Article

The influence of pH and temperature on water as a pressurized fluid in the extraction of bioactive myconutrients from chaga mushroom (*Inonotus obliquus*)

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Abstract: Mushrooms have long rich history in folk medicine, traditional and functional foods due to high content of dietary myco-nutrients. Currently, there is increased interest in finding appropriate food-grade green extraction systems capable of extracting these bioactive compounds from dietary mushrooms for applications in various food, pharmacological or nutraceutical formulations. Herein, we evaluated a modified Swiss water process (SWP) method using alkaline and acidic pH at low and high temperature under pressurized conditions as a suitable green food grade solvent to obtained extracts enriched with myco-nutrients (dietary phenolics, total antioxidants (TAA), vitamins, and minerals) from Chaga. Ultra-high performance liquid chromatography coupled to high resolution accurate mass tandem mass spectrometry (UHPLC-HRAMS-MS/MS) was used to assess the phenolic compounds and vitamin levels in the extracts, while inductively coupled plasma mass spectrometry (ICP-MS) was used to determine the mineral contents. Over twenty phenolic compounds were quantitatively evaluated in the extracts and the highest total phenolic content and antioxidant activity was observed at pH11.5 at 100°C. The most abundant phenolic compounds present in Chaga extracts included phenolic acids such as protocatechuic acid 4-glucoside (0.7-1.08μg/mL), syringic acid (0.62-1.18μg/mL), and myricetin (0.68-1.3μg/mL). Vitamins are being reported for the first time in Chaga. pH 2.5 at 100°C treatment shows superior effects in extracting the B vitamins whereas pH 2.5 at 60 and 100°C treatments were outstanding for extraction of total fat-soluble vitamins. Vitamin E content was the highest for the fat-soluble vitamins in the Chaga extract under acidic pH (2.5) and high temp. (100°C) and ranges between 50 to 175 μg/100g Chaga. Antioxidant minerals ranged from 85.94 μg/g (pH7 at 100°C) to 113.86 μg/g DW (pH2.5 at 100°C). High temperature 100°C and a pH of 2.5 or 9.5. The treatment of pH11.5 at 100°C was the most useful for recovering phenolics and antioxidants from Chaga including several phenolic compounds reported for the first time in Chaga. SWP is being proposed herein for the first time as a novel, green food-grade solvent system for the extraction of myco-nutrients from Chaga and have potential applications as a suitable approach to extract nutrients from other matrices. Chaga extracts enriched with bioactive myconutrients and antioxidants may be suitable for further use or applications in the food and nutraceutical industries.

Keywords: Chaga (*Inonotus obliquus*), Accelerated Solvent Extraction (ASE), myconutrients, Swiss water process (SWP), Green extraction, pH, Temperature

1. Introduction

Functional foods refer to food products or ingredients that are useful in providing known health benefits beyond basic nutritional requirements [1]. Mushrooms are well recognized as high quality sources of functional ingredients with respect to having enhanced levels of vitamins, proteins, and fiber [2]. Historically, mushrooms have rich traditions in folk medicine in many cultures across the globe, as well as popularity as food ingredient in many different cuisines. For example, the well characterized fruiting bodies are best known for their medical treatments or therapies in many Asian countries [3]. Recent studies have shown the health-promoting properties of mushroom to be associated with their ability to scavenge reactive oxygen species, decrease inflammation, protect against tumors, provide insulin resistance in type 2 diabetes, and bolster the body's immune system [3,4]. One of the most common dietary mushrooms used as a functional food ingredient and to have several health benefits in traditional and folk medicine is Chaga (Inonotus obliquus L.) [5]. Chaga mushroom is a fungus and a member of the Hymenochaetaceae family [6]   commonly found on birch trees within the colder (boreal) climates of North America, Northern Europe, and Asia [7]. As such Chaga distribution is reflective of the circumboreal forest ecosystem (**Fig. 1**) in the Holarctic regions of Canada, the USA, Asia, and Europe (mainly Russia, Japan, Siberia, and Korea). In these regions, Chaga is highly valued for its use as a therapeutic drink and in enhancing the immune system in traditional or folk medicine [8]. The health benefits ascribed to enhancing the immune system functions are associated with beta-glucans activities. Furthermore, Chaga is reported to suppress the growth of cancer cells via decreased expression of the beta-catenin pathway, regulation of gut bacteria and to its high antioxidant activity [5,9,10]. Previous studies have also reported that Chaga polyphenols and superoxide dismutase enzyme form a potent antioxidant system associated with reduced oxidative stress, diabetes, cancer, and cardiovascular disease risks [11,12].
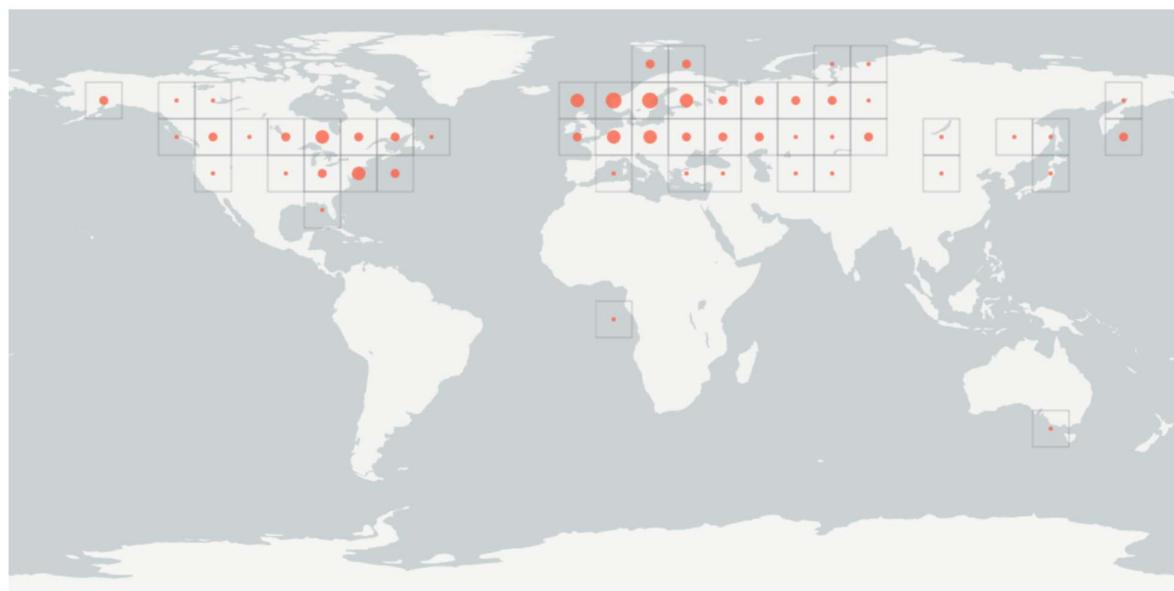

