell wall components of mushrooms more efficiently than hot water. Fu, Chen, Dong, Zhang, and Zhang (2010) reported that ultrasound-assisted extraction at 75 °C effectively extracted *β*-glucans in chaga mushroom. However, in the present study, the content of *β*-glucans was the lowest in the UE, suggesting ultrasonic temperature might be low to efficiently extract *β*-glucans.

The UE and EE were significantly ($p<0.05$) higher in total triterpenoids than the HWE and HE (Table 1). Previous studies reported that cavitation phenomenon induced by ultrasound or enzymatic hydrolysis could efficiently rupture mushroom cell walls and break bonds between triterpenoids and cellular components (Tohtahon et al., 2017; Song et al., 2016), facilitating triterpenoids in mushroom to be transferred to extraction solvent. The content of triterpenoids in the HE was significantly ($p<0.05$) lower than that in the HWE, indicating that triterpenoids might be destroyed by high temperature (Wu, Lin, and Chau, 2001).

Content of total phenolic compounds was the highest in the HE, followed by the HWE, UE, and EE (Table 1). Choi et al. (2006) reported that phenolic compounds in shiitake mushroom treated at 121 °C for 30 min were higher than at 100 °C for 30 min. Seo and Lee (2010) also reported that phenolic compounds in chaga mushroom extracts produced by supercritical water extraction increased with temperature, suggesting that temperature be critical in extracting phenolic compounds from chaga mushroom.

174 *3.2. Identification and quantification of phenolic compounds*

175 2,5-Dihydroxyterephthalic and vanillic acids in the CME were detected by the LC-MS
176 (Fig. 1) and GC-MS (spectra not presented), but not on the HPLC (Fig. 2). This is
177 presumably because contents of 2,5-dihydroxyterephthalic and vanillic acids in the CME
178 were low, so it might be detected only in the MS with lower detection limit than the HPLC.

179 Five phenolic compounds (gallic acid, protocatechuic acid, 3,4-dihydroxybenzaldehyde,
180 caffeic acid, and syringic acid) in the CME were quantified using their corresponding
181 standards (Table 2). Gallic and caffeic acids were the highest in the UE. This might be
182 because they were known to be destroyed by heat treatment (Boles, Crerar, Grissom, & Key,
183 1988; Kim, 2009). Syringic acid was the highest in the HE. Ju et al. (2010) reported that
184 contents of protocatechuic and syringic acids in chaga mushroom steam-treated at 121 °C for
185 3 h were higher than those in untreated chaga mushroom by converting bound phenolic
186 compounds to free forms. 3,4-Dihydroxybenzaldehyde was significantly (p<0.05) higher in
187 the HWE and HE than in the EE and UE.

188

189 **4. Conclusion**

190 β-Glucans were higher in the HE and EE than in the HWE. Total phenolic compounds
191 was higher in the HE than in the HWE. Total triterpenoids were higher in the EE and UE than
192 in the HWE. Since contents of β-glucans, triterpenoids, and phenolic compounds in the CME
193 were affected by extraction methods, a specific extraction method corresponding to a group
194 or each of target compounds may be needed to maximize extraction.

195

196 **Conflict of interest**: none

197

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Table 1
Glucans, triterpenoids, and phenolic compounds in freeze-dried chaga mushroom extracts.

	Total glucans[1] (%, w/w, dry basis)	α-Glucans[1] (%, w/w, dry basis)	β-Glucans (%, w/w, dry basis)	Total triterpenoids (mg UAE/g)	Total phenolic compounds (mg GAE/g)
HWE	3.81±0.34^{b}	2.66±0.42^{ab}	1.15±0.12^{c}	8.90±0.30^{c}	166±8.6^{b}
HE	5.88±0.06^{a}	2.71±0.09^{ab}	3.17±0.04^{a}	7.03±0.76^{d}	204±8.9^{a}
EE	5.86±0.34^{a}	3.09±0.07^{a}	2.77±0.27^{b}	24.3±0.05^{a}	72.0±1.2^{d}
UE	3.02±0.05^{c}	2.35±0.09^{b}	0.68±0.13^{d}	16.2±0.13^{b}	99.9±2.3^{c}

HWE, hot water extract; HE, high temperature and pressure extract; EE, enzyme extract; UE, ultrasound extract; UAE, ursolic acid equivalent; and GAE, gallic acid equivalent.
Values are means ± standard deviations (n=3).
[1]Free sugars might be included.
[a-d]Different superscripts in the same columns indicate significant differences (p<0.05).

