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Qualitative analysis of secondary metabolites of chaga mushroom (*Inonotus Obliquus*): phenolics, fatty acids, and terpenoids

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Abstract

Chaga mushroom is a black perennial fungus that usually parasites on adult birch tree trunks. It has been conventionally used as a health-promoting supplement and nutraceutical in different cultures for centuries. The desired clarification of the profile of chaga secondary metabolites responsible for various bioactive properties has been continuously pursued for decades but has only partially been unveiled. Meanwhile, in recent years, attention to food safety, quality stability, authentication, and sustainability of chaga products from the wild has become increasingly popular in the current commercial market and related small/medium-size food industry enterprises. Phenolic, hydroxylated fatty acid, and terpenoid compounds produced by sclerotia of chaga mushrooms are bioactive constituents with antioxidant, anti-microorganism, and anti-tumor activities. Some new secondary metabolites of chaga mushroom have occasionally been reported previously, and effects of environment (e.g., cultivation method, harvesting region) on compositional characteristics noted. However, these have rarely and systematically compared the compositions of their material with a reliable database of known secondary metabolites of chaga. Therefore, this study aimed to achieve a rapid screening and characterization of secondary metabolites of Newfoundland chaga. A total of 111 phenolic, 63 fatty/aromatic acid, and 108 terpenoid constituents was primarily identified using HPLC-ToF-MS (high-performance liquid chromatograph coupled with time-of-flight mass spectra), among which 161 were newly reported. In addition, an update of the compositional database of chaga was provided as supplementary materials to help utilization and development of Newfoundland chaga mushroom as edible-fungi. Conclusively, chaga mushroom is a very promising food supplement abundant in numerous fungal secondary metabolites that were rarely found in other edible materials, even though its safety (e.g., oxalate content) aspects is still in need of additional investigation for being considered as a viable commercial nutraceutical.

Keywords: Edible fungi; LC-ToF-MS; Compositional database; Qualitative analysis; Bioactive compounds.

1. Introduction

Chaga mushroom is an edible herbal fungus that is mostly distributed in the circumboreal region of the Northern Hemisphere. The medicinal/nutraceutical use of chaga has been recorded in different ancient cultures, including Ainu and Khanty in Northeastern Asia, and various First nations such as Wet'suwet'en, Chipewyan, Cree, and Gitxsan in North America. Chaga is a Latinized Russian word known as the sterile conk trunk rot of birch in Canada. The official binomial name of chaga is *Inonotus obliquus*, but other names, including *Phaeoporus obliquus*, *Polyporus obliquus*, or *Fuscoporia obliqua*, have also been sporadically used in the earlier literature (He et al., 2001; Reid, 1976).

Attributed by its global anecdotal evidence of medicinal proper-

ties, chaga has been used as a functional beverage (tea) or folk medicine (decoction, ointment) for the treatment of stomach diseases, intestinal worms, liver/heart ailments, dermatomycoses, joint pain, and different types of cancer in the East European countries for centuries (Babitskaya et al., 2002; Koyama, 2017; Lemieszek et al., 2011; Peng and Shahidi, 2020; Saar, 1991; Shashkina et al., 2006; Shikov et al., 2014). To date, numerous studies have claimed various bioactivities, together with related biomolecular mechanism of chaga, including antioxidant, antimicrobial, anti-cancer, hypoglycemic, antilipidemic, anti-inflammation, abirritative, immunoregulatory, and cardioprotective effects (Koyama et al., 2008; Patel, 2015; Peng and Shahidi, 2020; Shashkina et al., 2006; Zhong et al., 2009). Such a broad spectrum of biological/pharmacological functions implies the complexity of bioactive substances in chaga, although related clinical data are relatively scarce. As a result, the bioactive compounds of chaga have gradually been unveiled over the past 20 years. Many chaga-based supplements have been commercialized in the current nutraceutical market. To satisfy and sustain the increased commercial demand for chaga products, the artificial culture of chaga has been practiced for decades to overcome the long growth period of wild chaga (Ka et al., 2017; Sun et al., 2011; Zheng et al., 2010).

The diversity and content of bioactive components of this parasitic fungus varies greatly according to its nutritional and environmental conditions, including physical (e.g., UV and γ-radiation), biological (e.g., host), and chemical (e.g., pH, oxygen, heavy metals, and exogenous phytochemicals) factors. For instance, ergosterol became the dominant sterols in the cultured mycelium of chaga, rather than lanosterol and inotodiol in wild chaga. Other trace sterols of wild chaga, such as episterol, 24-methylene dihydrolanosterol, and ergosterol peroxide can not be found in cultured mycelium (Zheng et al., 2007). Similar phenomenon was observed among different wild types of chaga. For example, the Canadian and Ukrainian chaga collected by Géry et al. (2018) contained around 1% and 10% of betulin and betulinic acid content of French chaga, respectively. The total phenolic content of decoction and tincture of chaga from Thailand is around 2 and 5–10 times of those harvested from Russia and Finland, even though the DPPH scavenging efficacy of chaga tincture from Finland is about 9–20 times higher than those from Thailand and Russia. Meanwhile, the content of *p*-hydroxybenzoic acid of Finland and Thailand chaga is around 2 and 20 times higher than that in the Russian chaga, respectively, while gallic acid can be detected in samples from Thailand but not in those from Finland and Russia (Glamočlija et al., 2015). Apparently, chaga from different production sites usually show distinguishable chemical profiles. To achieve a comparable compositional profile of the artificial culture of chaga mycelia, various physiochemical stimulus have been applied to mimic growth environments which efficiently manipulates the production of chaga secondary metabolites (Zheng et al., 2010). More information on compositional and proportional difference, and the production monitoring of secondary metabolites of chaga is found in a recent review, in which around 220 compounds including 108 terpenoids, 64 small-molecule phenolics, 10 alkaloids, 17 amino acids, and various bioactive polymers such as polysaccharides-protein complex and allomelanins were summarized (Peng and Shahidi, 2020). In combination with newly updated 21 phenolics and 10 terpenoids from 2021 to 2022, it provides the database for the current study of primary compositional characterization of wild chaga harvested in Newfoundland (Abu-Reidah et al., 2021; Chang et al., 2022; Kou et al., 2021). In this article, numerous bioactive compounds including small-molecule phenolics, terpenoids, and hydroxylated fatty acids were qualitatively identified using full-scan ToF mass spectrometry. Intriguingly, more than half of these secondary metabolites can not be found in previous reports, implying the significance of quality discrepancy of chaga materials from different production regions and its potential influences on the products' performance and reliability. The establishment of reproducible qualification protocols of natural food supplements and related compositional databases can effectively promote the capability of companies and authorities in supervising their quality control, product standardization, and potential counterfeits. Therefore, this contribution aimed to provide a comparative analysis between small-molecule bioactive secondary metabolites from Newfoundland wild chaga and its counterparts reported elsewhere, as well as an updated database of phenolic, fatty acid, and terpenoid compounds.

2. Materials and methods

2.1. Solvents and reagents

HPLC grade reagents acetonitrile, ethanol, and methanol were purchased from Fisher Scientific, Ltd. (Ottawa, ON, Canada). Formic acid was purchased from ACROS Organics (Morris, IL, USA). Water was purified using a Milli-Q system, Millipore (Bedford, MA, USA).

2.2. Extraction of secondary metabolites from chaga mushroom

Chaga powder was obtained locally from Dr. Aubrey Anderson from Department of Fisheries and Oceans (DFO), St. John's, NL, Canada. For extraction, 500 mg chaga material was freeze-dried and then ultrasonically extracted twice with 10 mL solvent mixture consisting of water-ethyl acetate-acetone (20:40:40, v/v/v). The supernatants of the extracts were combined, 1 mL of which was filtered into LC sample vials without any further concentration.