Fig. 1. Global Geographical distribution of Chaga mushroom in the circumboreal regions

These health benefits of Chaga are also due to its high myco-nutrient content [13]. Chaga is reported to be a rich source of Macrominerals such as potassium (K), nitrogen (N), calcium (C) and a reasonable source of magnesium (Mg), chlorine (Cl), phosphorus (P), sodium (Na), rubidium (Rb), sulfur (S) and manganese (Mn) 14, as well as trace or antioxidant minerals such as iron (Fe), copper (Cu), zinc (Zn), selenium (Se), and the micronutrient iodine (I). Regarding vitamins, Chaga contains significant quantities of B vitamins such as vitamin B1 (Thiamine), vitamin B2 (Riboflavin), vitamin B3 (Niacin), vitamin D2 (ergocalciferol), vitamins A and vitamin K 15. Besides the abundance of these vitamins, Chaga also contains significant levels of dietary phenolics which also act as antioxidants [16]. Considering the rich myconutrient composition of Chaga, there is great interest in the food and nutraceutical industries to find suitable food-grade, green extraction techniques that will permit the recovery of high contents of these mycochemicals from Chaga for applications in functional foods, nutraceuticals and pharmacological formulations or innovations.

Many different methods have been used historically to extract myco-chemicals from a variety of natural sources. To this end, organic solvents such as ethanol, methanol, acetone, ethyl acetate, -or their mixture with water, dichloromethane, and hexane, either at ambient temperature or at the boiling point of the chosen solvent(s) are often used. Longer treatment times, higher temperatures, large quantities of solvent required, decomposition of thermo-labile myco-chemicals, environmental health and safety risks are challenges associated with some of the organic solvents typically employed for extracting bioactive compounds from Chaga.

Today, innovative extraction methods are explored continuously to overcome these challenges. The Swiss Water Extraction Method is an example of a green system that is considered food-grade, and represents an environmentally friendly technique combining water, temperature, osmosis, pressure, and extraction time to remove primarily caffeine from coffee beans [17]. This method was designed to reduce energy utilization, facilitate the use of alternative water-based solvents geared at producing high-quality safe extracts and isolates from a range of matrices [18]. Currently there is a paucity of information in the scientific literature concerning how pH modulates solvents' extraction efficiency using the Swiss Water Extraction method under pressurized conditions and even less is known when applied to extracting myco-chemicals from Chaga matrices. Recently, boiling water extraction was demonstrated to be successful in the extraction of bioactive mycochemicals from Chaga sourced from different geographical areas for various medicinal applications in oncology [19]. Boiling water extraction method represents the most common approach to obtain Chaga extracts, and tea in many cultures and laboratory applications currently. We hypothesize that pH will enhance the extraction efficiency of water-based extraction solvents under pressurized conditions and could be useful in extracting high levels of functional ingredients from Chaga mushrooms.

Herein, we proposed to use a modified version of the Swiss Water Extraction method under pressurized conditions at varying pH as a green solvent or potential food-grade approach suitable for recovering high levels of myconutrients from Chaga.

2. Materials and Methods

2.1. Chemicals

Water-soluble vitamins including thiamine hydrochloride (vitamin B1), riboflavin (vitamin B2), nicotinamide (vitamin B3), pantothenic acid (vitamin B5), pyridoxine (vitamin B6), folic acid (vitamin B9), and cobalamin (vitamin B12) were purchased from Sigma Aldrich (St. Louis, USA). Likewise, polyphenol standards including quercetin, trans-ferulic acid, ellagic acid, gallic acid, ǫ-hydroxybenzoic acid, salicylic acid, protocatechuic acid, caffeic acid and vanillic acid were acquired from Sigma Aldrich (St. Louis, USA). ICP-MS 43-element standard mix (IV-ICPMS-71A) was purchased from Inorganic Ventures (Christiansburg, Virginia, USA). Tris(2-carboxyethyl)phosphine hydrochloride (TCEP),

xylenol orange, ferrous ammonium sulfate hexahydrate, ortho-dianisidine dihydrochloride, meta-Phosphoric acid, glacial acetic acid (HPLC grade), methanol (HPLC grade), acetone (HPLC grade), acetonitrile (HPLC grade), 2,4,6-tripyridyl-s-triazine (TPTZ), ferric chloride hexahydrate, anhydrous sodium sulfate, ethanol (95% v/v), hydrogen peroxide (30%, v/v), sulfuric acid (98% v/v), sodium phosphate, hydrochloric acid (36% v/v), ortho-phosphoric acid (85% v/v), methylchroman-2-carboxylic acid (Trolox), sodium hydroxide, glycerol, sodium carbonate, and Folin-Ciocalteu reagent were purchased from Sigma Aldrich (Oakville, Ontario, Canada). Nitric Acid (TraceMetal™ Grade) and Dionex ASE™ Prep DE diatomaceous earth were obtained from Thermo-Fisher Scientific (Ottawa, Canada).