Table 2
Phenolic compounds (mg/g) in freeze-dried chaga mushroom extracts.

	Gallic acid	Protocatechuic acid	3,4-Dihydroxy benzaldehyde	Caffeic acid	Syringic acid
HWE	0.83±0.03^{d}	0.90±0.03^{c}	1.45±0.07^{b}	0.80±0.02^{b}	0.93±0.02^{b}
HE	0.91±0.01^{c}	1.07±0.01^{a}	1.67±0.02^{a}	0.72±0.02^{c}	1.54±0.03^{a}
EE	0.95±0.01^{b}	0.98±0.02^{b}	1.32±0.05^{c}	0.85±0.02^{b}	0.68±0.01^{c}
UE	1.05±0.00^{a}	1.11±0.04^{a}	1.30±0.05^{c}	0.99±0.07^{a}	0.90±0.07^{b}

HWE, hot water extract; HE, high temperature and pressure extract; EE, enzyme extract; and UE, ultrasound extract.
Values are means ± standard deviations (n=3).
$^{a-d}$Different superscripts in the same columns indicate significant differences (p<0.05).

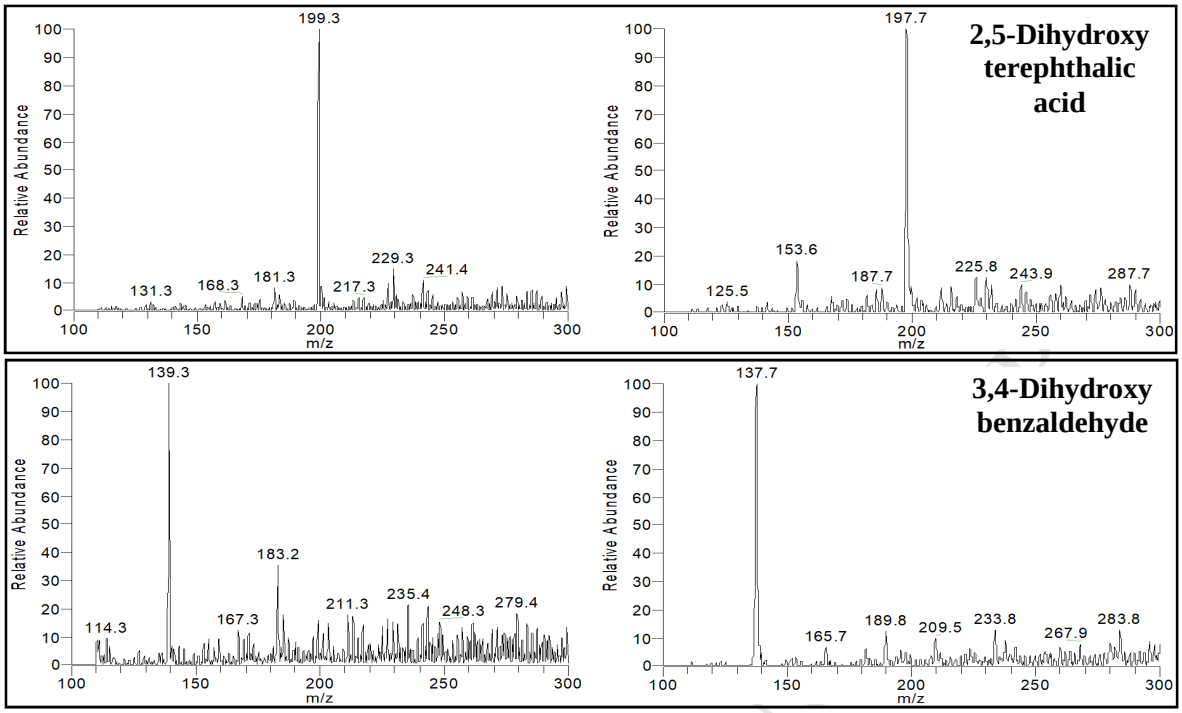

Fig. 1. LC-MS spectra of phenolic compounds in chaga mushroom extracts with positive (left) and negative (right) ion modes.