2.3. Qualitative analysis by HPLC-ESI-TOF-MS

The composition of extract was determined using high-performance liquid chromatography-electrospray ionization-time of flight-mass spectrometry (HPLC-ESI-TOF-MS), using an Agilent 1260 HPLC unit (Agilent Technologies, Palo Alto, CA, USA) with a UV diode array detector (UV-DAD) and a SUPERLCOSILTM LC-18 column (4.6 × 250 mm × 5 µm with guard column; Sigma-Aldrich, Oakville, ON, Canada) by gradient elution. The mobile phase consisted of 0.1% formic acid in deionized water (A) and 0.1% formic acid and 5% acetonitrile in methanol (B). The solvent gradient was as follows: 5–5% B, 0–6 min; 5–8% B, 6–16 min; 8–20% B, 16–40 min; 20–45% B, 40–75 min; 45–60% B, 75–85 min; 60–90% B 85–110 min; 90–100% B, 110–120 min; 100–100, 120–125 min; 100–5% B, 125–130 min. The column was thermostatically controlled at 40 °C, and the flow rate was set at 1 mL/min. The UV-visible absorbance of the peaks was monitored between 190 and 600 nm. All samples and standards were dissolved in methanol, and the sample injection volume was 5 µL. LC flow was further analyzed online by an orthogonal time-of-flight mass spectrometer (6230 TOF LC/MS system; Agilent Technologies, Palo Alto, CA, USA) equipped with an electrospray ionization source (ESI). The ESI source was operated in the negative ion mode, and full scan mass spectral data were acquired over a range from m/z 100 to 1,700. The MS conditions were as follows:

drying gas flow rate, 10 L/min; nebulizer pressure, 60 psi; drying gas temperature, 350 °C; ESI temperature, 400 °C; and capillary voltage, 110 V. The mass spectrum results were analyzed by the Agilent MassHunter Workstation (version B.05.01).

3. Results and discussion

Secondary metabolites include a large group of specific compounds required for survival and adaptation of plants, fungi, and bacteria in their environment. They can react to biotic stresses including mutualistic (e.g., pollination) or antagonistic interactions (e.g., disease-resistance and existence competition) with other organisms, or deal with abiotic stresses such as radiation and heavy metals. Macro fungi have a well-developed secondary metabolism producing a variety of low-molecular-weight secondary metabolites that render tremendous bioactivity and medicinal properties. However, fungi secondary metabolites remain mostly underexplored compared to plant-derived ones in spite of centuries' old application; chaga mushroom is one typical case.

The actual compositional profile of chaga extract is very complicated, which may arise from various pathways of secondary metabolism in wild chaga and its host so that a gradient with over 120 min elution duration was used to sufficiently separate them by LC C-18 system before the coupled ESI-ToF-MS analysis. The mass error for molecular ions of all identified compounds was within ±10 ppm. For example, compound A1 gave a deprotonated molecular ion $[M-H]^-$ at 181.0719, with the difference of −0.76 ppm from calculated $[M-H]^-$ at 181.0718, therefore indicating its molecular formula as $C_6H_{14}O_6$, which is tentatively identified as a sugar alcohol of hexose, such as sorbitol.

The eluted compounds are mainly non-nitrogenous compounds, including phenolics, terpenoids, and fatty acid derivatives, and this study focuses on the analysis of phenolic constituents. Figure s1–s3 shows the total ion chromatograph (TIC) of chaga extract. By comparison with the updated phenolic database (Table s1), our results classified these constituents of Newfoundland chaga into two categories, 'known' and 'newly detected compounds', along with their accurate MS[1] data given in Table 1, as discussed below.

3.1. Qualitative analysis of small phenolic molecules of chaga

Table 1 shows that the phenolic composition of chaga is a complex profile covering 111 compounds, although the actual content of soluble small-molecular-weight phenolics is the tip of the iceberg due to the abundance of (insoluble) phenolic polymers, especially melanin- and lignin-polysaccharide complexes (Wang et al., 2015; Wold et al., 2018). According to our Folin-Ciocalteu and antioxidant results of chaga extracts, the efficacy and mass equivalents of degradation products of insoluble phenolics are several times higher than those of its soluble counterpart (unpublished results). The phenolics of chaga include phenolic acids, flavonoids, coumarins, quinones, and styrylpyrones. Among the 111 phenolic compounds in Table 1, 49 of them are in accordance with previous studies, while the other 62 are newly detected.

3.1.1. Phenolic acid derivatives

For phenolic acids, a total of 37 compounds were identified, including 16 hydroxybenzoic acid derivatives (compounds No. P1/2/3/4/7/9/19/20/21/23/24/28/48/54/57/70/71/72) and 19 hydroxycinnamic acid derivatives (No. P12/13/14/16/17/26/34/47/50/52/53/68/74/88/89/91/92/93/110). Twenty-four of those structures have been previously been reported in chaga and the rest of the compounds, P23 ($C_{15}H_{20}O_9$), P48 ($C_{22}H_{26}O_{11}$), P50 ($C_{18}H_{22}O_{10}$), P52 ($C_{25}H_{30}O_{14}$), P53 ($C_{25}H_{32}O_{13}$), P54 ($C_{23}H_{28}O_{13}$), P68 ($C_{25}H_{30}O_{13}$), P71($C_{23}H_{26}O_{13}$), P72 ($C_{23}H_{26}O_{13}$), P89 ($C_{34}H_{40}O_{18}$), P91 ($C_{34}H_{40}O_{18}$), and P93 ($C_{34}H_{40}O_{18}$), were tentatively identified as syringic acid rhamnoside, agnuside, sinapoylquinic acid, lippioside II, oleuropein, picroside II, grandifloroside, helonioside B, mallophenol A, mallophenol A and its isomers, smilaside B, smilaside B isomer, and jaslanceoside B, respectively. Compared to the other phenolic acids or even any other phenolic compound, hydroxybenzoic acid (P7) was reported as the most dominating phenolic in chaga (Glamočlija et al., 2015). However, in this study, it was noticed that the dominating phenolics might indeed be dihydroxybenzaldehyde (P8) instead of hydroxybenzoic acid (P7), the former's log P is slightly lower than the latter and therefore appearing subsequently (8.812 min of P7 and 10.189 min of P8) with a very different relative abundance in TIC (total ion chromatogram). This result is in line with that of Abu-Reidah et al. (2021)'s recent quantification results of Newfoundland chaga. However, the protocatechuic acid glucoside, ellagic acid, methylellagic acid, and 2,5-dihydroxyterephthalic acid detected in Abu-Reidah et al. (2021)'s study were not found in our sample. However, the material used in our study was enriched with various benzoic and cinnamic acid derivatives. The phenolic acids with relatively higher abundance were compounds P52, P54, P68, P71, P72, P110, and especially syringic acid and its derivatives (P23/24/57/70).

3.1.2. Coumarin, xanthone, anthrone, and quinone derivatives

Eighteen coumarin, xanthone, anthrone, and quinone derivatives can be sporadically found in the sample. Except dihydroxycoumarin (P10), coumarin (P18), and inonotphenol A (P36), the derivatives including P15 ($C_9H_6O_3$), P31 ($C_{14}H_{10}O_5$), P32 ($C_{11}H_8O_5$), P33 ($C_{14}H_{16}O_7$), P39 ($C_{12}H_{12}O_5$), P42 ($C_{13}H_{12}O_5$), P43 ($C_{13}H_8O_7$), P45 ($C_{14}H_{10}O_4$), P51 ($C_{16}H_{18}O_{10}$), P57 ($C_{12}H_{10}O_4$), P58 ($C_{24}H_{18}O_9$), P66 ($C_{12}H_{14}O_8$), P78 ($C_{14}H_8O_7$), P85 ($C_{14}H_8O_8$), and P97 ($C_{22}H_{16}O_8$), are all newly detected and were tentatively characterized as hydroxycoumarin, dihydroxymethoxyxanthone, purpurogallin, phellodenol F, trimethoxycoumarin, pentahydroxyxanthone, acetyl dimethoxycoumarin, leucoquinizarine, fraxin, liqcoumarin, gaboroquinone A, fulvic acid, pentahydroxyanthraquinone, hexahydroxyanthraquinone, and hydramycin, respectively. Among these derivatives, the compounds with relatively higher abundance were compounds P15, P33, P39, P42, P45, P57, and P85.

