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Extraction, Chemical Composition, Antiradical Capacity, and Photoprotective Effect of *Inonotus obliquus* from Eastern Canada

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Abstract: To promote the rational use of *Inonotus obliquus* (chaga) from Eastern Canada, a mushroom consumed in infusion as a health drink, the extraction of its constituents was investigated. The extraction was carried out with water or ethanol at room temperature or by heating as well as following exposure to ultrasound. The extracts of the four seasons obtained with the four extraction methods were compared for their contents in carbon, nitrogen (N), sulfur (S), potassium (K), betulin, betulinic acid, potassium, flavonoids, and polyphenols. The antiradical effect as well as the photoprotective effects of all extracts were also investigated. The results show that there is no difference between the quantities extracted from the extracts of the 4 seasons. The results show a significant difference between the mass of the extracts obtained with water and ethanol. Betulinic acid was found to be more abundant in the extracts obtained with water while betulin was more abundant in the extracts obtained with ethanol. The mushroom and the extracts had high C contents, but low N and S contents, typical of protein-poor and carbohydrate-rich materials. Extracts were particularly rich in potassium, five times more than bananas. Heating favors the extraction of polyphenols and flavonoids. The aqueous extracts of chaga harvested in winter had the highest antiradical capacity. With a Sun Protection Factor (SPF) higher than 30 in the UVB wavelength, chaga extracts might be used as sunscreen. Extracts obtained with water had the highest SPF in general. The analysis of this mushroom further highlights this local product that deserves more attention for its potential benefits as a functional food/nutraceutical product. In addition to its nutritional values, this mushroom can also be used for its cosmetic qualities as it can be used as a sunscreen.

Keywords: *Inonotus obliquus*; chaga; potassium; antiradical; photoprotective effects

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1. Introduction

Modern lifestyle with low physical activity combined with poor nutrition has resulted in an epidemic of chronic diseases that can lead to many health problems [1]. Functional foods and nutraceuticals can influence specific functions of the body, thus providing additional health benefits through the support with beneficial ingredients [2–9]. The functional products and nutraceuticals industry is an innovative industry that is in constant growth and in continuous research of new products (refs). Among the products already investigated for their potential as functional foods and nutraceuticals, mushrooms are very good candidates due to their ability of adaptation and synergy with their host. *Inonotus obliquus* [10], also known as chaga, which grows on white or yellow birch trees in northern regions may be an excellent candidate given its adaptation to extreme climatic conditions and its history of use by the population in these regions.

Analyses of chaga extracts from various regions of the world have revealed that it is composed of bioactive compounds such as reticular chitin, β-glucans, polysaccharides,

flavonoids, terpenoids, and certain mineral salts [11–16]. Hence, research teams have shown the potential of these extracts as antioxidant, antiviral, antidiabetic, antiparasitic, anti-neuroinflammatory, antiarthritic, and anti-inflammatory [17–20]. Studies have shown that chaga can have an impact in the prevention and treatment of diseases, such as diabetes and hypertension [21]. Among the products contained in this mushroom, polyphenols, flavonoids, and some terpenes have demonstrated their bioactivities [17,22–25]. Betulinic acid and betulin, two terpenes with various biological properties, found in birch and chaga are of great importance especially for their anticancer properties [22].

In addition to the antioxidant/antiradical effect, polyphenols and flavonoids can absorb UV rays. Photoprotection against UV damage is an essential prophylactic and therapeutic element. Topical sunscreens contain molecules or molecular complexes that can absorb, reflect, or scatter UV photons. More and more people are allergic to certain sunscreens due to the presence of some chemicals [26]. To reduce DNA harm produced by UV radiation, anti-mutagens with UV-protective domains are being investigated. A diversity of plants and their constituents have been detected to contain anti-mutagenic potency [27,28]. Mushrooms can also have such properties that remain to be exploited.

Our work is the first on chaga collected in eastern Canada. In this contribution, we have explored the differences between the harvesting seasons of chaga. Based on any scientific basis, the chaga is harvested only in late spring or early fall. Three trees were selected for chaga harvesting during the four seasons of the year. The extracts of the four seasons obtained with four extractions procedures using water and ethanol were compared for their organic elemental composition and contents in potassium, betulin, betulinic acid, flavonoids, and polyphenols. The antiradical effect as well as the photoprotective effects of all extracts were also investigated.

2. Materials and Methods

Three trees were selected for chaga harvesting during the four seasons of the year (from spring 2020 to winter 2021) in the Grand-Falls region, New Brunswick, Canada. GPS coordinates of each tree were taken to be able to track it at each harvest (tree 1: N 47.15173, W 67.51067; tree 2: N 47.15083, W 67.51395; tree 3: N 47.14855, W 67.51200). The collected mushrooms were dried and ground into fine powders for further use. Folin–Ciocalteu's reagent, 2,2-bipyridyl, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), quercetin, gallic acid, betulinic acid, and betulin were purchased from Sigma-Aldrich (Oakville, ON, Canada).

2.1. Extraction

Chaga (1 g) was mixed with 10 mL of H_2O or EtOH and stirred for 24 h. The extraction was performed either at room temperature (RT) or with heating (70 °C for water and 50 °C for ethanol) and with or without ultrasound pretreatment (20 min sonification with an amplitude of 50 Hz). After 24 h, the supernatant was recovered by centrifugation (2500 rpm for 10 min for ethanolic extracts and 2800 rpm for 10 min for aqueous extracts). To optimize the solid–liquid extraction, the residue was mixed with 10 mL of the solvent and stirred for 24 h under the same conditions two more times. Supernatants were recovered by centrifugation as described above and combined with the previous ones. The aqueous extracts were lyophilized while the ethanolic extracts were evaporated under an airstream at RT and then dried under vacuum pump to a constant weight.

2.2. Betulin and Betulinic Acid Content

Betulin and betulinic acid contents were determined by liquid chromatography–mass spectrometry (LC-MS). The system consists of a high-performance liquid chromatography (HPLC, Agilent 1100) coupled to a triple quadrupole mass spectrometer (Ultivo Agilent, Santa Clara, CA, USA). The chromatographic separation was performed with a C18 column (InifinityLab Poroshell 120 EC-C18, 2.1 × 100 mm, particle size 2.7 micron). The injection volume was 1 µL of each extract. The chromatography was performed in isocratic mode and the mobile phase was composed of 85% acetonitrile and 15% water contain-

ing 0.1% formic acid and the flow rate of the mobile phase was 0.5 mL/min. The mass spectrometer was used in SIM (selected ion monitoring) mode. Betulin was detected at *m/z* 443.5 and betulinic acid at *m/z* 439.5 (Supplementary Materials Figure S1). Betulin and betulinic acid contents were determined based on the standard curves for betulin and betulinic acid (0.01–2.5 µg/mL in acetonitrile). The calibration curves (five data points) were linear with R^2 = 0.9992 for betulinic acid and R^2 = 1 for betulin. Examples of chromatograms of extracts obtained with water and ethanol are included in the Supplementary Materials (see Figures S2 and S3).

2.3. ICP Analysis

Metals concentrations were measured in chaga and its extracts by inductively coupled plasma mass spectrometer (ICP-MS) after their acidic mineralization: 50 mg of chaga or extract was digested in a mixture of 0.75 mL HCl (37%, Fisher Scientific, Ottawa, ON, Canada) and 1.25 mL HNO_3 (37%, Fisher Scientific, Ottawa, ON, Canada) at RT for at least 24 h. Samples were diluted by a factor 25 to allow their measurement by the iCAP-Q ICP-MS (Thermo Scientific; Bremen, Germany) interfaced with the ASX-520 auto sampler from CETAC Technologies (Omaha, NE, USA). The extracts were weighed before and after mixing with the acids. Indium 115 was used as an internal standard and the sequence systematically included metal standards, procedural blanks, reference materials, and samples. From the mass and the volume of acid added to the samples, the values obtained by ICP-MS were converted into concentration expressed in ng/mg.

2.4. Elemental Analysis

The dry weight percentages of carbon (%C), nitrogen (%N), and sulfur (%S) of the chaga and chaga extracts were measured with an Elementar vario ISOTOPE select elemental analyzer. Samples and standards were introduced to a combustion column held at 1150 °C containing WO_2 granulate and quartz chips. An external calibration was performed each day by analyzing sulfanilamide standards. Accuracy and precision were calculated by analyzing two sulfanilamide aliquots, treated as samples, a few times during each run. The measured (x%) elemental composition was different from the expected (y%) values, in percent difference ($100 \cdot |x - y| \%/y\%$), by <11% (mean = 3%) for %C, <13% (mean = 3%) for %N, and <39% (mean = 4%) for %S. The coefficients of variation were 3.2% for %C, 4.3% for %N, and 6.5% for %S.