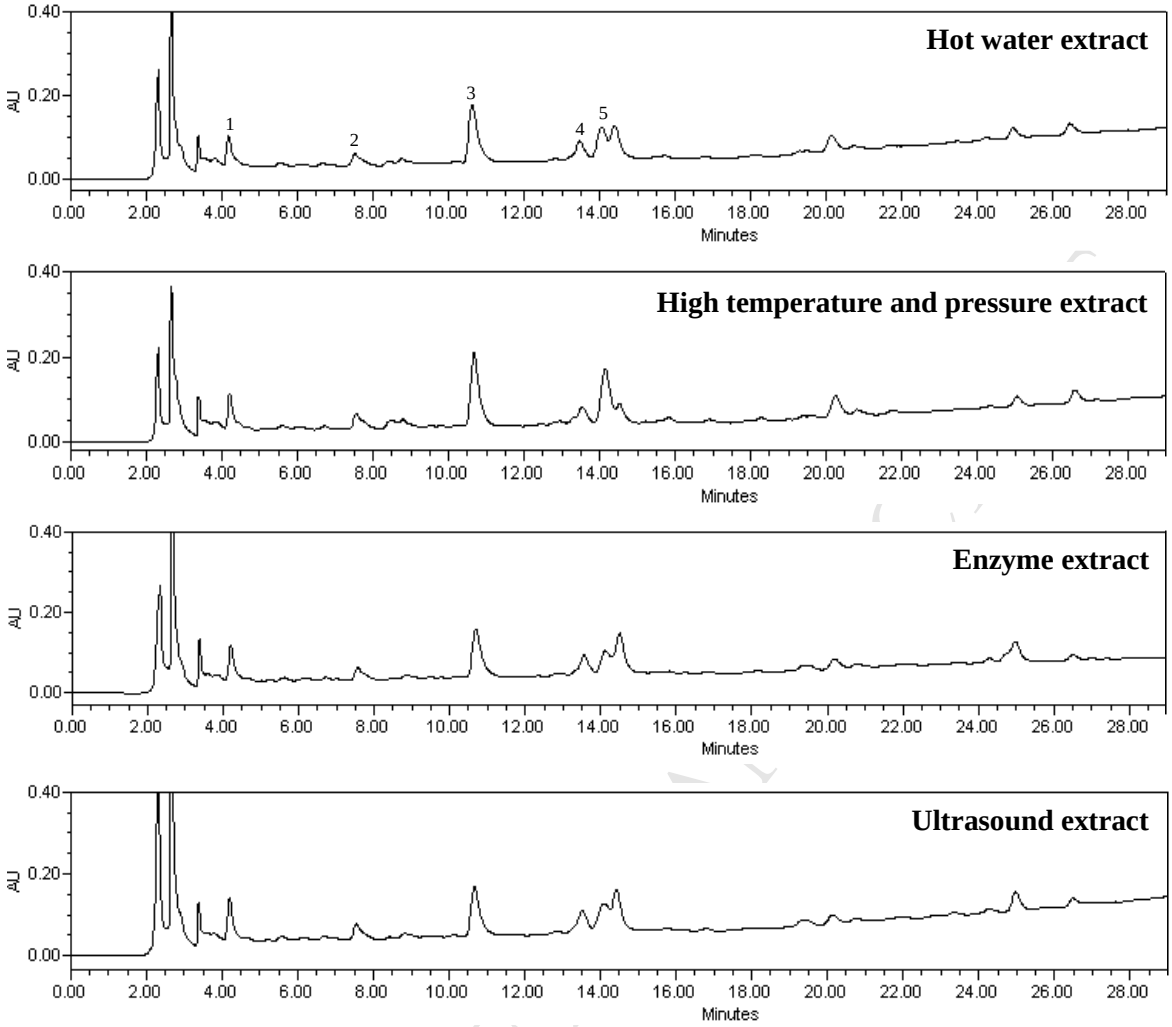

Fig. 2. HPLC chromatograms of phenolic compounds in chaga mushroom extracts (λ=280 nm). 1, gallic acid; 2, protocatechuic acid; 3, 3,4-dihydroxybenzaldehyde; 4, caffeic acid; and 5, syringic acid.

- Bioactive compounds in chaga extracts were determined.
- Contents of bioactive compounds were affected by extraction methods.
- High temperature and pressure extracted more β-glucans and phenolics than others.
- Enzyme extract had higher β-glucans and triterpenoids than hot water extract.