3.1.3. Flavonoid derivatives

Flavonoid derivatives are a diverse group of phenolics that wee also detected in the current chaga sample. A total of 30 flavonoids were detected while their ion abundances were generally in trace amounts compared with the rest of phenolic groups. Zheng et al. (2008) compared the phenolic contents of wild chaga and its mycelia cultures. Mycelia cultures of chaga mainly consisted of flavonoids [e.g., naringin, ECG (epicatechin gallate), kaempferol], and lesser amounts of styrylpyrones and melanins. However, for the wild chaga, flavonoids were determined in trace amounts, while styrylpyrones (e.g., phelligridin A/D and inoscavin A/B) and melanins were dominant. In our sample, we noticed a similar phenomenon. As aforementioned, among 85 previously reported phenolic compounds, 37 were not detected in the current sample, especially

Table 1. Chemical constituents (saccharides and phenolics) of chaga extract scanned by HPLC-ESI-TOF-MS

Compound no.	t_R (min)	Formula	$[M-H]^-/[2M-H]^-$ (m/z)			Compounds detected in current study		
			Measured	Calculated	Error (ppm)	Newly detected compounds	Known compounds	Reference
Saccharides (S)								
A1	3.054	$C_6H_{14}O_6$	181.0719	181.0718	−0.76		Sorbitol	Shin et al. (2001)
A2	3.071	$C_{12}H_{22}O_{11}$	341.1082 /683.2280 /387.1130	341.1089	2.15	Disaccharide	–	
A3	3.371	$C_{24}H_{38}O_{20}$	645.188	645.1884	0.57	Tetrasaccharide (Beta-D-Delta(4)-GlcpA-(1->4)-beta-D-Glcp-(1->4)-alpha-L-Rhap-(1->3)-beta-D-Glcp)	–	
Small-molecule Phenolics (P)								
Phenolic acid derivatives								
P1/2	2.606/4.463	$C_7H_6O_5$	169.0149	169.0142	−3.84	–	Trihydroxybenzoic acid and its isomer	Zheng et al. (2008); Glamočlija et al. (2015); Abu-Reidah et al. (2021)
P3	4.631	$C_8H_6O_5$	181.0151	181.0142	−4.69	–	Hydroxyisophthalic acid	Hwang et al. (2016)
P4	4.083	$C_7H_6O_4$	153.0201	153.0193	−4.98	–	Dihydroxybenzoic acid	Ju et al. (2010); Nakajima et al. (2007); Glamočlija et al. (2015); Abu-Reidah et al. (2021)
P7	8.812	$C_7H_6O_3$	137.0240	137.0244	3.03	–	Hydroxybenzoic acid/ salicylic acid	Glamočlija et al. (2015); Abu-Reidah et al. (2021)
P12/14/16	18.866/20.741 /23.446	$C_9H_8O_4$	179.035	179.0350	−0.1	–	Caffeic acid and its isomers	Nakajima et al. (2007); Abu-Reidah et al. (2021)
P13/26	20.360/32.721	$C_{10}H_{10}O_3$	177.0547	177.0557	5.72	–	4-Methoxycinnamic acid or mullein or 3,4-Dihydroxybenzalacetone	Nakajima et al. (2007); Kim et al. (2011); Abu-Reidah et al. (2021)
P17	24.275	$C_9H_8O_3$	163.0405	163.0401	−2.64	–	Coumaric acid	Abu-Reidah et al. (2021); Zheng et al. (2009); Kim et al. (2008)
P9/19	17.406/24.939	$C_8H_8O_4$	167.0345	167.035	2.87	–	Dihydroxyphenylacetic acid	Ju et al. (2010); Abu-Reidah et al. (2021)
							Homogentisic acid	Kim et al. (2008)
							Vanillic acid	Ju et al. (2010)
P20	26.515	$C_9H_8O_5$	195.029	195.0299	4.58	–	4-Methoxyisophthalic acid	Chang et al. (2022)

Table 1. Chemical constituents (saccharides and phenolics) of chaga extract scanned by HPLC-ESI-TOF-MS - *(continued)*

Compound no.	t_R (min)	Formula	$[M-H]^-/[2M-H]^-$ (m/z)			Compounds detected in current study		
			Measured	Calculated	Error (ppm)	Newly detected compounds	Known compounds	Reference
P21	28.208	$C_{14}H_{10}O_6$	273.0400	273.0405	1.68	–	6,6′-Dihydroxy-(1,1′-biphenyl)-3,3′-dicarboxylic acid	Hwang et al. (2016)
P23	30.547	$C_{15}H_{20}O_9$	343.1026	343.1035	2.49	Syringic acid rhamnoside	–	
P24/57/70	31.111/50.109/57.193	$C_9H_{10}O_5$	197.0449	197.0455	3.27	–	Hydroxy dimethoxybenzoic acid/Syringic acid	Mazurkiewicz (2006); Hwang et al. (2016); Abu-Reidah et al. (2021); Zheng et al. (2009)
P28	33.965	$C_{11}H_{14}O_5$	225.0777	227.0768	−3.77	–	Methylgallate	Chang et al. (2022)
P48	46.907	$C_{22}H_{26}O_{11}$	465.138	465.1402	4.8	Agnuside	–	
P34/47	41.050/46.873	$C_{10}H_{10}O_4$	193.051	193.0506	−1.89	–	cis/trans Ferulic acid	Kim et al. (2008); Abu-Reidah et al. (2021)
P50	48.433	$C_{18}H_{22}O_{10}$	397.1141	397.114	−0.2	Sinapoylquinic acid	–	
P52	48.533	$C_{25}H_{30}O_{14}$	553.1566	553.1563	−0.58	Lippioside II	–	
P53	49.196	$C_{25}H_{32}O_{13}$	539.1788	539.177	−3.31	Oleuropein	–	
P54	49.545	$C_{23}H_{28}O_{13}$	511.1436	511.1457	4.13	Picroside II	–	
P68	56.712	$C_{25}H_{30}O_{13}$	537.1631	537.1614	−3.22	Grandifloroside	–	
P71/72	57.608/57.907	$C_{23}H_{26}O_{13}$	509.1316	509.1301	−3.01	Mallophenol A and its isomer	–	
P74	59.367	$C_{11}H_{12}O_4$	207.0663	207.0663	−0.08	–	Sinapaldehyde	Chang et al. (2022)
P88	63.299	$C_{19}H_{20}O_4$	311.1285	311.1289	1.23	–	(9S)-Acerogenin M	Chang et al. (2022)
P89	63.747	$C_{34}H_{40}O_{18}$	735.2112	735.2142	4.06	Helonioside B	–	
P91/93	64.428/64.892	$C_{34}H_{40}O_{18}$	735.2113	735.2142	3.92	Smilaside B and its isomer	–	
P92	64.826	$C_{26}H_{30}O_{14}$	565.1556	565.1563	1.2	Jaslanceoside B	–	
P110	110.636	$C_{39}H_{56}O_5$	603.4082	603.4055	−4.47	–	Betulin-3-O-caffeate	Wold et al. (2020)
Coumarin, quinone, anthrone, and xanthone derivatives								
P10	18.817	$C_9H_6O_4$	177.0203	177.0193	−5.44	–	Dihydroxycoumarin	Ju et al. (2010)
P15	22.019	$C_9H_6O_3$	161.0246	161.0244	−1.13	Hydroxycoumarin	–	
P18	24.508	$C_9H_6O_2$	145.0301	145.0295	−4.09	–	Coumarin	Chang et al. (2022)
P31	37.864	$C_{14}H_{10}O_5$	257.046	257.0455	−1.76	Dihydroxy methoxyxanthone	–	
P32	37.914	$C_{11}H_8O_5$	219.0302	219.0299	−1.38	Purpurogallin	–	
P33	40.27	$C_{14}H_{16}O_7$	295.0831	295.0823	−2.61	Phellodenol F	–	
P36	41.697	$C_{12}H_{10}O_6$	249.0409	349.0405	−1.75	–	Inonotphenol A	Chang et al. (2022)

Table 1. Chemical constituents (saccharides and phenolics) of chaga extract scanned by HPLC-ESI-TOF-MS - *(continued)*