2.2. Preparation of Chaga extracts

The Chaga mushroom was powdered using a mechanical mill (Hamilton Beach, purchased from Amazon.ca). Then 5g samples were filled into 10 mL stainless-steel accelerated solvent extractor (ASE) cell sample holders and extracted using a Dionex 350 ASE (Thermo Scientific, Massachusetts, USA). Ionized water at different pH values (pH=2.5, 7.0, 9.5 and 11.5) was used as extraction solvents (SD501, Enagic, Toronto, Canada). The ASE extractions were conducted based on the following program: (number of cycles = 2; pressure = 1500 psi; static time = 5 min; flush ratio = 50% rinse, and temperature = 60^{0}C and 100^{0}C). The extracts recovered were lyophilized using a freeze dryer (FreeZone freeze dry system, Labconco, MO, U.S.A.) and the dried extracts stored in amber-colored vials at -20 ^{0}C until further chemical analysis.

2.3. Total antioxidant activity (TAA) analysis

The TAA of the mushroom extracts was measured colorimetrically using the ferric reducing antioxidant power (FRAP) method 20, with minor modifications. Briefly, 20 μL of each standard or sample was reacted with 180 μL of freshly made FRAP solution, and the resultant mixture incubated in the dark for 120 mins. Absorbance measurements were recorded at 593 nm on a Cytation imaging microplate reader (BioTek, Vermont, USA). The TAA extracts' values were determined by summing the lipophilic (LAA) and hydrophilic (HAA) antioxidant activity values based on a Trolox standard curve using the following concentrations (0.5, 1, 1.5, 2, 2.5, 3, 3.5 mM). The results for the Chaga extracts were expressed in μmol Trolox equivalent (TE)/g extract (Dry weight: DW).

2.4. Total phenolic content (TPC) analysis

The TPC of mushroom extracts was measured using the Folin-Ciocalteu reagent as described previously [21], with minor modifications. Thus, 25 μL of each standard or mushroom extract was mixed with 125 μL of a 10-fold diluted Folin-Ciocalteu reagent in microplate wells. Acidified ethanol (50 μL, 0.7% v/v) or sodium phosphate buffer (50 μL, 50 mM, pH 7.5) was added to analyze the lipophilic or hydrophilic phenolic content in the extracts, respectively. The resultant mixtures were incubated in darkness for 120 mins, and the absorbances measured at 755 nm on a Cytation imaging microplate reader (BioTek, Vermont, USA). The TPC of the mushroom extracts were calculated by summation of the hydrophilic phenolic content (HPC) and lipophilic phenolic content (LPC) and the concentration measured based on quercetin standard curve. The results were expressed as μmol quercetin equivalent/g extract (DW).

2.5. B-vitamin (B1, B2, B3, B5, B6, B9, and B12) analysis by Ultra-High-pressure Liquid Chromatography-Mass Spectrometry (UHPLC-MS)

The determination of B vitamins (water-soluble) in freeze-dried mushroom extracts was based on the method of Akhavan et al. [22]. Briefly, a stock solution of vitamin B standard mix (B2, B3, B5, B6, and B9) at a concentration of (1 mg/mL) was diluted serially with distilled water to obtain B vitamin calibration standards in the range of 5-100ug/mL Aliquots of the extracts (1mg) were diluted with distilled water (10 mL) and spiked with

hippuric acid as an internal standard. The resultant mixture was homogenized, then incubated in darkness for 45 minutes at 4°C, followed by centrifugation at 15000 rpm for 15 mins at 0°C. The supernatant was collected, pooled, and filtered into amber-colored vials for analysis by UHPLC-HRAMS-MS/MS. For the analysis of Vitamin B, a Luna C18 column (100 × 2.0 mm I.D., particle size: 3 μm, pore diameter: 100 Å) purchased from Phenomenex (CA, USA) was employed. The solvent system used was as follows: solvent A consisted of 10 mM of ammonium formate and 0.1 % Formic Acid, while solvent B consisted of 100% acetonitrile. Chromatographic separation was performed at 35°C (column oven temperature) with a flow rate of 0.3 mL/min, and 10 μL of the extract was injected into the system. The following solvent gradient was used for separating the vitamins: ramp at 20 mins with 55% solvent B; next transition to 22 min. 0% Solvent B and 100 % of Solvent A. The Orbitrap ESI source was run in positive ion mode for data acquisition. The following optimized parameters were used for the Orbitrap mass spectrometer: sheath gas flow rate of 51; an auxiliary gas flow rate: 0; ion spray voltage: 3.20 kV; capillary temperature: 300°C; S-lens RF: 100 V; capillary voltage: 35 V; mass range: 50-500 m/z; full scan mode at a resolution of 60,000 m/z; top-3 data-dependent MS/MS at a resolution of 30,000 m/z; and collision energy of 35 (arbitrary unit); injection time 22 min; isolation window: 2.0 m/z; automatic gain control target: 0.250; with dynamic exclusion setting of 30.0s. The mass spectrometer was externally calibrated to 1 ppm using ESI negative and positive calibration solutions (Thermo Scientific, MO, USA) before usage.