2.5. Antioxidant Activity

Chaga extract solution (100 µL, 10 mg/mL in methanol) was mixed with 3 mL of the DPPH solution (6×10^{-5} M in methanol). After incubation in the dark at 37 °C for 20 min, the absorbance of 100 µL was measured at 515 nm with a microplate reader (Vario Scan spectrophotometer, Thermo-Fisher Scientific, San Jose, CA, USA). Ascorbic acid (10 mg/mL in methanol) with a concentration of 10 mg/mL and that of each extract (10 mg/mL) was used as positive control. The experiment was performed in triplicate. The radical scavenging activity was calculated using the following formula [29]:

$$\%\text{Inhibition} = [(A_{\text{control}} - A_{\text{test}})/A_{\text{control}}] \times 100$$

2.6. Total Phenolic Content

The total phenolic content (TPC) was determined with the Folin–Ciocalteu method with minor modifications [30–32]. Chaga extract solution (100 µL; 1 mg/mL in methanol) was mixed with 2 mL of 2% Na_2CO_3 and vortexed for 3 min. Then, 100 µL of 50% Folin–Ciocalteu was added to the mixture. After incubation in the dark at 25 °C for 30 min, the absorbance of 100 µL was measured at 700 nm with a microplate reader (Vario Scan spectrophotometer, Thermo-Fisher Scientific, San Jose, CA, USA). The total polyphenolic content was calculated based on the standard curve for gallic acid (3–0.25 mg/mL) and

expressed as mg of gallic acid equivalents (GAE) per g of dry chaga (mg GAE/g chaga). The calibration curves (five data points) were linear with $R^2 = 0.999$.

2.7. Total Flavonoid Content

The total flavonoid content was determined by the colorimetric method using aluminum chloride with minor modifications [30]. Chaga extract solution (100 µL; 1 mg/mL in methanol) was mixed with 1.5 mL of 95% ethanol. Then, 100 µL of 10% $AlCl_3$ and 100 µL of 1 M potassium acetate were added. After incubation in the dark at 25 °C for 30 min, the absorbance of 100 µL was measured at 700 nm with a microplate reader (Vario Scan spectrophotometer, Thermo-Fisher Scientific, San Jose, CA, USA). Total flavonoid content was calculated based on the quercetin standard curve (0.5–10 mg/mL) and expressed as mg quercetin equivalents (QcE) per g of dry chaga (mg QcE/g chaga). The standard curves (five data points) were linear with $R^2 = 0.995$.

2.8. Photoprotective Effects

Sun protection factors ($SPF_{(290–320)}$ and $SPF_{(290–400)}$) and UVA protection factor (PF-UVA) were determined in vitro for each extract (10 mg/mL in methanol) at 5 nm intervals by ultraviolet–visible spectrophotometry in a mini quartz cuvette [33–38]. MeOH and water: MeOH (1:1) were used as blanks for ethanol and water extracts, respectively.

$SPF_{(290–320)}$ was calculated using the following equation:

$$SPF_{(290–320)} = CF \times \sum_{290}^{320} EE(\lambda) \times I(\lambda) \times Abs(\lambda)$$

Here, CF: correction factor; EE(λ): erythmogenic effect of radiation with wavelength λ; Abs(λ): absorbance at wavelength λ; I(λ): intensity of solar light at wavelength λ. The values of EE(λ) and I(λ) are constants [39,40].

$SPF_{(290–400)}$ was calculated using the following equation:

$$SPF_{(290–400)} = \frac{\sum_{290}^{400} S(\lambda) \times EA(\lambda)}{\sum_{290}^{400} S(\lambda) \times EA(\lambda) \times T(\lambda)}$$

EA(λ): erythemal action spectrum: S: Solar spectral irradiance: (λ): spectral transmittance value at the given wavelength. By Diffey and Robson the S × EA values were determined [37,41].

PF-UVA or $SPF_{(320–400)}$ was calculated according to the following equation:

$$PF\text{-}UVA = \frac{\sum_{320}^{400} S(\lambda) \times EA(\lambda)}{\sum_{320}^{400} S(\lambda) \times EA(\lambda) \times T(\lambda)}$$

A(λ), S, and T(λ) are as above [41].

2.9. Statistical Analysis

Statistical analyses were performed with GraphPad Prism 6 software (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Extraction

The quantities of chaga aqueous extracts obtained under different conditions showed no significant variations from one season to another (Figure 1).

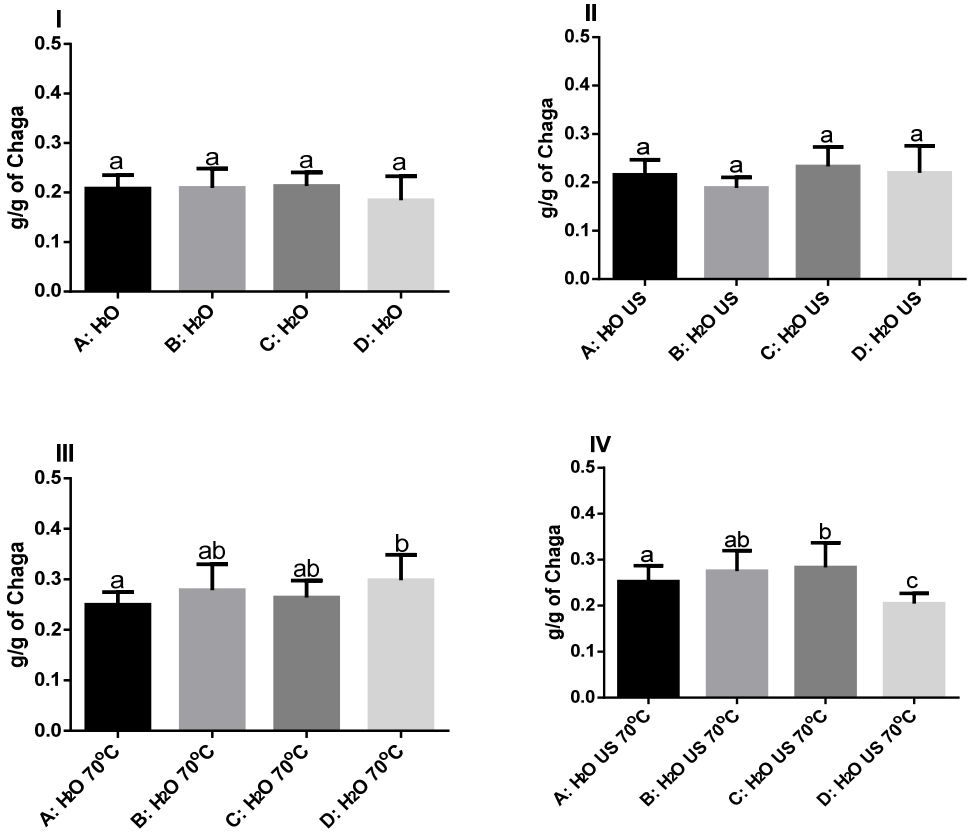

Figure 1. Extracts (g/g of chaga) obtained with water for each extraction condition according to the harvest season ((**A**): spring; (**B**): summer; (**C**): fall; (**D**): winter). Data are expressed as means ± SEM of at least three independent experiments each performed in triplicate. Values without one common superscript are significantly different determined by one-way ANOVA with Tukey's multiple comparison test ($p < 0.05$), (A: spring; B: summer; C: fall; D: winter; US: ultrasound; (**I**): RT; (**II**): US, RT; (**III**): 70 °C; (**IV**): US, 70 °C).

For each season, the extraction with heating seems to be more efficient (Figure 2). On the other hand, the extraction by heating preceded by an ultrasound treatment was more effective only for the samples collected during summer and fall seasons (Figure 2B,C).

The extraction using ethanol as the solvent is almost 10 times less efficient than with water since in general about 0.02–0.03 g/g of chaga ethanolic extracts are obtained each time (Figure 3) compared to 0.2–0.3 g/g of chaga aqueous counterparts (Figure 1). In addition, the amounts extracted from one season to the next are not significantly different if only the weight of the extracts is considered (Figure 3). This constitutes a similar trend with what is observed when water is used as the extracting solvent (Figure 2).