Compound no.	t_R (min)	Formula	$[M-H]^-/[2M-H]^-$ (m/z)			Compounds detected in current study		
			Measured	Calculated	Error (ppm)	Newly detected compounds	Known compounds	Reference
P39	42.228	$C_{12}H_{12}O_5$	235.0615	235.0612	−1.28	Trimethoxycoumarin	–	
P42	44.866	$C_{13}H_8O_7$	275.0188	275.0197	3.36	Pentahydroxyxanthone	–	
P43	44.949	$C_{13}H_{12}O_5$	247.06	247.0612	4.83	Acetyl dimethoxycoumarin	–	
P45	46.425	$C_{14}H_{10}O_4$	241.0514	241.0506	−3.17	Leucoquinizarine	–	
P51	48.466	$C_{16}H_{18}O_{10}$	369.0833	369.0827	−1.57	Fraxin	–	
P57	50.225	$C_{12}H_{10}O_4$	217.0511	217.0506	−2.14	Liqcoumarin	–	
P58	50.739	$C_{24}H_{18}O_9$	449.0882	449.0878	−0.88	Gaboroquinone A	–	
P66	55.518	$C_{12}H_{14}O_8$	307.0465	307.0459	−1.81	Fulvic acid	–	
P78	60.23	$C_{14}H_8O_7$	287.0212	287.0197	−5.12	Pentahydroxyanthraquinone	–	
P85	65.971	$C_{14}H_8O_8$	303.0139	303.0146	2.44	Hexahydroxyanthraquinone	–	
P97	67.779	$C_{22}H_{16}O_8$	407.0785	407.0772	−3.09	Hydramycin	–	
Flavonoids								
P22	29.535	$C_{15}H_{10}O_8$	317.0311	317.0303	−2.54		Myricetin	Abu-Reidah et al. (2021)
P29	34.496	$C_{19}H_{20}O_6$	343.1184	343.1187	0.91	Tetramethoxyflavanone	–	
P30/37	34.927/41.813	$C_{15}H_{10}O_7$	301.0349	301.0354	1.58	–	Quercetin and its isomer	Zheng et al. (2009); Abu-Reidah et al. (2021)
P35	41.282	$C_{19}H_{18}O_6$	341.1021	341.1031	2.81	Tetramethoxyflavone	–	
P38	42.095	$C_{18}H_{16}O_6$	327.0878	327.0874	−1.18	Hydroxy trimethoxyflavone	–	
P41	43.406	$C_{15}H_{10}O_6$	285.0405	285.0412	−2.58	–	Kaempferol	Zheng et al. (2009)
P44	46.259	$C_{26}H_{28}O_{14}$	563.1385	563.1406	3.77	Vicenin-1	–	
P46	46.608	$C_{24}H_{24}O_{14}$	535.1094	535.1093	−0.13	Haploside A	–	
P55	49.594	$C_{19}H_{18}O_5$	325.1078	325.1081	1.06	Moracin P	–	
P59	51.121	$C_{19}H_{20}O_5$	327.1237	327.1238	0.3	Tetramethoxychalcone	–	
P60/63	51.95/52.975	$C_{22}H_{24}O_{12}$	479.1220	479.1195	−5.21	Noidesol and its isomer	–	
P61	52.730	$C_{16}H_{12}O_5$	283.0626	283.0612	−4.94	–	Fortuneletin/5,7-dihydroxy-3′-methoxyflavone	Zheng et al. (2009)
P62	52.936	$C_{28}H_{34}O_{15}$	609.1820	609.1825	0.81	–	Rutin	Zheng et al. (2009)
P67	56.397	$C_{22}H_{26}O_{10}$	449.145	449.1453	0.71	Auriculoside	–	
P69	57.177	$C_{20}H_{22}O_{10}$	421.1156	421.114	−3.74	Polydine	–	
P73	58.255	$C_{18}H_{16}O_5$	311.0928	311.0925	−0.97	Trimethoxyflavone	–	
P75	59.666	$C_{28}H_{32}O_{15}$	607.1681	607.1668	−2.07	Diosmin	–	

Table 1. Chemical constituents (saccharides and phenolics) of chaga extract scanned by HPLC-ESI-TOF-MS - *(continued)*

Compound no.	t_R (min)	Formula	$[M-H]^-/[2M-H]^-$ (m/z)			Compounds detected in current study		
			Measured	Calculated	Error (ppm)	Newly detected compounds	Known compounds	Reference
P77	60.163	$C_{43}H_{42}O_{13}$	765.2562	765.2553	−1.22	Iryantherin F	–	
P80/81	61.275/61.690	$C_{34}H_{42}O_{18}$	737.2318	737.2298	−2.66	Brutieridin and its isomer	–	
P83	62.387	$C_{16}H_{14}O_7$	317.0658	317.0667	2.76	Dihydrorhamnetin	–	
P84	62.868	$C_{15}H_{12}O_5$	271.0621	271.0612	−3.32	–	Naringenin	Zheng et al. (2009)
P85	62.868	$C_{19}H_{16}O_6$	339.088	339.0874	−1.73	Acetylafromosin	–	
P86	62.951	$C_{16}H_{12}O_7$	315.0505	315.051	1.67	–	Methoxy tetrahydroxyflavone/ Isorhamnetin	Zheng et al. (2008); Zheng et al. (2009); Abu-Reidah et al. (2021)
P87	63.25	$C_{25}H_{20}O_{10}$	479.0996	479.0984	−2.56	Rhodiolin	–	
P90	64.096	$C_{31}H_{36}O_{15}$	647.2011	647.1981	−4.56	Embigenin 2″-(2‴-acetylrhamnoside)	–	
P94	65.606	$C_{16}H_{12}O_6$	299.058	299.0561	−6.29	Methoxyl trihydroxylflavonol	–	
P96	67.696	$C_{15}H_{10}O_6$	285.0424	285.0407	−7.13	–	Kaempferol or Luteolin	Zheng et al. (2009)
Styrylpyrones								
P25	32.239	$C_{13}H_6O_8$	288.9982	288.9990	2.76	Phelligridin J	–	
P27	33.334	$C_{13}H_{10}O_5$	245.0457	245.0455	−0.62	–	Hispidin	Abu-Reidah et al. (2021)
P40	43.256	$C_{28}H_{18}O_5$	433.1185	433.1081	−0.81	–	Methylinoscavin C	Zheng et al. (2011)
P64/106	54.555/86.329	$C_{25}H_{18}O_9$	461.0884	461.0878	−1.29	Inoscavin A and inonobulin C	–	
P65	55.136	$C_{25}H_{20}O_9$	463.1017	463.1035	3.78	–	Davallialactone	Zhao et al. (2015b)
P79	60.711	$C_{13}H_8O_6$	259.0253	259.0248	−1.88	Phelligridin A	–	
P82	62.171	$C_{22}H_{16}O_7$	391.0825	391.0823	−0.44	Phelliribsin A	–	
P98	74.117	$C_{23}H_{14}O_{10}$	449.0503	449.0514	2.49	–	Inonoblin B	Lee et al. (2007)
P99	79.344	$C_{21}H_{16}O_8$	395.0776	395.0772	−0.91	Inoscavin D	–	
P100	80.024	$C_{22}H_{18}O_8$	409.0953	409.0929	−5.87	Methylinoscavin D	–	
P101	83.126	$C_{25}H_{14}O_{10}$	473.0508	473.0514	1.31	–	Phelligridin E	Lee et al. (2007)
P102	83.939	$C_{20}H_{12}O_8$	379.0459	379.0459	0.11	–	Phelligridin D	Lee et al. (2007)
P103	84.835	$C_{23}H_{16}O_8$	420.0787	419.0772	−3.47	–	Inoscavin C	Zhao et al. (2015b)
P104	85.814	$C_{32}H_{18}O_{12}$	593.0725	593.0725	0.08	–	Phelligridin G	Lee et al. (2007)
P106	86.329	$C_{25}H_{18}O_9$	461.0884	461.0878	−1.29	–	Inonobulin C	Lee et al. (2007)
P107	87.142	$C_{20}H_{12}O_7$	363.0505	363.0510	1.45	–	Phelligridin C	Lee et al. (2007)
Others								

Table 1. Chemical constituents (saccharides and phenolics) of chaga extract scanned by HPLC-ESI-TOF-MS - *(continued)*

Compound no.	t_R (min)	Formula	$[M-H]^-/[2M-H]^-$ (m/z)			Compounds detected in current study		
			Measured	Calculated	Error (ppm)	Newly detected compounds	Known compounds	Reference
P5	7.335	$C_7H_6O_4$	153.0201	153.0193	−4.98	–	2,6-Dimethoxyphenol	Mazurkiewicz (2006)
P6	7.352	$C_6H_6O_2$	109.0290	109.0295	4.57	–	Resorcinol	Mazurkiewicz (2006)
P8	10.189	$C_7H_6O_3$	137.0239	137.0244	3.75	–	Dihydroxybenzaldehyde	Nakajima et al. (2007); Zheng et al. (2008); Kim et al. (2011); Liu et al. (2014)
P11	18.833	$C_8H_8O_2$	135.0449	135.0452	1.86	Hydroxy phenylacetaldehyde	–	
P49	47.355	$C_{22}H_{28}O_{10}$	451.1610	451.1610	−0.06	–	Erythro-4,7,9,9′-tetrahydroxy-3,5,3′,5′-tetramethoxy-8-O-4′-neolignane	Chang et al. (2022)
P76	59.666	$C_{20}H_{22}O_9$	405.1221	405.1191	−7.37	Astringin	–	
P108	91.406	$C_{19}H_{22}O_3$	297.1487	297.1496	3.08	–	(−)-(S)-acerogenin B	Chang et al. (2022)
P109	109.657	$C_{26}H_{38}O_2$	381.2797	381.2799	0.53	Desmethyl tocotrienol	–	
P111	113.938	$C_{28}H_{42}O_2$	409.3118	409.3112	−1.45	β/γ-Tocotrienol	–	

various flavonoid derivatives such as EGCG (epigallocatechin gallate), ECG, EGC (epigallocatechin), naringin, apigenin, eriocitrin, rhoifolin, isorhamnetin-3-*O*-rutinoside, and narirutin. However, several other non-flavonoid phenolics, including resveratrol, phellxinye A, inonoblin A, methylinoscavin A/B, and phelligridin C/F were also absent. For the 31 detected flavonoid derivatives, there were 18 flavones(ols) (compounds P22/30/37/41/44/46/60/61/62/63/73/75/86/87/90/94/96), 5 flavanones(ols) (compounds P29/80/81/83/84), 3 flavans(ols) (compounds P67/69/83) as well as aurones (compound P55), chalcones (P59), isoflavones (P85), and lignoflavonoids (P77). Twenty compounds in the list were newly detected. Compound P69 ($C_{20}H_{22}O_{10}$) showed the highest relative abundance, and then were compounds P38 ($C_{18}H_{16}O_6$), and P55 ($C_{19}H_{18}O_5$).