2.6. Fat-Soluble Vitamins (A, E, D3, K1, K2, E) analysis by UHPLC-HRAM-MS/MS

The determination of fat-soluble vitamins present in the freeze-dried mushroom extracts was based on the method of Ramadan and coworkers with minor modifications [23]. Briefly, mushroom extracts were homogenized in 1 mL of methanol: chloroform (1:1) containing hippuric acid as an internal standard. The resultant mixture was incubated for 30 mins in darkness at 4°C followed by sonication for an additional 30 mins at 4°C in a sonication water bath. Thereafter, the samples were homogenized, centrifuged (15,000 rpm for 15 mins at 0°C), then the supernatant was collected into amber-colored vials for LC-MS analysis. A polar acclaim C18 column (150 × 4.6 mm I.D., particle size: 5 μm, pore diameter: 120 Å; Thermo Fisher Scientific, ON, Canada) was used for the resolution of the fat-soluble vitamins. The solvent system used consisted of 5 mM ammonium acetate. The separation was carried out at 40°C (column compartment temperature) with a flow rate of 0.3 mL/min, and 10 μL of the extract was injected into the machine and elution done isocratically for 32 mins. The Orbitrap MS was operated in the positive ESI ion mode during data acquisition. The following optimized parameters were used for the Orbitrap MS : sheath gas flow rate: 25; ion spray voltage: 500 kV; an auxiliary gas flow rate: 2; S-lens RF: 80.00 V; mass range: 60-900 m/z; capillary temperature: 320°C; capillary voltage: 10.0 V; full scan mode at a resolution of 60,000 m/z; top-3 data-dependent MS/MS at a resolution of 30,000 m/z; and collision energy of 35 (arbitrary unit); injection time of 15 min; isolation window: 2.0 m/z; automatic gain control target: 0.250; with dynamic exclusion setting of 30.0s. The mass spectrometer system was externally calibrated to 1 ppm using ESI positive and negative calibration solutions (Thermo Scientific, MO, USA) before usage.

2.7. Phenolic compounds analysis by UHPLC-HRAM-MS/MS

Aliquots (1 mL) of 10 % Formic acid: water (v/v) was added to 1 mg freeze-dried extracts in centrifuge tubes. The resulting mixture was homogenized, incubated in darkness for 15 mins and then centrifuged at 15,000 rpm for 15 minutes at 0°C. The supernatant was pooled into amber-colored vials for UHPLC-HRAM-MS/MS analysis. A standard mix of select phenolics were used to create a standard curve covering the following concentration range: 5-100 ug/mL (ppm). The UHPLC-HRAMS/MS analysis was conducted on an LTQ Orbitrap XL mass spectrometer (Thermo Scientific, MO, USA) with an automated Dionex UltiMate 3000 UHPLC system controlled by the Chromeleon software. Resolution

of the phenolics present in the sample was performed on a Luna C18 column (100 × 2.0 mm I.D., particle size: 3 μm, pore diameter: 100 Å) purchased from Phenomenex (CA, USA). The solvent system used was as follows: Solvent A consisted of H_2O (containing 0.1 %, v/v formic acid), and Solvent B consisted of acetonitrile (consisting of 0.1%, formic acid). Chromatographic separation was done at 25°C (column temperature) with a flow rate of 0.3 mL/min, and 5 μL of the extract was injected into the machine. The following solvent gradient was used for separating the polyphenols: 0-2 min with 0-10% solvent B; 2-15 min applying 10-45% solvent B; 15-30 min using 45-90% solvent B; 30-35 min using 90-10% solvent B; The Orbitrap MS was operated in the negative ESI ion mode. The optimized parameters used for the Orbitrap mass spectrometer were: sheath gas flow rate : 25; ion spray voltage: 5.0 kV; an auxiliary gas flow rate: 2; capillary temperature: 320°C; capillary voltage: 10 V; S-lens RF:80 V; mass range:50-1500 m/z; capillary voltage: 10 V; full scan mode of 60,000 m/z resolution; top-6 data-dependent MS/MS acquisition at a resolution of 30,000 m/z and collision energy of 35 (arbitrary unit); isolation window: 2.0 m/z, automatic gain control target: 2 e5 with dynamic exclusion setting of 30.0s and injection time of 15 min. The MS was externally calibrated to 1 ppm using ESI positive and negative calibration solutions (Thermo Scientific, MO, USA) before usage.

2.8. Extraction and analysis of minerals by inductively coupled plasma-mass spectrometry (ICP-MS)

Aliquots (1 mg) of freeze-dried Chaga Mushroom extracts were digested in 10 mL of trace nitric acid (70% v/v) using a Multiwave Go-microwave digestion system (Anton Paar GmbH, Graz, Austria). The microwave digester was operated under the following conditions: temperature ramped to 180°C for 10 minutes, then held at this temperature (180°C) constantly for an additional 20 minutes. A calibration curve (ranging from 1 to 500 μ L-1) was prepared using IV-ICPMS-71A standard mixture (InorganicTM Ventures, Inc; Christiansburg, VA 24073, USA). Aliquots of the digestates were diluted with 2% nitric acid and spiked with rhodium-103 (final concentration = 10 μL-1) as the internal standard. The ICP-MS analysis was conducted using the following parameters: auxiliary gas flow of 0.79 L/min, nebulizer gas flow of 1.01 L/min, plasma gas flow of 14 L/min, RF power of 1548 W, detector mode KED, dwell time of 0.01s using argon gas at 99.99% purity.

2.9. Statistical Analysis

One-way ANOVA was used to assess the effects of extraction pH and temperature on the antioxidant activity, phenolic compounds, vitamins, and mineral content in the mushroom extracts. When the means were significantly different between pH and temperature combinations, the means were compared with Fisher's Least Significant Difference (LSD), $\alpha = 0.05$. Pearson's correlation coefficients were used to determine the linear association between subclasses of myco-chemicals (phenolics, vitamins, and minerals) and their contributions to the antioxidant activity of the extracts. Outliers were detected using the Grubbs test. Principal component analysis was used to discern similarities between the extraction conditions (pH and temperatures) and the myco-nutrients recovered. Statistical analyses were performed with XLSTAT (Addinsoft, Paris, France). Figures were prepared using XLSTAT and SigmaPlot 13.0 software programs (Systat Software Inc., San Jose, CA).