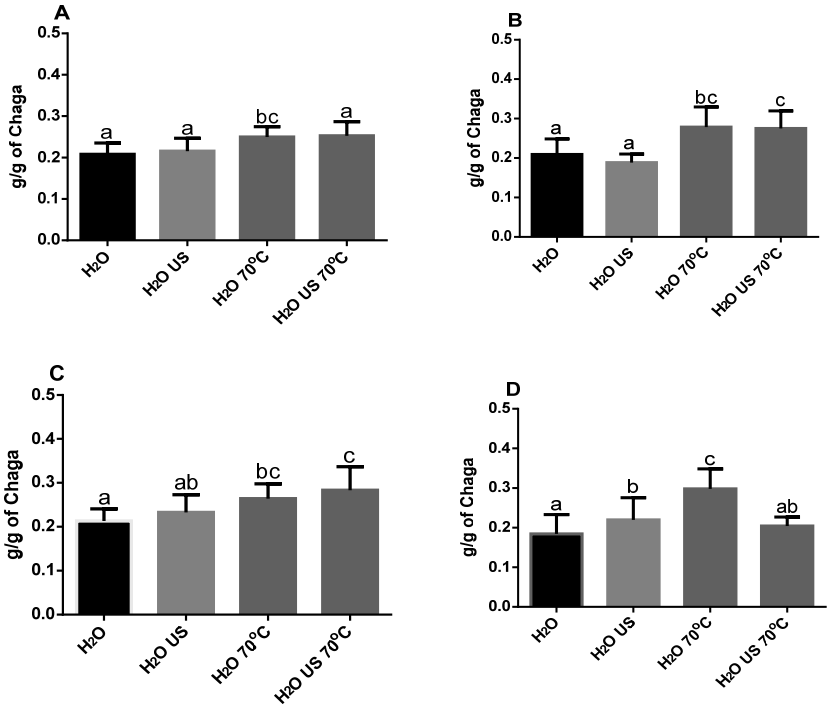

Figure 2. Extracts (g/g of chaga) obtained with water for each season according to the extraction conditions. Data are expressed as means ± SEM of at least three independent experiments each performed in triplicate. Values without one common superscript are significantly different determined by one-way ANOVA with Tukey's multiple comparison test ($p < 0.05$), ((**A**): spring; (**B**): summer; (**C**): fall; (**D**): winter; US: ultrasound).

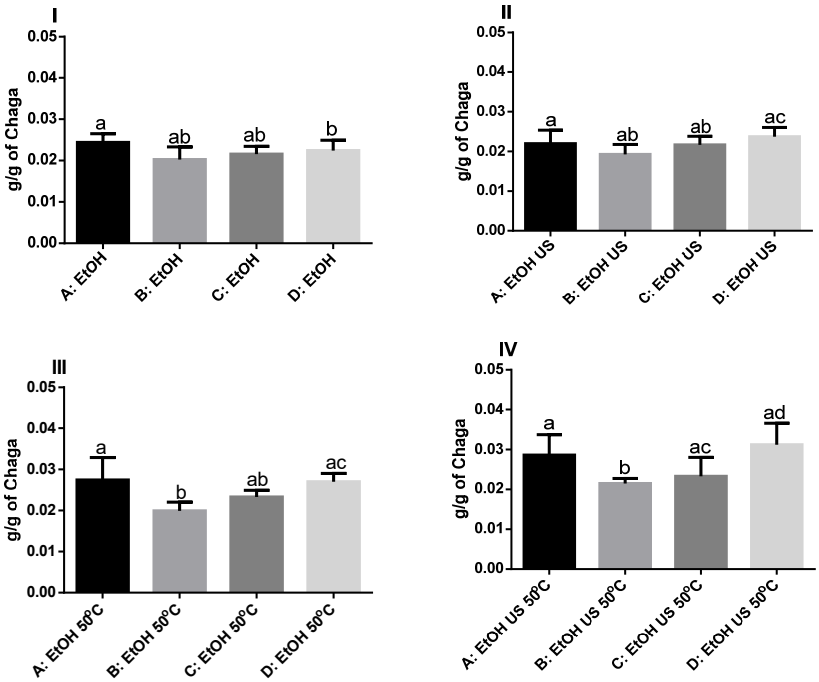

Figure 3. Extracts (g/g of chaga) obtained with ethanol for each extraction condition according to the harvest season ((**A**): spring; (**B**): summer; (**C**): fall; (**D**): winter). Data are expressed as means ± SEM of at least three independent experiments each performed in triplicate. Values without one common superscript are significantly different determined by one-way ANOVA with Tukey's multiple comparison test ($p < 0.05$), (A: spring; B: summer; C: fall; D: winter; US: ultrasound; (**I**): RT; (**II**): US, RT; (**III**): 50 °C; (**IV**): US, 50 °C).

In contrast to extractions with water, neither heating nor ultrasound treatment appeared to influence the yield of extraction with ethanol except for the mushroom harvested in winter (Figure 4).

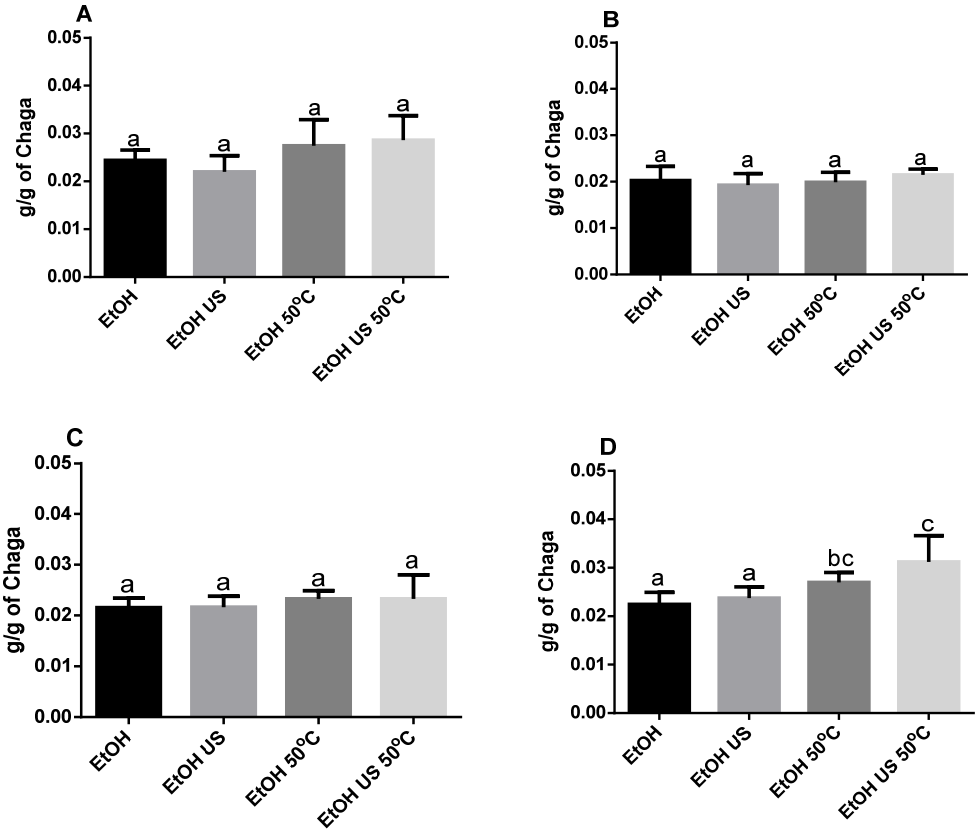

Figure 4. Extracts (g/g of chaga) obtained with ethanol for each season according to the extraction conditions. Data are expressed as means ± SEM of at least three independent experiments each performed in triplicate. Values without one common superscript are significantly different determined by one-way ANOVA with Tukey's multiple comparison test ($p < 0.05$), (**A**): spring; (**B**): summer; (**C**): fall; (**D**): winter; US: ultrasound.

3.2. Betulinic Acid and Betulin Content

Regardless of the season or the extraction protocol, betulin was not detected in any of the aqueous extracts except for the mushroom harvested in the fall season that shows a very small amount (0.6 ng/mL) in the extract obtained by heating (Table 1). On the other hand, betulinic acid was abundantly detected in all aqueous extracts. Moreover, heating as well as ultrasound treatment prior to the extraction increases the quantity extracted of this product (Table 1). Furthermore, extracts of mushrooms harvested in summer, fall, and winter seem to be the most enriched in betulinic acid (Table 1). Among all the aqueous extracts obtained with no heating nor ultrasound, the fall one is by far the richest in betulinic acid (360.8 ng/mL vs. 40.6 ng/mL or less for the other samples). These findings suggest that fall is the ideal season to harvest chaga with the highest yield in betulinic acid while keeping the extraction protocol simple and easy to carry out.

Table 1. Concentrations of betulinic acid and betulin in aqueous extracts.