3.1.4. Styrylpyrone derivatives

Styrylpyrones, also known as hispidin derivatives, are a rare group in plant-derived phenolics. They have a skeleton core of C6-C2-C5 and can be found in macrofungi from genus *Inonotus* and *Phellinus* or primitive angiosperm from families *Piperaceae*, *Lauraceae*, *Annonaceae*, *Ranuculaceae* and *Zingiberaceae* (Lee and Yun, 2011). Detailed demonstration of the structural diversity, biosynthetic pathways, bioactivities, and corresponding mechanisms of styrylpyrones are found elsewhere (Lee and Yun, 2011).

A total of 16 styrylpyrones were found in the current sample, 10 of them including hispidin (compound P27), methylinoscavin C (P40), davallialactone (P65), inonoblin B (P98), phelligridin E (P101), phelligridin D (P102), inoscavin C (P103), phelligridin G (P104), inonoblin C (P106), and phelligridin C (P107) are known styrylpyrones from previous compositional studies of chaga. The newly detected styrylpyrones P25, P64, P79, P99, and P100, were tentatively identified as phelligridin J, inoscavin A, phelligridin A, phelliribsin A, inoscavin D, and methylinoscavin D, respectively. The compounds with relatively higher abundance were P102, P107, and P79. The other 6 previously known styrylpyrones including inonoblin A, phelligridin F, phelligridin H, methylinoscavin A/B, inoscavin B, and methyl davallialactone, could not be found in the current sample.

3.1.5. Other phenolic compounds

Other phenolics, including the derivatives of simple phenols (compounds P5/6), phenolic aldehydes (P8/11), neolignans (P49), stilbenes (P76), diarylheptanoids (P108), and tocopherols (P109/111), were found in the samples we examined. As mentioned earlier, compound P8 was dominant in its relative abundance compared to any other chaga phenolics in TIC, implying the antimicrobial and anti-cancer effects of phenolic extracts of chaga. Furthermore, the high content of *α*- and *β*-tocopherols previously reported in Newfoundland chaga could not be found in the current sample while the desmethyl tocotrienol and *β*/*γ*-tocotrienol were present at the end of the current elution time. Thus, either a longer elution time or a more hydrophilic mobile phase is recommended in future studies.

3.2. Qualitative analysis of aromatic acids, fatty acids, and related derivatives

A total of 63 derivatives of aromatic acids and fatty acids were tentatively identified. Except three compounds F1, F2, and F28, which were characterized as aromatic acids (phthalic acid, ben-

zoic acid, and di-iso-octyl phthalate), the other 60 compounds were all aliphatic acid derivatives including monocarboxylic acids and dicarboxylic acids as well as their hydroxylated derivatives. Twenty-one of them were hydroxylated fatty acids (compounds F3/7/8/9/10/13/14/17/26/35/39/40/44/45/48/49/54/56/58/60/63) , 5 are dicarboxylic acid (compounds F4/5/6/11/39), and the left 37 compounds (F12/15/16/18/19/20/21/22/23/24/26/27/29/30/31/32/33/34/36/37/38/41/42/43/46/47/50/51/52/53/55/57/59/61/62) were unmodified saturated/unsaturated fatty acids. The chain length of fatty acid derivatives ranged from 12 carbons (compound F12, lauric acid) to 27 carbons (compound F63, hydroxyheptacosanoic acid) in the current sample. The longest saturated monocarboxylic acid was docosanoic acid (compound F55, $C_{22}H_{44}O_2$) and longest unsaturated monocarboxylic acid was hexacosenoic acid (F62, $C_{26}H_{50}O_2$) which were eluted at the very end of the gradients (119.728 and 121.570 min), while in the past study using GC-MS, the longest saturated monocarboxylic acid detected was melissic acid ($C_{30}H_{60}O_2$) (Table s2). This was also the case for the elution of tocopherols; For LC-MS used in this study, the more extended elution gradient with a more hydrophilic organic phase was required for achieving detection of such hydrophilic compounds (melissic acid, C30:2). Hydroxylation and decarboxylation significantly increased the hydrophilicity and shortened the elution time. For fatty acids and their derivatives, 21 compounds (F1/2/3/4/5/7/8/9/10/11/13/14/16/26/35/39/45/46/49/56/63) were newly found, and the rest of the 41 fatty acids and 1 aromatic acid derivatives have been reported in the past. In both the current and previous studies, hydroxylated fatty acid derivatives, the common oxygenated metabolites of fatty acids during bacterial or fungal fermentation and a vital component of some fungal cell walls, have been abundantly detected. As given in Table 2 and supplementary database (Table s2) of chaga secondary metabolites, the fatty acid derivatives were mainly reported by Shcherbakov et al. (2022) and Sun et al. (2011). Although both studies used GC-MS to identify the fatty acid composition, different characterization results were reported for the same molecular formula. For example, in Shcherbakov et al. (2022)'s study, $C_{14}H_{28}O_2$ (compound F15) was identified as myristic acid/tetradecanoic acid while it was reported as ethyl dodecanoate in Sun et al. (2011)'s study. Similar cases apply to $C_{16}H_{32}O_2$ (compounds F25/33/41/48/52), $C_{17}H_{34}O_2$ (F30/32), $C_{19}H_{38}O_2$ (F37/38), and $C_{20}H_{40}O_2$ (F43), as shown in Table 2. Only using tandem mass or standard comparison can help to distinguish and confirm their accurate structures. In this study, oleic acid (F27, $C_{18}H_{34}O_2$), hydroxydocosanoic acid (F44, $C_{22}H_{44}O_3$), hydroxytricosanoic acid (F48, $C_{23}H_{46}O_3$), hydroxytetracosanoic acid (F54, $C_{24}H_{48}O_3$), hydroxypentacosanoic acid (F58, $C_{25}H_{50}O_3$) showed predominant relative abundance, followed by stearic acid (F24, $C_{18}H_{36}O_2$), hydroxyhexacosanoic acid (F60, $C_{26}H_{52}O_3$), linoleic acid (compounds F22, $C_{18}H_{32}O_2$), palmitic acid and its isomers (F25/33/41/48/52, $C_{16}H_{32}O_2$), hydroxyarachidic acid (F35, $C_{20}H_{40}O_3$), hydroxyheneicosanoic acid (F39, $C_{21}H_{42}O_3$), hydroxytricosenoic acid (F40, $C_{23}H_{44}O_3$).

3.3. Qualitative analysis of terpenoids of chaga

A total of 108 terpenoids were tentatively identified from the current chaga sample, but only 30 of them

can be recognized based on previous reports (Table s3, 129 known terpenoids in chaga) and the rest of the 78 were found as new terpenoids. In this study, except for the last compound T108 ($C_{30}H_{50}O$, 122.267 min), most of the terpenoids eluted by the current gradient were terpenes substituted with multi-oxygenated functional groups, and various terpenes and their mono-oxygenated derivatives reported previously could not be detected due to limitation of current elution period. Meanwhile, apart from compounds T1 (monoterpenoid glycoside), T8/25/55 (sesquiterpenoids), T35/38/41/45 (diterpenoids), T15(tetraterpenoid), T87 (pentaterpenoid), the rest of the 98 terpenoids identified from our sample were all triterpenoids and steroids. The compound T77 ($C_{30}H_{48}O_3$) showed the highest relative abundance within all the terpenoids, followed by compounds T75 ($C_{30}H_{46}O_3$), T76 ($C_{39}H_{56}O_5$), T83 ($C_{31}H_{50}O_3$), T84 ($C_{39}H_{56}O_4$), T93 ($C_{39}H_{58}O_5$), T103 ($C_{32}H_{52}O_6$), and T106 ($C_{39}H_{58}O_4$). It is remarkable that several hydroxycinnamoyl esters of triterpenoids, including compounds P110, T76, T80, T82, T84, T93, T101, T104, and T106, were detected, which highlights new antioxidant contributors other than pure phenolics, lignin, and melanin in chaga extracts considering their high relative abundance in TIC. However, compared to phenolic and fatty acid derivatives, the tentatively deduced structures of terpenoid compounds are more equivocal based on the primary mass data because of their higher molecular weights, which usually lead to many possible isomers. Therefore, the names of newly identified terpenoids in Table 3 were mainly from previous terpenoid studies of other fungi and only provided as preliminary results. Furthermore, numerous *in vivo* and *in vitro* studies of bioactive properties of purified chaga terpenoids have previously been reported and reviewed (Peng and Shahidi, 2020).