3. Results and discussion

The current interest in medicinal mushrooms for their unique and promising health promotive benefits such as anti-inflammatory, anticancer, anti-diabetic, and antioxidant properties is remarkable [24]. Furthermore, consumers have high interests in natural sources of dietary antioxidants for use in their daily food intake. Herein, we demonstrated a modified Swiss water method consisting of elevated temperature and pH under pressurized condition using accelerated solvent extraction as a suitable food grade solvent system to extract myco-chemicals from Chaga mushroom. Chaga mushroom is

considered an enriched source of natural antioxidants. As such, we evaluated the lipo-philic, hydrophilic, and total phenolics as well as the antioxidant content in the extracts obtained.

3.1. Total Polyphenols Content (TPC) and Antioxidant activity (AA) of myconutrients from Chaga Extract

Principal component analysis demonstrated the TPC and TAA clustered together with the extracts obtained at pH 11.5 at 100°C. The TPC and TAA were also grouped with the HPC, and HAA under these extraction conditions, while the LPC clustered in quadrant 4 of the PCA biplot with extracts obtained at pH 2.5/60°C, pH 2.5/100°C and pH 7/100°C. This grouping captured 96.22% of the total variability in the data set, with PC1 explaining 81.45%, and PC2 accounting for 14.77% of variability in data (**Fig 2A**). TPC and HPC were grouped in the first quadrant (Q-1) with pH11.5-100°C treatment, implying that they are best extracted using high pH and temperature from chaga matrices under pressurized conditions. Furthermore, this pH and temperature combination appears to extract the hydrophilic portion comprising the highly polar phenolic compounds, and this may be noted from the ANOVA analysis, which demonstrates HPC is the major contributor to the TPC. Interestingly, the composition of the solvent system is water based and was not anticipated to extract any significant component of lipophilic polyphenols or antioxidants. However, we observed that although most of the polyphenols and antioxidants extracted were hydrophilic in nature, the lower solvent pH (2.5 and 7) was effective in extracting the lipophilic phenolics from the chaga matrix, while higher pH (11.5) at 100°C was effective in extracting LAA from Chaga under pressurized conditions using water as an accelerated solvent (**Fig 2 A& B**). We observed the TAA varied significantly with treatment and ranged from 98.40 to 381.30 μmol TE/g DW, with the highest TAA present in the extract was acquired at pH 11.5 and a temperature of 100°C. Similarly, the TPC ranged between 102.27±1.39 to 273.06±2.19 μM QE/g Chaga DW, with the highest TPC also observed in the extracts acquired at pH 11.5 and a temperature of 100°C (**Fig 2; Table S1 & S2**). These results demonstrate extraction with pH 11.5 at 100°C has unique conditions and extraction potential towards the acquisition of hydrophilic and lipophilic antioxidants in Chaga mushroom extract using water as an accelerated solvent under pressurized conditions. Hwang and coworkers (2019)25 reported that phenolic compounds extracted increased in mushrooms treated at 100°C. Seo and Lee (2010) [26] also noted that phenolic components in Chaga extracts produced by supercritical water increased with temperature, implying that temperature appears to be vital in phenolics extraction from Chaga Mushroom. It is worth mentioning that the use of pH, temperature, and pressure can change the physiochemical nature of water to extract organic components that are generally not soluble in aqueous solvents, which is clearly shown from the extraction of the lipophilic phenolic components in our study. The values we observed for TPC and TAA in Chaga extracts are consistent with values observed in the literature [25]. However, huge variation is noted in the literature for the phenolics content, and antioxidant activities measured in Chaga. The variation in antioxidant activities and phenolics content reported in the literature are reported to be influenced by source geography, temperature, carrier birch type, time of harvest, and part used for extraction [27].

The correlational analysis between TAA and TPC showed that a strong antioxidant activity came from the HAA ($r=0.76$, $p< 0.0001$) (**Fig. 2A and Fig. 6**). Interestingly, relatively considerable levels of both LAA and LPC were observed in the extract, considering that an aqueous-based solvent system was used (**Fig. 2B**). The elevated temperature and pH used for the extraction under pressurized conditions appear to contribute to the extraction of lipophilic antioxidants and phenolics in this extraction system. Finally, the correlation coefficient ($r=0.78$) between TAA and TPC, suggested that approximately 78% of the extracts' total antioxidants may be attributed to the TPC present in the extract, which was driven by the high association of the HPC with the TAA ($r=0.81$). These findings

suggest that the hydrophilic phenolics are a major contributor to the TAA of the extracts obtained in this extraction system.

A

Biplot: phenolic and Antioxidant activity (axes F1 and F2: 96.22 %)