Saison/Extraction Conditions	Betulinic Acid (ng/mL)	Betulin (ng/mL)
A/H_2O	40.6	nd [a]
A/H_2O US	160.2	nd
A/H_2O 70 °C	150.1	nd
A/H_2O US 70 °C	133.9	nd
B/H_2O	13.6	nd
B/H_2O US	405.7	nd
B/H_2O 70 °C	246.2	nd
B/H_2O US 70 °C	229.4	nd
C/H_2O	360.8	nd
C/H_2O US	351.3	nd
C/H_2O 70 °C	346.8	0.6
C/H_2O US 70 °C	369.7	nd
D/H_2O	11.7	nd
D/H_2O US	334.5	nd
D/H_2O 70 °C	384.5	nd
D/H_2O US 70 °C	389.0	nd

[a] not detected. A: spring, B: summer, C: fall, D: winter, US: ultrasound.

Unlike their aqueous analogs, the ethanolic extracts contain tiny quantities of betulin, spanning from a few to tens of ng/mL. Besides, for some seasons, and extraction conditions, betulin was more abundant than betulinic acid. Mushrooms harvested in the fall and the winter seem to be the most concentrated in betulinic with amounts of 90 ng/g betulin for the extract obtained even at room temperature (Table 2).

Table 2. Concentrations of betulinic acid and betulin in ethanolic extracts.

Saison/Extraction Conditions	Betulinic Acid (ng/mL)	Betulin (ng/mL)
A/EtOH	22.62	13.45
A/EtOH US	28.13	7.41
A/EtOH 50 °C	22.10	4.75
A/EtOH US 50 °C	7.57	4.02
B/EtOH	12.94	3.97
B/EtOH US	4.833	70.87
B/EtOH 50 °C	15.85	26.19
B/EtOH US 50 °C	7.66	9.16
C/EtOH	21.93	53.93
C/EtOH US	16.29	53.93
C/EtOH 50 °C	8.21	10.61
C/EtOH US 50 °C	16.88	87.47
D/EtOH	29.2	90.49
D/EtOH US	23.94	68.45
D/EtOH 50 °C	25.42	51.73
D/EtOH US 50 °C	13.21	27.90

A: spring, B: summer, C: fall, D: winter, US: ultrasound.

3.3. ICP/MS Analysis

Analysis using ICPMS revealed that mushrooms harvested throughout the year were rich in potassium as shown in Figure 5. The potassium concentrations were not significantly different in the twelve samples analyzed. In addition to potassium, manganese (Mn), magnesium (Mg), and calcium (Ca) were also detected. K was 125 times more abundant than Mn, 63 times more abundant than Mg, and 42 times more abundant than Ca.

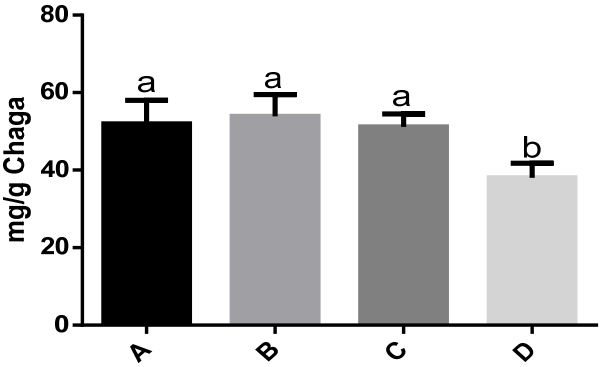

Figure 5. Concentration of K in Chaga (mg/g) for each season. Data are expressed as means ± SEM of at least three independent experiments each performed in triplicate. Values without one common superscript are significantly different determined by one-way ANOVA with Tukey's multiple comparison test ($p < 0.05$), (A: spring; B: summer; C: fall; D: winter).

In addition to K, Ca, Mg, and Mn, traces of the following metals were detected: Li, Be, Na, Mg, Al, P, Ca, Ti, V, Cr, Fe, Co, Ni, Cu, Zn, As, Rb, Sr, Y, Cd, Cs, Ba, La, Ce, Eu, and Pb.

The concentration of potassium was higher in the extracts obtained with water than those obtained with ethanol (Supplementary Materials Figure S4). The potassium concentration in aqueous extracts was 24 to 68 times higher than in those obtained with ethanol. Extraction conditions had no effect on the concentration of potassium in the extracts, except for summer extracts obtained after heating and pretreatment with ultrasound. Compared to the other three seasons, extracts from the winter were the least rich in potassium (Figure 6).

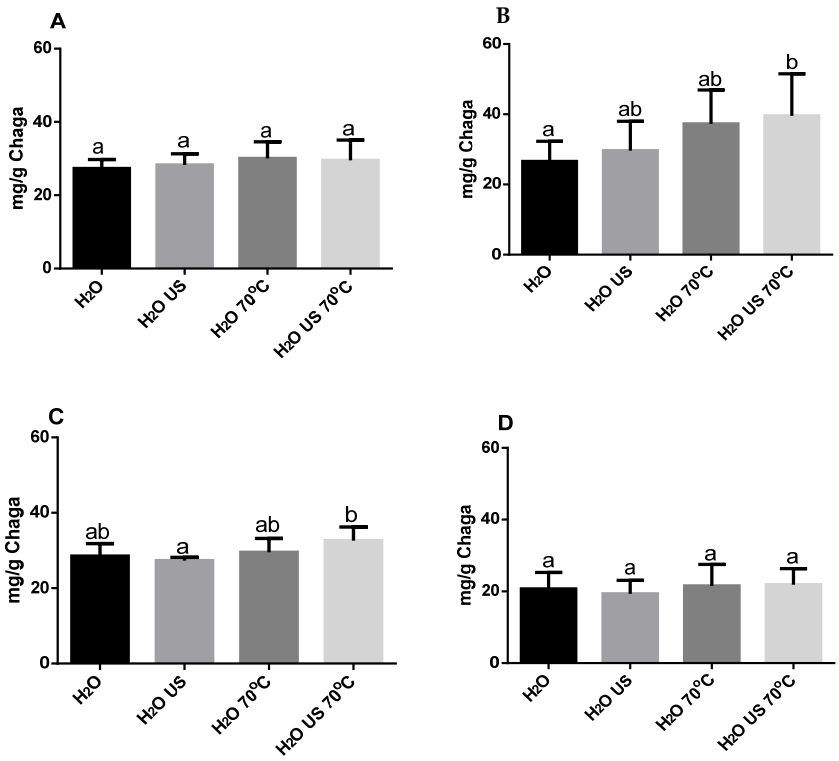

Figure 6. Concentration of K in chaga extracts obtained with water for each season (mg/g). Data are expressed as means ± SEM of at least three independent experiments each performed in triplicate. Values without one common superscript are significantly different determined by one-way ANOVA with Tukey's multiple comparison test ($p < 0.05$), (**A**): spring; (**B**): summer; (**C**): fall; (**D**): winter; US: ultrasound.

3.4. Elemental Analysis

Table 3 shows that the chaga mushroom was made of 42.4 to 44.9% C, indicative of mostly organic material, while it remains very poor in N and S. Moreover, the respective proportions of C, N, and S were almost constant during the year around. The only noticeable seasonal changes were for %S witnessing an increase in spring (season A) and a decrease in winter (season D). Finally, %N was also slightly higher in spring (season A) when compared to the other seasons.

Table 3. Elemental analysis (%dry weight) of the chaga mushroom.

Season	%C	%N	%S	C/N [a]
A	43.44	0.43	0.075	118
B	42.97	0.40	0.045	125
C	42.38	0.39	0.040	127
D	44.87	0.40	Bd [b]	131

[a] Atomic ratio, [b] Below detection, A: spring; B: summer; C: fall; D: winter.

Generally, aqueous extracts of chaga exhibited similar C, N, and S contents than the mushroom itself (Table 4). The first exception was for C/H_2O extract that had the lowest %C, probably because it was not completely desalted. The other exceptions were for the B/H_2O and D/H_2O US extracts that had greater %S than the other samples.

Table 4. Elemental analysis (%dry weight) of chaga aqueous extracts.

Season/Extraction Conditions	%C	%N	%S	C/N [a]
A/H_2O	51.12	0.36	0.070	166
A/H_2O US	43.80	0.35	0.055	146
A/H_2O 70 °C	44.30	0.35	0.040	148
A/H_2O US 70 °C	42.27	0.42	0.040	117
B/H_2O	41.79	0.37	0.12	132
B/H_2O US	40.50	0.35	0.065	135
B/H_2O 70 °C	45.69	0.35	0.045	152
B/H_2O US 70 °C	41.33	0.34	0.025	142
C/H_2O	22.86	0.22	0.010	121
C/H_2O US	42.09	0.35	0.020	140
C/H_2O 70 °C	46.80	0.37	0.040	148
C/H_2O US 70 °C	43.90	0.38	0.030	135
D/H_2O	41.14	0.28	0.070	171
D/H_2O US	44.71	0.36	0.19	145
D/H_2O 70 °C	46.42	0.36	0.045	150
D/H_2O US 70 °C	55.23	0.40	0.055	161

[a] Atomic ratio, A: spring; B: summer; C: fall; D: winter; US: ultrasound.