4. Conclusion

This study tentatively screened and identified over two hundred and eighty phenolic, fatty acid, and terpenoids compounds through literature review and comparison with previously reported studies of chaga. This priliminary screening of non-nitrogenous small-molecule phenolics, fatty acids, and terpenoids by HPLC-ToF-MS shows that Newfoundland chaga exhibits a unique secondary metabolites' profile demonstrating its potential for further development as a high-quality food supplement and nutraceutical. On the other hand, although compositional studies of chaga were initiated half of a century ago, many major bioactive compounds are still waiting to be identified. Due to the parasitic feature and environmental sensitivity of chaga mushroom, its secondary metabolic pathways and related bioactive stability of commercial products can be significantly skewed and jeopardized. Therefore, strategies for artificial cultivation and standardization of chaga culturing/farming and sustainable production may be the way for future production and marketing of chaga. Additional new secondary metabolites are waiting to be screened, separated, and identified in a targeted manner for Newfoundland chaga. However, further studies are also required for identification of nitrogenous compounds (e.g., peptides and alkaloids) and polymeric compounds (e.g., polysaccharides, lignin, and melanin) of chaga and to examine their myriad of physicochemical properties and bioactivities. Moreover, to a great extent, the use of chaga has usually been randomly guided by the folk experience, and the reported cases showing potential adverse health effects (on kidney) due to extremely high quantity of oxalic acid in chaga (up to 14% of dried material) have recently provoked serious safety concerns in unguided long-term administration of wild chaga and its products (Kwon et al., 2022; Lee et al., 2020; Peng and Shahidi, 2020). The standardized quality control based on fast detection technologies, the advancement of oxalate-free processing protocols, and the dosage guideline based on quantification of the bioactives present and their safe level of use and sufficient preclinical/clinical data for its chronic toxicity are most urgently needed.

Table 2. Chemical constituents (fatty acids, aromatic acids, and related derivatives) of chaga extract scanned by HPLC-ESI-TOF-MS

Compound no.	t_R (min)	Formula	$[M-H]^-/[2M-H]^-$ (m/z)			Compounds detected in current study		
			Measured	Calculated	Error (ppm)	Newly detected compounds	Known compounds	Reference
Fatty acids, aromatic acid, and related derivatives (F)								
F1	10.902	$C_8H_6O_4$	165.019	165.0193	2	Phthalic Acid	–	
F2	14.918	$C_7H_6O_2$	121.0300	121.0295	−4.07	Benzoic acid	–	
F3	92.219	$C_{18}H_{34}O_5$	329.2359	329.2333	−7.73	Trihydroxyoctadecenoic acid	–	
F4	95.471	$C_{17}H_{26}O_4$	293.1764	293.1758	−1.93	Heptadecatrienedioic acid	–	
F5	96.081	$C_{18}H_{32}O_4$	311.2206	311.2228	6.99	Octadecadienedioic acid	–	
F6	96.848	$C_{18}H_{34}O_4$	313.2366	313.2384	5.83	–	Octadecanedioic acid	Shcherbakov et al. (2022)
F7/8/10	99.917/100.448 /101.228	$C_{18}H_{30}O_3$	293.2100	293.2122	7.54	Hydroxylinolenic acid and its isomers	–	
F9	100.730	$C_{18}H_{32}O_3$	295.2287	295.2279	−2.01	Hydroxylinoleic acid	–	
F11	101.875	$C_{19}H_{32}O_4$	323.2211	323.2228	5.19	Nonadecadienedioic acid	–	
F12	102.340	$C_{12}H_{24}O_2$	199.1713	199.1704	−4.73	–	Lauric acid	Shcherbakov et al. (2022)
F13	103.169	$C_{18}H_{34}O_3$	297.2433	297.2435	0.73	Hydroxyoleic acid	–	
F14	105.940	$C_{15}H_{30}O_3$	257.2111	257.2122	4.33	Hydroxypentadecanoic acid	–	
F15	107.699	$C_{14}H_{28}O_2$	227.2014	227.2017	1.11	–	Myristic acid	Shcherbakov et al. (2022)
							Ethyl dodecanoate	Sun et al. (2011)
F16	107.848	$C_{20}H_{30}O_2$	301.2188	301.2173	−4.95	Eicosapentaenoic acid		
F17	108.296	$C_{16}H_{32}O_3$	271.2293	271.2279	−5.26	–	2-Hydroxyhexadecanoic acid	Shcherbakov et al. (2022)
F18	108.595	$C_{16}H_{30}O_2$	253.2185	253.2173	−4.71	–	Palmitoleic acid	Shcherbakov et al. (2022)
F19	108.910	$C_{20}H_{38}O_4$	341.2692	341.2697	1.56	–	Eicosanedioic acid	Shcherbakov et al. (2022)
F20	109.308	$C_{18}H_{30}O_2$	277.2161	277.2173	4.33	–	Linolenic acid	Kahlos et al. (1989); Shcherbakov et al. (2022)
F21	109.790	$C_{15}H_{30}O_2$	241.2176	241.2173	−1.22	–	Pentadecanoic acid	Shcherbakov et al. (2022)
F22	109.922	$C_{18}H_{32}O_2$	279.2334	279.233	−1.59	–	Linoleic acid	Sun et al. (2011)
F23	110.553	$C_{17}H_{32}O_2$	267.2323	267.2330	2.44	–	Heptadecenoic acid	Shcherbakov et al. (2022)
F24	110.768	$C_{18}H_{36}O_2$	283.2642	283.2643	0.19	–	Stearic acid	Shcherbakov et al. (2022)
F25/33/ 41/48/52	111.747/114.634 /116.957/118.152 /118.965	$C_{16}H_{32}O_2$	255.233	255.233	−0.18	–	Palmitic acid	Shcherbakov et al. (2022)
							Ethyl tetradecanoate	Sun et al. (2011)
F26	112.029	$C_{18}H_{36}O_3$	299.2608	299.2592	−5.43	Hydroxystearic acid	–	
F27	112.278	$C_{18}H_{34}O_2$	281.2501	281.2486	−5.3	–	Oleic acid	Shcherbakov et al. (2022)
F28	112.71	$C_{18}H_{34}O_2$	281.2505	281.2486	−6.72	–	Vaccenic acid	Shcherbakov et al. (2022)
F29	112.942	$C_{24}H_{38}O_4$	389.2698	389.2697	−0.17	–	Di-iso-octyl phthalate	Sun et al. (2011)

Table 2. Chemical constituents (fatty acids, aromatic acids, and related derivatives) of chaga extract scanned by HPLC-ESI-TOF-MS - *(continued)*