B

Total antioxidants and phenolics vs. treatments
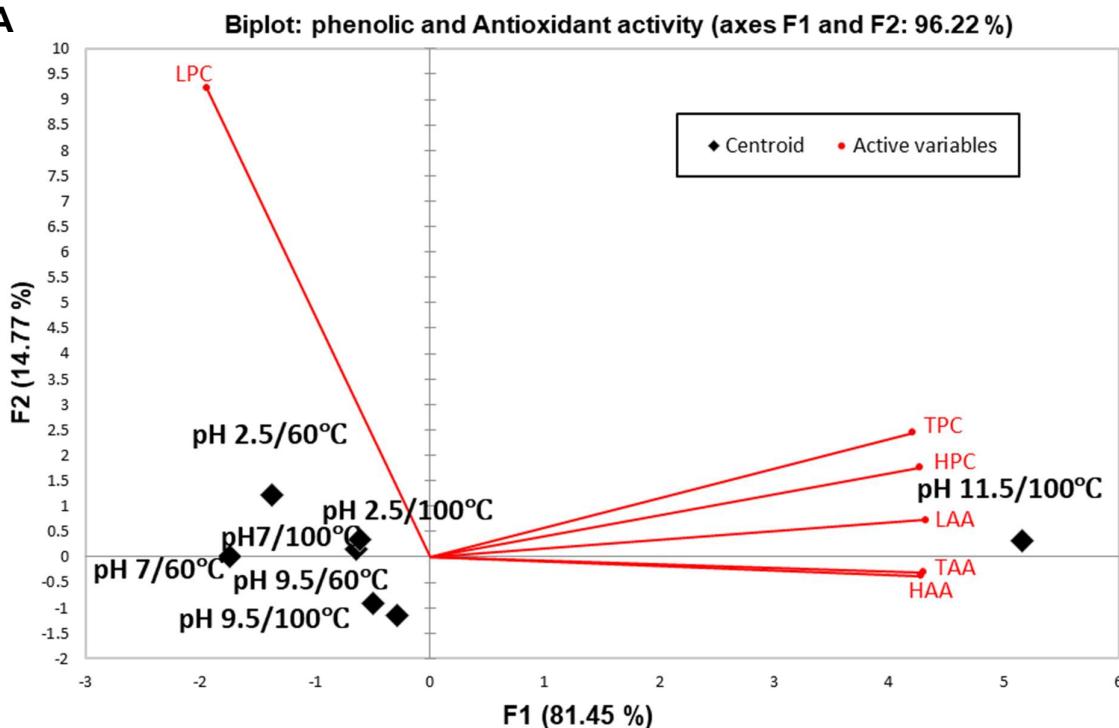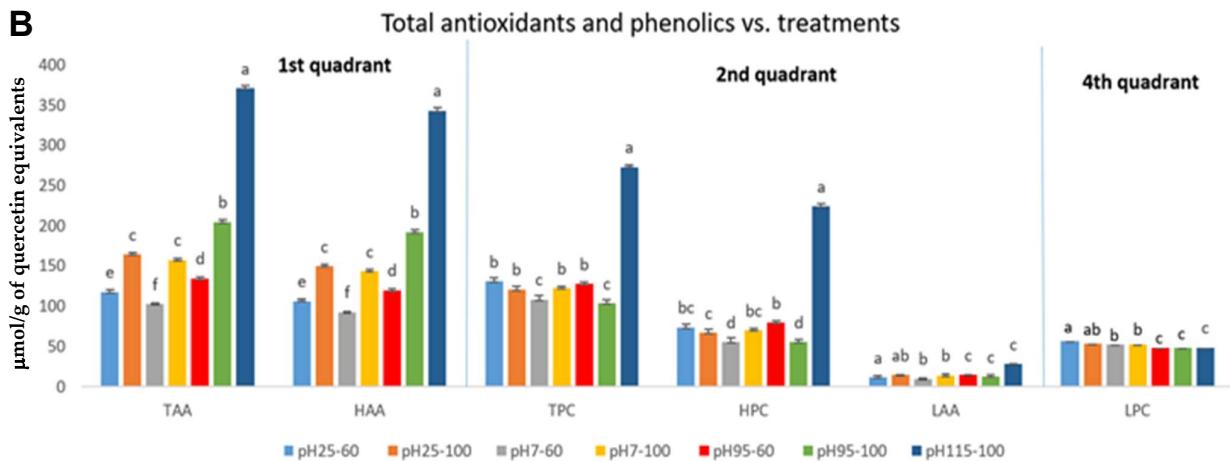
3.2. Assessment of the extraction conditions on the myco-chemicals recovery in chaga extracts using the modified Swiss method under pressurized conditions.

Mushrooms have a long history in folk medicine and ethnopharmacological uses for their health promotive benefits. The rich myco-nutritional composition is attributed to these health promotive benefits [28]. Evaluation of the extracts obtained in this study demonstrated Chaga is a good source of vitamins, dietary polyphenols, antioxidant, trace, and macro minerals.