Compared to the chaga mushroom and aqueous extracts, the ethanolic extracts were generally enriched in C since %C exceeds 60.7% except for 4 extracts having %C between 43.7 and 45.0% (Table 5). These ethanolic extracts had similar N contents than the mushroom and aqueous extracts, but often very low S contents (except for A/ EtOH US 50 °C). The atomic C/N ratios indicated that the organic molecules extracted with ethanol using various conditions were often depleted in N (e.g., C/N > 200) and S when compared to the chaga mushroom and aqueous extracts. However, the average atomic C/N ratio for the ethanolic extracts from spring (season A) was significantly lower (*t*-test, $p < 0.05$) than that of the other seasons.

Table 5. Elemental analysis (%dry weight) of chaga ethanolic extracts.

Season/Extraction Conditions	%C	%N	%S	C/N [a]
A/EtOH	62.79	0.51	bd [b]	144
A/EtOH US	43.67	0.38	0.02	134
A/EtOH 50 °C	45.72	0.41	bd	130
A/EtOH US 50 °C	45.08	0.34	0.07	155
B/EtOH	64.38	0.36	bd	209
B/EtOH US	64.79	0.28	bd	270
B/EtOH 50 °C	44.39	0.41	0.01	126
B/EtOH US 50 °C	61.31	0.32	bd	224
C/EtOH	60.71	0.31	0.03	228
C/EtOH US	62.08	0.34	bd	213
C/EtOH 50 °C	62.19	0.31	bd	234
C/EtOH US 50 °C	65.87	0.29	bd	265
D/EtOH	63.40	0.28	bd	264
D/EtOH US	62.38	0.26	0.03	280
D/EtOH 50 °C	63.63	0.26	bd	286
D/EtOH US 50 °C	61.75	0.23	bd	313

[a] Atomic ratio, [b] Below detection, A: spring; B: summer; C: fall; D: winter; US: ultrasound.

3.5. Antioxidant Activity

Regardless of the extraction conditions, the DPPH-radical scavenging ability of the extracts was about 40–50%. Except for the extracts obtained following exposure to ultrasound and under heating, the extracts of the autumn had the lowest ability (Figure 7).

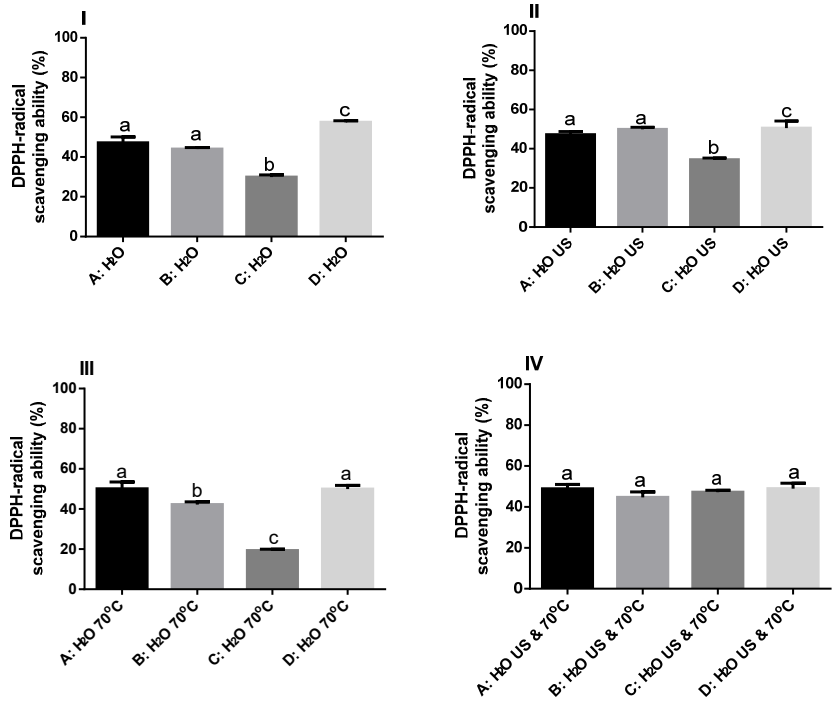

Figure 7. DPPH-radical scavenging ability (%) of chaga extracts (10 mg/mL) obtained with water for each season according to the extraction conditions. Data are expressed as means ± SEM of at least three independent experiments each performed in triplicate. Values without one common superscript are significantly different determined by one-way ANOVA with Tukey's multiple comparison test ($p < 0.05$), (A: spring; B: summer; C: fall; D: winter; US: ultrasound; (**I**): RT; (**II**): US, RT; (**III**): 70 °C; (**IV**): US, 70 °C).

Fall was the season that yields the aqueous extracts with the lowest DPPH-radical scavenging ability compared to the other seasons while winter was the season that gives rise to the extracts with the highest DPPH-radical scavenging ability approaching, in this case, 60% (Figure 8).

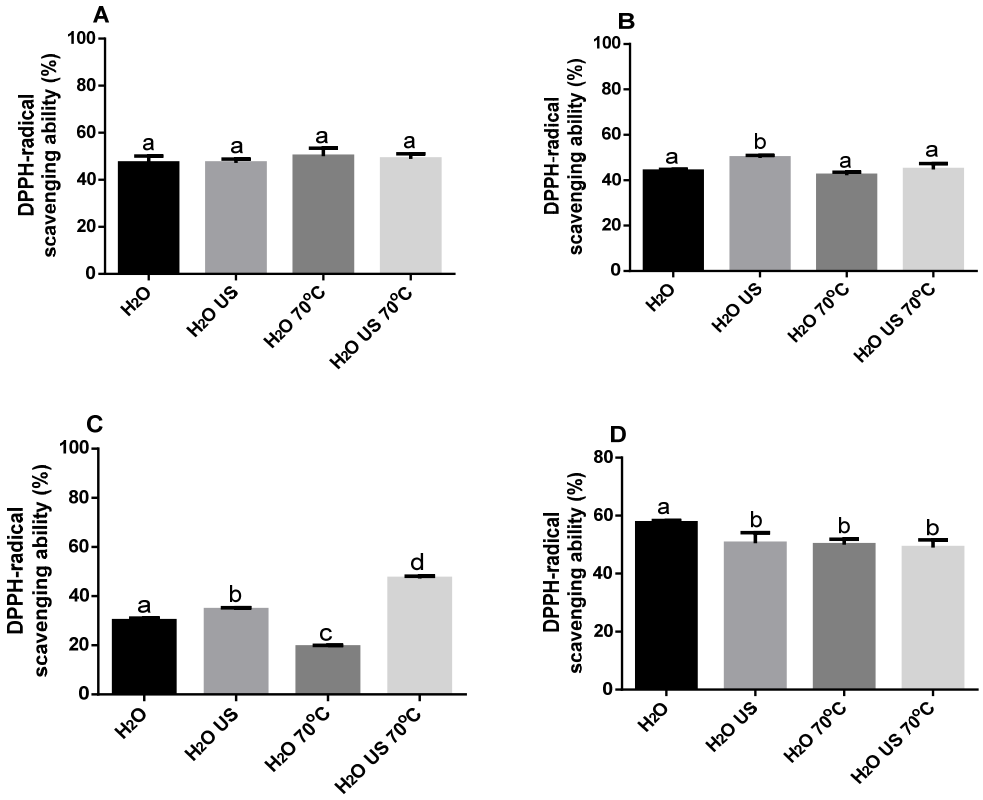

Figure 8. DPPH-radical scavenging ability (%) of chaga aqueous extracts (10 mg/mL) obtained under extraction conditions for each season. Data are expressed as means ± SEM of at least three independent experiments each performed in triplicate. Values without one common superscript are significantly different determined by one-way ANOVA with Tukey's multiple comparison test ($p < 0.05$). (**A**): spring; (**B**): summer; (**C**): fall; (**D**): winter; US: ultrasound.

Compared to water, ethanol has proven to be a good solvent for the extraction of constituents with a better DPPH-radical scavenging ability (Figures 9 and 10). As shown in Figure 9, regardless of the season, there were no significant differences under the same extraction conditions.