Compound no.	t_R (min)	Formula	$[M-H]^-/[2M-H]^-$ (m/z)			Compounds detected in current study		
			Measured	Calculated	Error (ppm)	Newly detected compounds	Known compounds	Reference
F30/32	112.809/113.357	$C_{17}H_{34}O_2$	269.2509	269.2486	−8.5	–	Heptadecanoic acid/ margaric acid	Shcherbakov et al. (2022)
							Ethyl pentadecanoate	Sun et al. (2011)
F31	113.191	$C_{20}H_{36}O_2$	307.2627	307.2643	5.04	–	Ethyl linoleate	Sun et al. (2011)
F34/42 /47/53	114.900/117.090 /118.069/119.015	$C_{18}H_{36}O_2$	283.2648	283.2643	−1.92	–	Ethyl hexadecanoate	Sun et al. (2011)
F35	114.983	$C_{20}H_{40}O_3$	327.2895	327.2905	2.95	Hydroxyarachidic acid	–	
F36	115.198	$C_{20}H_{38}O_2$	309.2785	309.2799	4.52	–	Isopropyl linoleate	Sun et al. (2011)
F37/38	115.746/116.211	$C_{19}H_{38}O_2$	297.2783	297.2799	5.39	–	Nonadecanoic acid	Shcherbakov et al. (2022)
							Ethyl heptadecanoate	Sun et al. (2011)
F39	116.277	$C_{21}H_{42}O_3$	341.3057	341.3061	1.22	Hydroxyheneicosanoic acid		
F40	116.393	$C_{23}H_{44}O_3$	367.3219	367.3218	−0.36	–	Hydroxytricosenoic acid	Shcherbakov et al. (2022)
F43	117.438	$C_{20}H_{40}O_2$	311.2939	311.2956	5.3	–	Arachidic acid	Shcherbakov et al. (2022)
							Ethyl octadecenoate	Sun et al. (2011)
F44	117.538	$C_{22}H_{44}O_3$	355.3228	355.3218	−2.89	–	Hydroxydocosanoic acid	Shcherbakov et al. (2022)
F45	117.588	$C_{24}H_{46}O_3$	381.3386	381.3374	−3.09	Hydroxytetracosenoic acid	–	
F46	117.704	$C_{22}H_{42}O_2$	337.3118	337.3112	−1.76	Docosenoic Acid	–	
F48	118.533	$C_{23}H_{46}O_3$	369.3390	369.3374	−4.27	–	Hydroxytricosanoic acid	Shcherbakov et al. (2022)
F49	118.666	$C_{25}H_{48}O_3$	395.3560	395.3531	−7.39	Hydroxypentacosenoic acid		
F50	118.681	$C_{21}H_{42}O_2$	325.3132	325.3112	−6.12	–	Heneicosanoic acid	Shcherbakov et al. (2022)
F51	118.743	$C_{23}H_{44}O_2$	351.3272	351.3269	−0.98	–	Tricosenoic acid	Shcherbakov et al. (2022)
F54	119.595	$C_{24}H_{48}O_3$	383.3551	383.3531	−5.28	–	Hydroxytetracosanoic acid	Shcherbakov et al. (2022)
F55	119.728	$C_{22}H_{44}O_2$	339.3273	339.3269	−1.31	–	Docosanoic Acid/ behenic acid	Shcherbakov et al. (2022)
F56	119.778	$C_{26}H_{50}O_3$	409.3723	409.3687	−8.73	Hydroxyhexacosenoic acid	–	
F57	119.911	$C_{24}H_{46}O_2$	365.3448	365.3425	−6.27	–	Tetracosenoic acid	Shcherbakov et al. (2022)
F58	120.525	$C_{25}H_{50}O_3$	397.371	397.3687	−5.73	–	Hydroxypentacosanoic acid	Shcherbakov et al. (2022)
F59	120.641	$C_{23}H_{46}O_2$	353.3446	353.3425	−5.91	–	Tricosanoic acid	Shcherbakov et al. (2022)
F60	121.420	$C_{26}H_{52}O_3$	411.3865	411.3844	−5.17	–	Hydroxyhexacosanoic acid	Shcherbakov et al. (2022)
F61	121.537	$C_{24}H_{48}O_2$	367.3600	367.3582	−5.01	–	Lignoceric acid	Shcherbakov et al. (2022)
F62	121.570	$C_{26}H_{50}O_2$	393.3735	393.3738	0.77	–	Hexacosenoic acid	Shcherbakov et al. (2022)
F63	122.316	$C_{27}H_{54}O_3$	425.3995	425.4000	1.22	Hydroxyheptacosanoic acid	–	

Table 3. Chemical constituents (terpenoids) of chaga extract scanned by HPLC-ESI-TOF-MS

Compound no.	t_R (min)	Formula	$[M-H]^-/[2M-H]^-$ (m/z)			Compounds detected in current study		
			Measured	Calculated	Error (ppm)	Newly detected compounds	Known compounds	Reference
Terpenoids (T)								
T1	3.287	$C_{18}H_{22}O_{11}$	413.1093	413.1089	−0.88	Asperuloside	–	
T2	48.665	$C_{20}H_{24}O_{10}$	423.1301	423.1297	−1.01	Ginkgolide B	–	
T3	67.746	$C_{28}H_{42}O_8$	505.2837	505.2807	−5.94	Kidjoladinin	–	
T4	73.171	$C_{28}H_{46}O_8$	509.3153	509.312	−6.48	Palythoalone A	–	
T5/6/7	74.897/75.710 /78.796	$C_{31}H_{50}O_9$	565.3401	565.3382	−3.34	24-Methylene-lanosta-7,9(11)-dien-3*β*-ol and its isomers	–	
T8	79.41	$C_{31}H_{50}O_8$	549.3435	549.3433	−0.38	Laccijalaric ester II	–	
T9	80.007	$C_{30}H_{46}O_8$	533.3123	533.312	−0.58	Platycogenic acid A	–	
T10	80.372	$C_{30}H_{48}O_7$	519.3338	519.3327	−2.06	Platycodigenin	–	
T11	81.816	$C_{30}H_{52}O_8$	539.3579	539.3589	1.93	Longilene peroxide	–	
T12/22	81.965/86.810	$C_{38}H_{46}O_4$	565.3321	565.3323	0.41	Dichapetalin A and its isomers	–	
T13	82.28	$C_{30}H_{48}O_7$	519.3337	519.3327	−1.87	Trachelosperogenin B	–	
T14/16/21	83.110/84.835 /86.698	$C_{30}H_{46}O_7$	517.3205	517.3171	−6.6	Perulactone	–	
T15	83.326	$C_{38}H_{46}O_2$	533.3474	533.3484	1.83	1′,2′,5′-Tridemethyl-*β*,phi-carotene-3′-carboxylic acid	–	
T17	85.167	$C_{30}H_{48}O_6$	503.339	503.3378	−2.35	–	Inonotusal H	Kou et al. (2021)
T19	85.532	$C_{31}H_{48}O_8$	547.3283	547.3276	−1.2	Stereonsteroid D	–	
T20/26	86.163/90.261	$C_{31}H_{50}O_8$	549.3453	549.3433	−3.65	Methyl 3-(*β*-D-allopyranosyloxy) chol-5-en-24-oate	–	
T23	87.639	$C_{30}H_{50}O_8$	537.3453	537.3433	−3.73	Secodehydrothyrsiferol	–	
T24/28/34/56	88.170/90.759 /93.662/102.323	$C_{30}H_{48}O_5$	487.3467	487.3429	−7.78	–	Inonotusol D or C or E	Liu et al. (2014)
							Inonotusane E	Zhao et al. (2016)
T25	89.979	$C_{15}H_{22}O_4$	265.1427	265.1445	6.89	Fascicularone J	–	
T27	90.726	$C_{29}H_{44}O_7$	503.2977	503.3014	7.39	Capitasterone	–	
T29	91.140	$C_{28}H_{40}O_6$	471.2776	471.2752	−5.06	Ajugin B	–	
T30	91.754	$C_{28}H_{38}O_6$	469.2595	469.2596	0.13	Withanone	–	
T31/32	91.754/92.932	$C_{31}H_{50}O_7$	533.3481	533.3484	0.52	Viburnol F	–	
T33	93.214	$C_{28}H_{42}O_6$	473.2917	473.2909	−1.77	Cynanchogenin	–	
T35	93.762	$C_{20}H_{24}O_3$	311.1656	311.1653	−1.06	Triptophenolide	–	

Table 3. Chemical constituents (terpenoids) of chaga extract scanned by HPLC-ESI-TOF-MS - *(continued)*

Compound no.	t_R (min)	Formula	$[M-H]^-/[2M-H]^-$ (m/z)			Compounds detected in current study		
			Measured	Calculated	Error (ppm)	Newly detected compounds	Known compounds	Reference
T36	93.994	$C_{29}H_{46}O_7$	505.3151	505.3171	3.91	Ajugasteron B	–	
T37	94.210	$C_{30}H_{46}O_6$	501.3191	501.3222	6.1	Medicagenic Acid	–	
T38	94.426	$C_{28}H_{40}O_5$	455.2801	455.2803	0.43	Tolypodiol	–	
T39	94.658	$C_{28}H_{42}O_5$	457.295	457.2959	2.07	Strophasterol C	–	
T40/43	95.089/95.753	$C_{30}H_{46}O_5$	485.3301	485.3272	−5.86	3*β*,15,223-trihydroxylanosta-7,9(11),24-trien-26-oic acid/ Methyl ganolucidate	–	
T41	95.355	$C_{23}H_{34}O_7$	421.2232	4212232	−0.05	Nigakilactone J	–	
T42	95.388	$C_{28}H_{42}O_5$	457.2957	457.2959	0.54	5*β*,6*β*-epoxy-3*β*,7*α*,9*α*-trihydroxy-(22E,24R)-ergosta-8(14),22-dien-15-one	–	
T44	95.852	$C_{30}H_{44}O_6$	499.3050	499.3065	3.02	Ganolucidic acid D	–	
T45	96.383	$C_{21}H_{26}O_3$	325.1806	325.1809	0.98	Neridienone A	–	
T46/53/57/63	97.130/101.378/ 102.257/105.708	$C_{30}H_{46}O_4$	469.3346	469.3323	−4.82	–	Inonotsulide B	Taji et al. (2007)
							Inonotusane F/ Chagabusone A	Baek et al. (2018)
T47/49	98.540/99.951	$C_{30}H_{50}O_5$	489.3611	489.3585	−5.2	(7b,15a)-7,15-dihydroxysoyasapogenol B	–	
T48	98.806	$C_{22}H_{28}O_3$	339.1988	339.1966	−6.56	17*β*-Hydroxy-2-(hydroxymethyl)-17*α*-pregna-1,4-dien-20-yn-3-one	–	
T50	100.365	$C_{27}H_{42}O_4$	429.303	429.301	−4.57	–	Inonotusane C	
T51	101.129	$C_{30}H_{46}O_4$	319.2272	319.2279	2.09	–	17-Hydroxy-ent-atisan-19-oic acid	Ying et al. (2014)
T52	101.477	$C_{21}H_{28}O_2$	311.2023	311.2017	−2.07	–	Inonotusic acid	Liu et al. (2014)
T54	101.709	$C_{27}H_{40}O_4$	427.2853	427.2854	0.19	Dimethyl 4*β*,14*α*-dirnethyl-3-nor-5*α*-pregn-8-ene-4*α*,20*α*-dicarboxylate	–	
T55	102.307	$C_{15}H_{22}O_3$	249.1481	249.1496	6.07	–	3β-Hydroxycinnamolide	Ying et al. (2014)
T58	103.203	$C_{30}H_{46}O_4$	469.3364	469.3323	−8.65	Polyporenic acid C	–	
T59	103.551	$C_{29}H_{44}O_4$	455.3185	455.3167	−3.98	8*α*-epidioxyergosta-6,22-dien-3*β*-formate	–	
T60/61/62 /64/65	104.099/104.596/ 105.194/105.924 /106.654	$C_{30}H_{48}O_4$	471.3501	471.348	−4.48	–	Inonotsulide A	Taji et al. (2007)