3.2.1. Minerals' composition

The minerals recovered in the chaga extracts were grouped as antioxidant minerals, trace, macro, and heavy metals (**Fig. 3 & Table 1**) according to their dietary or nutritional relevance.   The results suggest significant quantities of antioxidant minerals (Mn, Cu, Zn and Se), macronutrients (Na, Mg, P, S, K, and Ca), trace minerals (B, Cr, and Co) and to a lesser extent toxic heavy metals (As) were present in the extracts (**Table 1 & Fig. 3**). However, the heavy metals extracted are significantly below the tolerable limits recommended for safe human consumption 29(Institute of Medicine (US) Panel on Micronutrients, 2001). Principal component analysis was used to discern the extraction conditions' effectiveness in recovering the different classes of minerals from Chaga matrices using the modified Swiss method. In the first quadrant of the PCA biplot, the total microminerals and trace minerals were grouped with pH 2.5 and pH 9.5 at the higher temperature treatments (100°C). These treatments seem to promote the extraction of macronutrients and trace minerals. The total antioxidant minerals were grouped in the fourth quadrant of the PCA biplot with a pH of 11.5 and high temperature (100°C). This may indicate that antioxidant minerals can be better extracted by high temperature and pH from Chaga using the modified Swiss method. Furthermore, pH 2.5 at 60°C was better for extracting heavy metals like Arsenic. This segregation accounted for 97.10% of the total variability in the data (**Fig. 3a**). Demšar plots were used to display the ranked performances of the extraction conditions for superior recovery of the minerals in the extracts (**Fig. 3b**). Zn was the predominant antioxidant mineral present in the extract with values ranging from 67.37±1.29 to 105.87±3.05 µg/g DW of chaga, with the highest concentration observed in the extracts collected at pH 2.5 and 9.5 at a temperature of 100°C (Table 1). Mn, Fe, Cu, and Se were the other antioxidant minerals present in the Chaga extracts. A pH of 2.5 at low temperature (60°C) was effective for the extraction of Mn from Chaga (9.85±3.05 µg/g DW). This was several folds higher than the values observed at the other pH and temperature combinations (0.0 to 0.539.85±3.05 µg/g DW). Conversely, a pH of 9.5 at temperatures of 60°C and 100°C, as well as a pH of 2.5 at a temperature of 60°C was the most effective in the recovery of Se from chaga matrices. This indicate pH is a very important determinant in modulating the level of selenium extracted from Chaga using the modified Swiss method. We next assessed the contributions of the antioxidant minerals to the total antioxidant activity measured in the extract using Pearson's correlation. The total antioxidant minerals were moderately correlated (r =0.52) with the TAA in the extracts recovered (**Fig 6**). The results of the mineral contents expressed in µg/g DW of Chaga mushroom is presented in table 1 along with the recommended daily allowances (RDA's) [29]. The antioxidant minerals form a very important part of the total minerals found in Chaga. These minerals are often required in low quantities in human nutrition to meet the RDAs [30] and typically supplemented in many food formulations. The recommended dietary allowance (RDA) for Cu, Mn, and Zn has been established. An infusion of Chaga mushroom made from 10 g dry weight (DW) (teaspoon full) could meet the demand for Cu, Mn, Zn and Se which is set at 5%, 10%, 10%, and 4%, respectively [29].

a.   **Biplot: Mineral Analysis of Chaga**
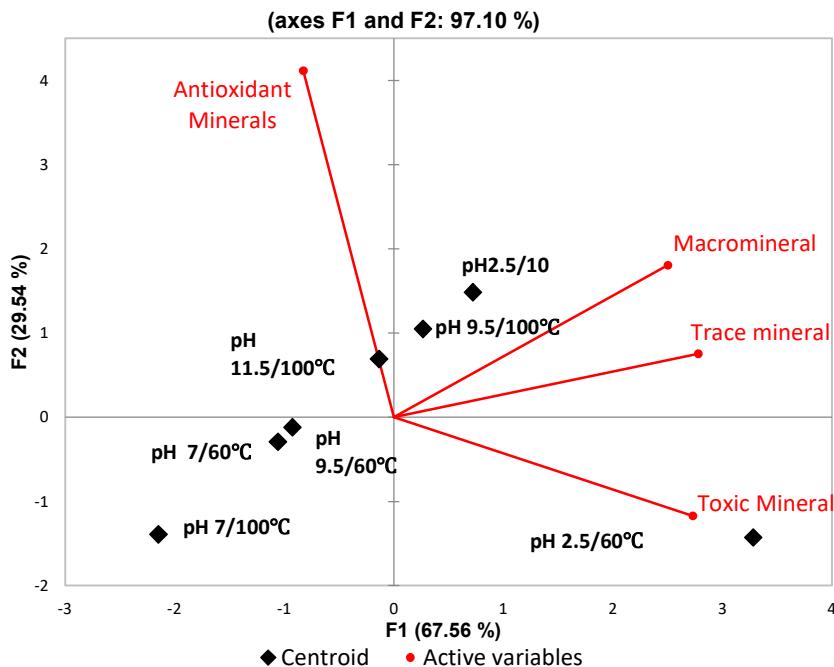
b.   **Demsar plots: Mineral extraction under different temperature and pH conditions**
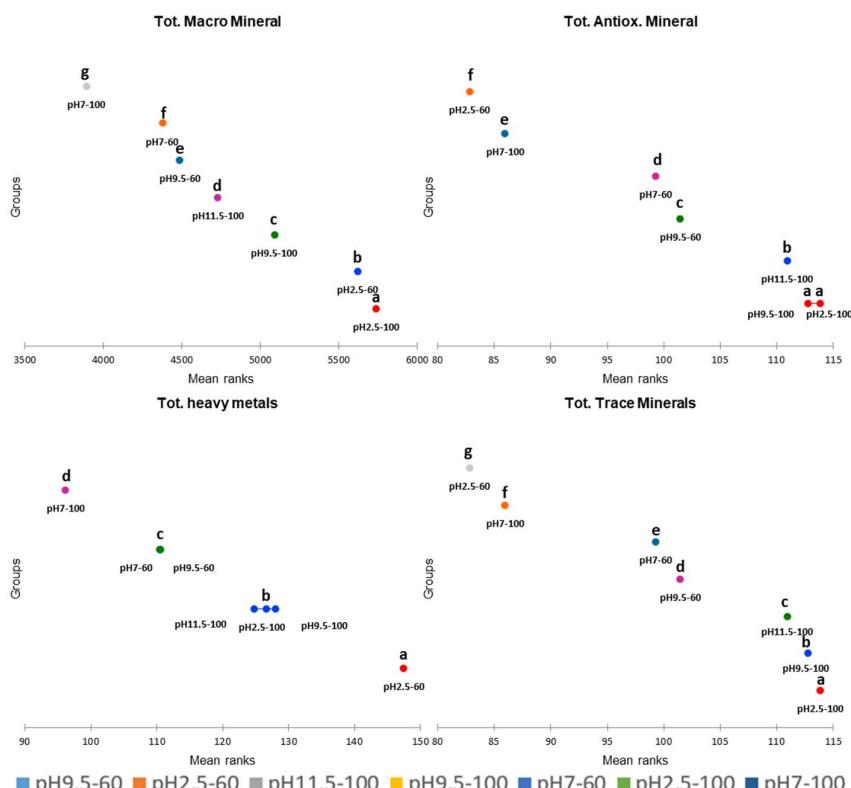
Fig. 3 a. Principal Component Analysis (PCA) illustrating the relationships between mineral contents in Chaga samples as affected by solvent pH (2.5, 7, 9.5, 11.5) and extraction temperatures (60°C and 100°C). **b.** Demsar plots showing the ranks of the mineral

extracted as a function of the extraction temperature and pH treatments. Means that have the same subscripts indicate no significant difference between extraction conditions. LSD=0.05, n=4 per experimental replicate. The antioxidant activities are reported as μmol/g of Trolox equivalents. The minerals content is represented as μg/g of Chaga DW.