Comparison of the extraction conditions within the same season revealed that there is no significant difference between the extraction methods. Heating or ultrasonic treatment prior to extraction had no effect on DPPH-radical scavenging ability (Figure 10).

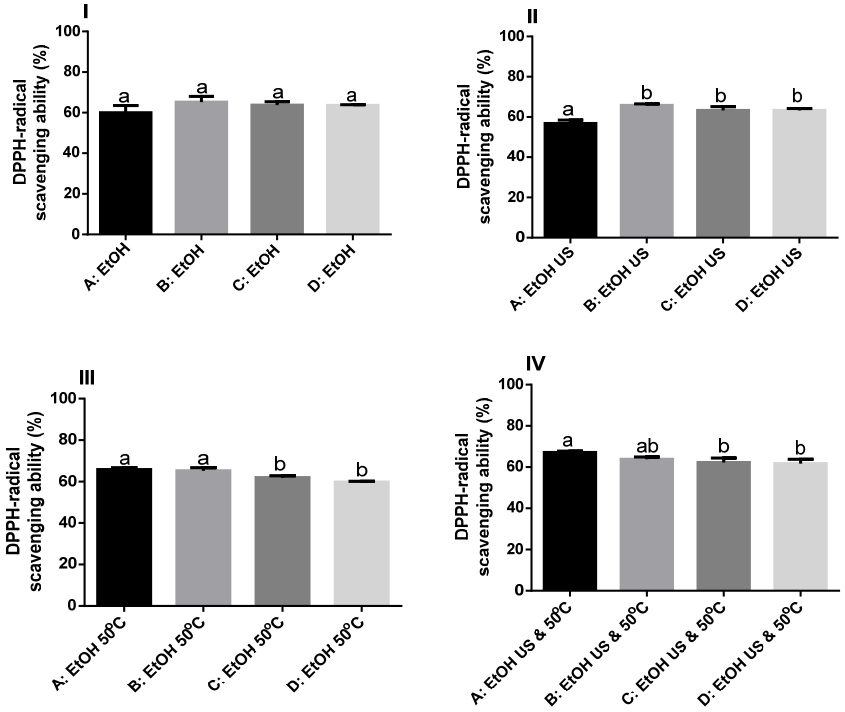

Figure 9. DPPH-radical scavenging ability (%) of chaga ethalonic extracts (10 mg/mL) obtained for each season under the extraction conditions. Data are expressed as means ± SEM of at least three independent experiments each performed in triplicate. Values without one common superscript are significantly different determined by one-way ANOVA with Tukey's multiple comparison test ($p < 0.05$), (A: spring; B: summer; C: fall; D: winter; US: ultrasound; (**I**): Rt; (**II**): US, RT; (**III**): 50 °C; (**IV**): US, 50 °C).

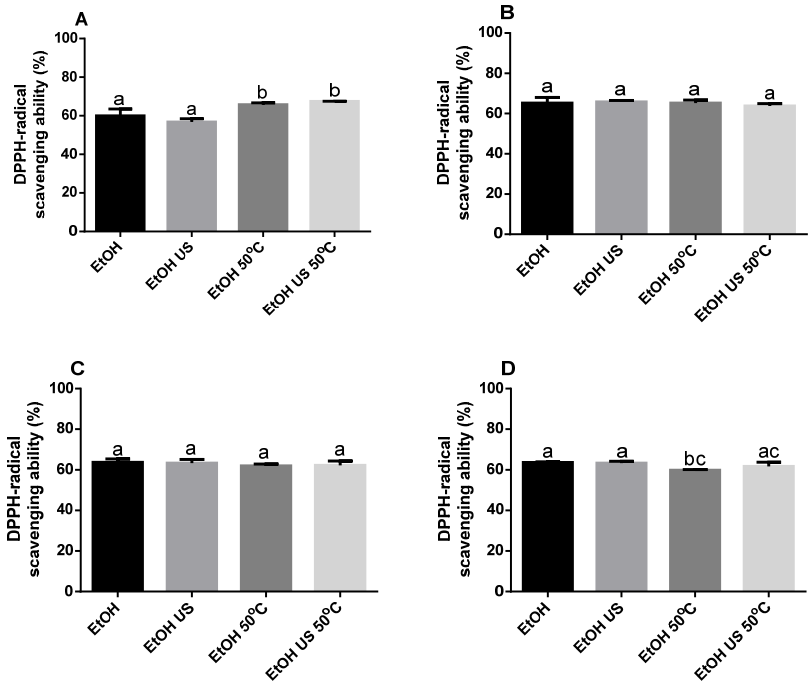

Figure 10. DPPH-radical scavenging ability (%) of chaga extracts (10 mg/mL) obtained with ethanol according to the extraction conditions for each season. Data are expressed as means ± SEM of at least three independent experiments each performed in triplicate. Values without one common superscript are significantly different determined by one-way ANOVA with Tukey's multiple comparison test ($p < 0.05$), (**A**): spring; (**B**): summer; (**C**): fall; (**D**): winter; US: ultrasound.

3.6. Total Flavonoid Content

It was possible to extract flavonoids with water under all investigated extraction conditions. Heating or ultrasonic pretreatment increased each time extraction yields as shown in Table 6. Extracts from the fall and winter seasons were the richest in flavonoids.

Table 6. Total flavonoid content of chaga aqueous extracts.

Season/Extraction Conditions	mg QcEq/g Chaga
A/H_2O	187.44 ± 4.14
A/H_2O US	219.16 ± 3.19
A/H_2O 70 °C	347.61 ± 2.44
A/H_2O US 70 °C	320.45 ± 8.47
B/H_2O	256.57 ± 8.31
B/H_2O US	246.89 ± 8.93
B/H_2O 70 °C	373.02 ± 9.61
B/H_2O US 70 °C	340.55 ± 9.58
C/H_2O	302.26 ± 4.79
C/H_2O US	412.49 ± 9.86
C/H_2O 70 °C	402.47 ± 8.30
C/H_2O US 70 °C	603.68 ± 7.75
D/H_2O	368.07 ± 5.66
D/H_2O US	380.87 ± 1.66
D/H_2O 70 °C	453.27 ± 1.29
D/H_2O US 70 °C	520.70 ± 3.98

A: spring; B: summer; C: fall; D: winter; US: ultrasound.

Ethanol allowed the extraction of flavonoids in a lower quantity than that obtained with water. As for water, heating or pretreatment with ultrasound slightly favors the extraction of flavonoids (Table 7).

Table 7. Total flavonoid content for chaga ethanolic extracts.

Season/Extraction Conditions	mg QcEq/g Chaga
A/EtOH	26.18 ± 1.37
A/EtOH US	14.28 ± 0.93
A/EtOH 50 °C	11.86 ± 0.51
A/EtOH US 50 °C	17.27 ± 2.43
B/EtOH	6.23 ± 1.20
B/EtOH US	7.62 ± 0.64
B/EtOH 50 °C	9.64 ± 1.04
B/EtOH US 50 °C	9.01 ± 0.85
C/EtOH	13.96 ± 1.56
C/EtOH US	15.32 ± 4.42
C/EtOH 50 °C	17.89 ± 1.70
C/EtOH US 50 °C	23.98 ± 5.26
D/EtOH	26.85 ± 1.82
D/EtOH US	22.85 ± 2.50
D/EtOH 50 °C	30.25 ± 0.51
D/EtOH US 50 °C	37.41 ± 0.96

A: spring; B: summer; C: fall; D: winter; US: ultrasound.

As for the extraction with water, the winter extracts were quite rich in flavonoids compared to the other seasons (Table 7).

3.7. Total Phenolic Content

As for the extraction of flavonoids, water extracts polyphenols from chaga (Table 8). Heating seems to have a more important effect than pretreatment with ultrasound. There does not seem to be any difference between the four seasons even if the winter season seems to have a few more polyphenols.

Table 8. Total phenolic content for chaga aqueous extracts.

Season/Extraction Conditions	mg GAEq/g Chaga
A/H_2O	523.50 ± 7.25
A/H_2O US	545.60 ± 2.06
A/H_2O 70 °C	789.22 ± 5.55
A/H_2O US 70 °C	923.63 ± 32.6
B/H_2O	603.03 ± 3.12
B/H_2O US	532.22 ± 4.72
B/H_2O 70 °C	974.10 ± 2.11
B/H_2O US 70 °C	919.03 ± 4.00
C/H_2O	582.27 ± 2.24
C/H_2O US	872.87 ± 4.38
C/H_2O 70 °C	951.61 ± 6.91
C/H_2O US 70 °C	1181.6 ± 7.16
D/H_2O	578.76 ± 2.15
D/H_2O US	822.26 ± 3.44
D/H_2O 70 °C	1050.9 ± 4.57
D/H_2O US 70 °C	1056.3 ± 3.63

A: spring; B: summer; C: fall; D: winter; US: ultrasound.