Table 3. Chemical constituents (terpenoids) of chaga extract scanned by HPLC-ESI-TOF-MS - *(continued)*

Compound no.	t_R (min)	Formula	$[M-H]^-/[2M-H]^-$ (m/z)			Compounds detected in current study		
			Measured	Calculated	Error (ppm)	Newly detected compounds	Known compounds	Reference
							Saponaceoic acid I	Zhao et al. (2016)
							Inonotusol I	Kou et al. (2021)
							Inonotsulide A	Taji et al. (2008a)
							Saponaceoic acid I	Zhao et al. (2016)
T66	107.832	$C_{39}H_{58}O_5$	605.4253	605.4211	−6.85	Karavoate M	–	
T67	108.612	$C_{31}H_{48}O_4$	483.3514	483.348	−7.05	Methyl polyporenic acid C	–	
T68/77/81/89/96/105/107	108.595/112.179/112.892/114.203/114.601/116.874/118.915	$C_{30}H_{48}O_3$	455.3526	455.3531	1.03	–	Trametenolic acid	Kim et al. (2011)
							Ganodecochlearin A	Zhao et al. (2016)
							Oleanolic acid/ Betulinic acid	Zhao et al. (2015a)
							Lanosta-7,9(11),23E-trien-3β,22R,25-triol	Taji et al. (2008b)
							Inonotusol G	Liu et al. (2014)
							Inonotsutriol C	Taji et al. (2008a)
							Inonotsuoxodiol A	Handa et al. (2010)
T69	109.093	$C_{25}H_{36}O_2$	367.2658	367.2643	−4.2	Variecolin	–	
T70	109.491	$C_{28}H_{44}O_6$	475.3091	475.3065	−5.43	(20S,22R,24R)-16,22-epoxy-3*β*,14*α*,23*β*,25-tetrahydroxyergost-7-en-6-one	–	
P110	110.636	$C_{39}H_{56}O_5$	603.4082	603.4055	−4.47	–	Betulin-3-O-caffeate	Wold et al. (2020)
T72	110.785	$C_{28}H_{42}O_4$	441.3040	441.3010	−6.71	Eringiacetal B	–	
T74	111.067	$C_{28}H_{44}O_5$	459.3131	459.3116	−3.26	Polyporusterone G	–	
T71/75	109.574/111.349	$C_{30}H_{46}O_3$	453.3381	453.3374	−1.5	–	3β-Hydroxylanosta-8,24-diene-21,23-lactone	Shin et al. (2000)
							3β-Hydroxylanosta-7,9(11),24-trien-21-oic acid	Zhao et al. (2015a)
							Inonotsulide C	Taji et al. (2007)
T76	111.714	$C_{39}H_{56}O_5$	603.4077	603.4055	−3.64	Celastrusin A	–	

Table 3. Chemical constituents (terpenoids) of chaga extract scanned by HPLC-ESI-TOF-MS - *(continued)*

Compound no.	t_R (min)	Formula	$[M-H]^-/[2M-H]^-$ (m/z)			Compounds detected in current study		
			Measured	Calculated	Error (ppm)	Newly detected compounds	Known compounds	Reference
T78/79	112.428/112.71	$C_{32}H_{50}O_4$	497.3628	497.3636	1.67	6α-Hydroxypolyporenic acid C and its isomers	–	
T80/84	112.826/113.556	$C_{39}H_{56}O_4$	587.4124	587.4106	−3.09	Lupeol caffeate	–	
T82	113.076	$C_{39}H_{54}O_5$	601.3909	601.3898	−1.75	3-(*p*-Coumaroyl) ursolic Acid	–	
T83	113.224	$C_{31}H_{50}O_3$	469.3711	469.3687	−5.06	–	Methyl trametenolate	Shin et al. (2000)
T85	113.772	$C_{29}H_{46}O_6$	489.3209	489.3222	2.58	(17R, 20R)-7β,20,23,29-tetrahydroxy-28-norlupane-3,16-dione	–	
T86	113.821	$C_{27}H_{46}O_4$	433.3322	433.3323	0.31	2,3-Secocholestan-2,3-dioic acid	–	
T87	113.888	$C_{55}H_{73}O_4$	796.5408	796.5436	3.52	Unknown pentaterpenoids	–	
T88	114.153	$C_{41}H_{54}O_5$	625.3909	625.3898	−1.68	Furostan-3,26-diyl dibenzoate	–	
T90/95	114.286/114.568	$C_{43}H_{73}O_{11}$	764.5114	764.508	−4.43	Unknown triterpenoid glycoside	–	
T91	114.352	$C_{29}H_{46}O_5$	473.3250	473.3272	4.74	Trichiol C	–	
T73/92	110.785/114.369	$C_{30}H_{48}O_6$	503.3396	503.3378	−3.54	–	Inonotusol A or Inonotusol B	Liu et al. (2014)
T93/104	114.385/116.824	$C_{39}H_{58}O_5$	605.4219	605.4211	−1.24	3β,20(s),26-trihydroxydammar-24-ene-3-O-*p*-coumarate and its isomers	–	
T94	114.568	$C_{38}H_{67}O_7$	634.4824	634.4814	−1.57	Unknown triterpenoid glycoside	–	
T97	114.767	$C_{40}H_{60}O_{12}$	731.3989	731.4012	3.14	Unknown triterpenoid glycoside	–	
T98	115.298	$C_{39}H_{68}O_{10}$	695.4752	695.474	−1.76	5α-Cholestanyl 3-O-(α-L-fucopyranosyl)-β-D-Galactopyranoside	–	
T99	115.547	$C_{35}H_{58}O_8$	605.4092	605.4059	−5.45	Unknown triterpenoid glycoside	–	
T100	115.646	$C_{40}H_{48}O_4$	591.3457	591.348	3.86	7,8,7′,8′-Tetradehydroastaxanthin	–	
T101/106	115.995/118.301	$C_{39}H_{58}O_4$	589.4284	589.4262	−3.67	β-Sitosteryl ferulate and its isomers	–	
T102	116.559	$C_{31}H_{50}O_5$	501.3557	501.3585	5.67	Ganoderiol I	–	
T103	116.592	$C_{32}H_{52}O_6$	531.3683	531.3691	1.53	Fomitopinic acid B	–	
T108	122.267	$C_{30}H_{50}O$	425.3792	425.3789	−0.73	–	Lanosterol	Kim et al. (2011)

Supplementary Material

Supplementary materials for this article are available at https://doi.org/10.31665/JFB.2022.17304.

Figure s1. TIC (total ion chromatograph) of chaga extract, 0-80 min.

Figure s2. TIC (total ion chromatograph) of chaga extract, 80-109 min.

Figure s3. TIC (total ion chromatograph) of chaga extract, 109-133 min.

Table s1. Known phenolic small molecules and polymers of chaga and their purification/identification.

Table s2. Polysaccharides, fatty acids, and other compounds of chaga and their purification/identification.

Table s3. Known terpenes and terpenoids of chaga and their purification/identification.

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