As for flavonoids, the rate of extraction of polyphenols from chaga with ethanol is lower than with water (Table 9). The polyphenol contents of the extracts from the winter were slightly higher than in the other seasons (Table 9).

Table 9. Total phenolic content for chaga ethanolic extracts.

Season/Extraction Conditions	Mg GAEq/g Chaga
A/EtOH	52.40 ± 0.91
A/EtOH US	32.65 ± 0.18
A/EtOH 50 °C	57.46 ± 7.19
A/EtOH US 50 °C	136.56 ± 0.54
B/EtOH	34.80 ± 2.40
B/EtOH US	57.08 ± 1.38
B/EtOH 50 °C	25.45 ± 0.74
B/EtOH US 50 °C	34.13 ± 0.98
C/EtOH	38.93 ± 1.25
C/EtOH US	38.55 ± 2.23
C/EtOH 50 °C	52.54 ± 0.58
C/EtOH US 50 °C	76.72 ± 1.35
D/EtOH	55.93 ± 1.10
D/EtOH US	50.80 ± 1.30
D/EtOH 70 °C	62.02 ± 1.52
D/EtOH US 70 °C	66.95 ± 2.18

A: spring; B: summer; C: fall; D: winter; US: ultrasound.

3.8. Photoprotective Effects

The in vitro $SPF_{(290–320)}$, $SPF_{(290–400)}$, and $PF\text{-}UVA_{(320–400nm)}$ of water extracts (10 mg/mL) and ethanol extracts (10 mg/mL) of chaga mushroom of the four seasons are presented in Tables 10 and 11. Aqueous and ethanolic extracts obtained under all investigated ex-

traction conditions have an $SPF_{(290–320)}$ upper than 30 (Tables 10 and 11). For the water extracts, heating and ultrasonic pretreatment did not have any effect on the amount of sun photoprotection factor of the extracts obtained in spring and autumn.

Table 10. Sun protection factors for chaga aqueous extracts.

Season/Extraction Conditions	$SPF_{(290–320)}$	$SPF_{(290–400)}$	$PF\text{-}UVA_{(320–400)}$
A/H_2O	34.02 ± 1.03	23.22 ± 5.23	16.70 ± 3.75
A/H_2O US	32.49 ± 0.06	17.03 ± 2.84	14.97 ± 0.19
A/H_2O 70 °C	33.48 ± 0.57	21.13 ± 2.35	15.47 ± 0.99
A/H_2O US 70 °C	32.33 ± 0.55	16.44 ± 1.83	13.95 ± 1.13
B/H_2O	33.47 ± 0.74	20.62 ± 3.09	17.68 ± 2.54
B/H_2O US	32.95 ± 0.56	18.89 ± 2.40	16.26 ± 2.62
B/H_2O 70 °C	34.72 ± 0.51	27.08 ± 2.81	20.63 ± 2.34
B/H_2O US 70 °C	34.97 ± 0.20	28.34 ± 0.42	22.52 ± 0.29
C/H_2O	35.88 ± 0.32	31.97 ± 1.78	21.82 ± 1.19
C/H_2O US	34.59 ± 0.54	27.93 ± 3.36	23.84 ± 3.02
C/H_2O 70 °C	32.97 ± 0.44	18.33 ± 1.40	15.02 ± 1.05
C/H_2O US 70 °C	34.88 ± 0.42	26.00 ± 1.51	18.14 ± 0.66
D/H_2O	34.61 ± 0.95	26.32 ± 5.03	20.14 ± 3.97
D/H_2O US	34.64 ± 0.0.86	25.21 ± 3.50	18.78 ± 1.48
D/H_2O 70 °C	34.46 ± 1.02	24.26 ± 4.35	18.06 ± 2.53
D/H_2O US 70 °C	35.33 ± 0.62	28.67 ± 2.69	19.37 ± 1.31

A: spring; B: summer; C: fall; D: winter; US: ultrasound.

Table 11. Sun protection factors of chaga ethanolic extracts.

Season/Extraction Conditions	$SPF_{(290–320)}$	$SPF_{(290–400)}$	$PF\text{-}UVA_{(320–400)}$
A/EtOH	35.21 ± 0.39	29.95 ± 3.13	22.8 ± 2.71
A/EtOH US	31.31 ± 0.63	13.42 ± 1.73	12.50 ± 1.48
A/EtOH 50 °C	31.27 ± 0.51	13.05 ± 1.47	11.85 ± 1.30
A/EtOH US 50 °C	33.79 ± 0.32	22.73 ± 1.09	19.80 ± 2.99
B/EtOH	32.21 ± 0.23	15.98 ± 0.41	14.40 ± 0.49
B/EtOH US	31.54 ± 0.26	13.37 ± 0.61	11.4 ± 0.58
B/EtOH 50 °C	33.50 ± 0.32	19.83 ± 1.39	15.14 ± 1.10
B/EtOH US 50 °C	31.74 ± 0.28	14.66 ± 0.87	14.66 ± 0.56
C/EtOH	33.08 ± 0.49	18.55 ± 1.35	15.2 ± 0.40
C/EtOH US	31.81 ± 0.14	14.71 ± 0.37	13.4 ± 0.20
C/EtOH 50 °C	32.67 ± 0.15	17.11 ± 0.62	14.0 ± 1.11
C/EtOH US 50 °C	32.64 ± 0.28	17.74 ± 0.93	15.8 ± 0.50
D/EtOH	33.91 ± 0.26	21.97 ± 0.68	17.03 ± 0.35
D/EtOH US	33.47 ± 0.35	21.10 ± 1.06	18.27 ± 0.20
D/EtOH 50 °C	34.66 ± 0.83	25.5 ± 3.48	18.90 ± 1.04
D/EtOH US 50 °C	32.27 ± 2.19	21.31 ± 1.96	19.46 ± 2.17

A: spring; B: summer; C: fall; D: winter; US: ultrasound.

Aqueous extracts obtained from summer and winter at 70 °C with ultrasonic pretreatment had a higher SPF than the extracts obtained in the mentioned seasons. In addition, according to Table 10, the highest amount of $SPF_{(290–320)}$, $SPF_{(290–400)}$, and $PF\text{-}UVA_{(320–400)}$ is related to aqueous extracts obtained in the fall.

Heating and using the ultrasonic pretreatment for ethanol extracts obtained from spring and autumn did not have any effect on their SPF (Table 11). Ethanol extracts obtained at 50 °C from chaga mushrooms harvested in summer and winter had higher SPF than other extracts obtained in the same seasons. As shown in Table 11, we have the best $SPF_{(290–320)}$, $SPF_{(290–400)}$, and $SPF_{(320–400)}$ for ethanol extracts obtained in spring. With the

investigations carried out on the SPF, the protection level of aqueous extracts was better than ethanolic extracts.

4. Discussion

4.1. Extraction

Unlike other kinds of mushrooms, the chaga is attached to its host all year round. Based on no scientific basis, harvesting is only conducted in late spring or early fall to avoid the beginning of the sap flow or before the snow. Therefore, three trees were selected for chaga harvesting during the four seasons of the year. As expected, extractions by heating were the more efficient (Figures 1–4). We chose temperatures lower than the boiling points of the two solvents used to avoid overheating during the extraction which can cause degradation of some constituents. As chaga is consumed in the form of a tea, we opted for a temperature of 70 °C for water which is close to the temperature of a cup of water heated a few minutes in the microwave before adding the chaga. The fact that the quantities extracted with water are greater than those obtained with ethanol reveal the presence of more hydrophilic constituents in the chaga.

4.2. Betulinic Acid and Betulin Content

The quantification of betulinic acid and betulin in the aqueous extracts of chaga revealed that water selectively extracted only betulinic acid and practically no betulin (Table 1). The presence of the carboxyl group may explain the affinity of this compound for water. Betulinic acid has important biological activities such as anticancer [42–45], antimicrobial, [46] and anti-HIV [47] activities. A chaga tea could thus allow the absorption of this bioactive product. As already reported, betulin was extracted with ethanol from chaga together with betulinic acid. The amounts of betulinic acid were lower than those extracted with water. Betulin was identified as one of the anti-hyperuricemia bioactive compounds in chaga mushroom [48].

4.3. ICP/MS Analysis

Potassium is an essential mineral for good health, especially for healthy muscles, bones, kidneys, and nerves. It is important to consume a variety of potassium-rich foods [49]. Western diets have resulted in a decrease in potassium intake through reduced consumption of vegetables and fruits [50]. Vegetable and fruits consumption is associated with a decreased risk of stroke, probably because of their high potassium content [51].

In the search for a variety of foods rich in potassium, chaga can be a good alternative since a simple tea with a teaspoon (1.5 g) of dried mushrooms can provide up to 52.5 mg of potassium, which is not negligible. Compared to the most potassium-rich vegetables and fruits (Table 12), consuming 1 g of chaga mushroom is equivalent to absorbing about 20 to 35 mg of K, which is higher than consuming 1 g of a raw banana which provides 3.58 mg of potassium or 1 g of a cooked potato which provides 5.73 mg of potassium. The consumption of 1 g of chaga brings even more than 1 g of dehydrated bananas, which brings 14,91 mg of potassium.

Table 12. Comparison of potassium-rich foods with chaga extracts.

Source	Potassium (mg/g) [a]
Banana, raw	3.58
Banana dehydrated or banana powder	14.91
Potato, skin, baked	5.73
chaga extracts (H_2O, all conditions, four seasons)	20–35

[a] Canadian Nutrient File (https://food-nutrition.canada.ca/cnf-fce/, accessed on 4 April 2023).

4.4. Elemental Analysis

The organic elemental composition of chaga mushroom (i.e., low N and S) is typical of protein-poor and carbohydrate-rich materials. Mattila et al. found very similar N content

(0.40 to 0.45%) in four cultivated mushrooms [52]. However, these values are higher than in vegetables, indicative of a greater protein content (~2% on a fresh-weight basis, [52,53]). The elemental composition of the chaga mushroom was consistent during the year. Water extracts generally had similar C, N, and S contents than the mushroom itself suggesting very little fractionation of N- and S-containing molecules. No consistent trend was noticed between the different conditions. In contrast, ethanol preferentially extracted organic molecules depleted in N and S, and maybe other heteroatoms such as O, explaining the measured enrichment in C in most cases. Again, no consistent trend was noticed between the different conditions. The only seasonal trend observed was with the ethanol extracts of season A containing organic molecules that were slightly more enriched in N (lower C/N). This change can be caused by the mushroom composition slightly more enriched in N in season A and maybe by a change in mushroom conditions (growth, climatic conditions, etc.).

4.5. Antioxidant Activity

Antioxidants are considered important nutraceuticals with protective effects on several diseases [54]. There are increasing concerns and recommendations for consumers to use natural antioxidants from plant sources instead of synthetic antioxidants, which have been restricted because some have been found to be toxic and carcinogenic. The consumption of fruits and vegetables with natural antioxidants was reported to lower the incidences of cardiovascular diseases, diabetes, and cancer [55,56]. Antioxidant capacity can be tested using a wide variety of methods [57]. In the present study, the antioxidant activity of the chaga extracts was evaluated in terms of their free radical scavenging capacity by DPPH. Phenolics and flavonoids, in general, constitute a major group of compounds, which act as primary antioxidants [58] and are known to react with hydroxyl radicals [59], superoxide anion radicals [60], and lipid peroxy radicals [61]. Aqueous extracts and others obtained with ethyl acetate of chaga have been shown to contain polyphenols and flavonoids [19,62,63]. These extracts also have antioxidant activity [19,62–64]. In agreement with these results, all our extracts, aqueous or not, indeed had a capacity to inhibit free radicals with some differences between seasons for the aqueous ones. For the extracts obtained with water, a correlation between the contents of polyphenols and flavonoids and the DPPH-radical scavenging ability seems to be confirmed for the mushrooms harvested in winter but not for the extracts obtained with ethanol. Antioxidant activity can also come from nonphenolic compounds such as vitamins and carotenoids [65]. The synergistic effect between all components with antioxidant power should not be underestimated.

4.6. Photoprotective Effects

For a sunscreen to be powerful in prohibiting skin spoil, it ought to have a large range of absorption between 290 nm and 320 nm [66,67]. The effectiveness and safety of some artificial sunscreens can be drastically reduced due to allergic reactions [68]. The use of natural products can be advantageous since they come from organisms that have developed effective protection mechanisms against the deleterious side effects of oxidative stress and ultra-violet radiation. This is why many naturals are receiving considerable attention for their use as sunscreen [69]. All the chaga water and ethanol extracts (10 mg/mL) studied have absorption in the UVB and UVA wavelength bands and therefore can directly eliminate UV photons. They can block more than 90 percent of ultraviolet rays (Tables 10 and 11) [70]. According to the FDA [71], sunscreen products can be categorized by their SPF values as follows: “minimum sunscreen products” if the SPF is between 2 and less than 12, “moderate sunscreen products” when the SPF is 12 to less than 30, while “high sunscreen products” if the SPF value is 30 [72]. These results let us to deduce that the extract of the chaga mushroom may be more efficient than other commercial cosmetic products. This effect may be explained by the presence of a variety of phenolic and flavonoid compounds, which have been regarded as a photoprotective agent against UV radiation. Thus, chaga mushroom extract can be used as a sunscreen ingredient in formulations designed to rule out sunburn. Studies have shown that extracts from natural plants improve the SPF of sunscreen

formulation. Fernandes et al. show that plant extracts exhibit photoprotective property when supplemented in sunscreen formulations can protect against UV radiation [73,74]. According to Nunes et al., in the ethanolic extract with a concentration of 0.2 mg/mL obtained from the leaves and bark of *Amburana cearenses* and *Curatella americana*, the SPF values are 12.60–14.74 [75]. In experiments performed by Cefali et al., they reported an SPF equal to 35 for blueberries hydroalcoholic extract [76]. The photoprotection activity of *A. Paniculata* plant extract with SPF equal to 15, shows the possibility of using this extract as a sunscreen agent [77]. *S. Platensis* extract has high absorption in the wavelength range of 290–320 nm. Its SPF value would be 30.37 $\pm$ 0.30. The above results indicate the optical protective effects of *S. Platensis* extract against UVB radiation on human skin. Thus, *S. Platensis* extract can be considered a source of antioxidant capable UVB adsorbents. It can also act as an additive in sunscreen, which reduces the harmful effects of UVB rays [78]. In another study of five types of native Brazilian bamboo extracts, SPF levels were reported to be between 34 and 86 [79]. According to the data obtained in this research, aqua and ethanol extracts of chaga with a dilution of 10 mg/mL have an SPF higher than 30 in the UVB wavelength. For this reason, it can be considered as a promising active ingredient because it offers high sun protection factors in small dilutions. However, to evaluate the efficacy of chaga extract, it is preferable that an in vivo test is performed.

5. Conclusions

In the present study, the active compounds of *Inonotus Obliquus* from three different trees in Eastern Canada were extracted with water and ethanol under four different extraction conditions. The three trees were monitored throughout the year to investigate the effect of seasonal changes on the extracts. The results show that there is no difference between the extracted amounts of the four seasons. The results show a significant difference between the mass of the extracts obtained with water and ethanol. Betulinic acid is more abundant in the extracts obtained with water while betulin is more abundant in the extracts obtained with ethanol. In addition to being rich in carbon and nitrogen, the extracts are particularly rich in potassium. Compared to the same portion of bananas, chaga is five times richer in potassium. Quantification of polyphenols and flavonoids showed that the extraction parameters influence their concentrations with higher contents during heating extraction.

The extracts obtained with water from mushrooms harvested in winter have the highest antiradical capacity. With a sun protection higher than 30 in the UVB wavelength, the chaga extracts can be perfectly used as sunscreen. The comparison of the photoprotective effect of the extracts reveals that those obtained with water have in general a stronger effect.

The present study is the first of its kind to investigate the effect of the harvesting season of chaga in eastern Canada. The choice of harvesting season can now be based on scientific facts. The analysis of this mushroom further highlights this local product that deserves more attention for its potential benefits as a functional food and nutraceutical product. In addition to its nutritional values, *Inonotus Obliquus* can also be used for its cosmetic qualities as a sunscreen.

Supplementary Materials: Supporting information (Figures S1–S4) can be downloaded at: https://www.mdpi.com/article/10.3390/nutraceuticals3030029/s1. Figure S1. HPLC chromatograms of betulinic acid and botulin; Figure S2. HPLC chromatograms of extract obtained with water (A-H_2O); Figure S3. HPLC chromatograms of extract obtained with ethanol (D/ EtOH); Figure S4. Concentration of K in Chaga extracts obtained with ethanol for each season (mg/g). Data are expressed as means $\pm$ SEM of at least three independent experiments. Values without one common superscript are significantly different determined by one-way ANOVA with Tukey's multiple comparison test ($p < 0.05$), (A: spring; B: summer; C: fall; D: winter; US: ultrasound).

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