Arsenic (As) was the only heavy metal observed in the extract and the values ranged between 0.81 ± 0.04 to 1.63 ± 0.05 with the highest-level present at pH 2.5 and a temperature of 60°C. It is important to note the level of As detected in the extract is low. As is lethal in humans in the range of 2-10 mg/kg/day for an average size human [29]. The values reported in these extracts are below the safe levels reported for human consumption. The U.S. Food and Drug Administration (FDA) has established tolerance levels for As to be permissible at levels ranging from 0.5 to 2 ppm in different foods [31].

Both the macro and trace minerals clustered with pH 2.5 and a temperature of 100°C in the PCA biplot (**Fig. 3a**) suggesting this condition was optimal for the extraction of these minerals from Chaga using the Modified Swiss method. Ca, Na and Mg were the predominant macro minerals present in the extracts and their levels were significantly impacted by an extraction pH of 2.5 and 9.5 at 100°C, where the highest levels for these minerals were observed. The other Macrominerals Ca and P recovery were highest at a pH of 2.5 and temperature 60°C. We observed Ca, Na and Mg were the predominant Macrominerals present in the extracts with significantly higher levels recovered (Table 1). Similarly, boron the predominant trace minerals, was more successfully recovered when extracted at pH 2.5 and a temperature of 60°C. These results clearly demonstrate that a pH of 2.5 or 9.5 at 100°C are useful for extracting macro and trace minerals from Chaga using the modified Swiss method.

Table 1. Mineral Content in Chaga (*Inonotus Obliquus*) extracts as a function of solvent pH and temperature under pressurized conditions

Classification of Minerals	pH 2.5 at 60°C	pH 7 at 60°C	pH 9.5 at 60°C	pH 2.5 at 100°C	pH 7 at 100°C	pH 9.5 at 100°C	pH 11.5 at 100°C	RDA's*
				pH Treatment and Temperature				
Antioxidant minerals								
55 Mn	9.85± 0.04 [a]	0.53± 0.00 [b]	0.38± 0.01 [c]	0.34± 0.01 [d]	0.28± 0.14 [e]	0.11± 0.08 [f]	0.00	2 (mg)
63 Cu	2.10± 0.15 [f]	3.84± 0.25 [d]	4.24± 0.08 [c]	4.27± 0.19 [c]	3.45± 0.31 [e]	4.38± 0.78 [b]	4.82± 0.04 [a]	1 (mg)
64 Zn	67.37± 1.29 [g]	91.86± 4.90 [e]	93.20± 2.53 [d]	105.87± 3.05 [a]	79.75± 0.42 [f]	104.75± 0.37 [b]	103.05± 2.84 [c]	10 (mg)
80 Se	3.46± 0.07 [ab]	3.05± 1.76 [c]	3.63± 0.42 [a]	3.37± 0.93 [b]	2.42± 0.18 [d]	3.51± 0.46 [ab]	3.04± 0.11 [c]	55 (μg)

Macrominerals

23 Na	1439.35± 5.91 [c]	1406.28± 5.16 [d]	1414.55± 11.03 [cd]	**1612.63± 1.21 [a]**	1228.21± 0.68 [e]	**1583.92± 2.11 [ab]**	**1580.22± 23.89 [b]**	800 (mg)
24 Mg	787.45± 2.15 [d]	1028.89± 1.58 [b]	1036.58± 1.35[b]	**1155.43± 10.34 [a]**	891.44± 1.24 [c]	**1159.06± 7.20 [a]**	**1161.53± 6.87 [a]**	375 (mg)
31 P	896.61± 0.60 [a]	339.63± 0.00 [c]	335.85± 15.82 [c]	370.48± 2.40[b]	290.58± 0.46 [d]	378.80± 9.31 [b]	335.65± 3.00 [c]	700 (mg)
39 K	**2004.07±19.96 [a]**	940.47± 4.41 [e]	1026.71± 9.75 [d]	**1853.77± 27.08 [b]**	902.02± 7.62 [e]	1211.62± 2.28 [c]	899.74± 4.81 [e]	2000 (mg)
44 Ca	480.10± 7.83 [d]	660.32± 3.67 [b]	671.57± 3.90 [b]	**745.19± 6.58 [a]**	577.58± 5.58 [c]	**756.34± 11.04 [a]**	**750.47± 3.73 [a]**	800 (mg)

Trace Minerals

11 B	64.25± 17.44 [a]	11.25± 2.57 [c]	8.97± 3.86 [d]	12.81± 5.68 [bc]	10.21± 0.36 [d]	15.26± 11.78 [b]	13.84± 0.00 [b]	
52 Cr	3.94± 0.49	0.00	0.00	0.00	0.00	0.00	0.00	40 (µg)

Toxic Minerals

75 As	1.63± 0.04 [a]	0.94± 0.01 [d]	0.97± 0.02 [c]	1.03± 0.01 [b]	0.81± 0.03 [e]	1.04± 0.09 [b]	1.04± 0.01 [b]	

*RDA's recommended daily allowances. Values were obtained from "Commission Regulation (EC) No 629/2008".

Note: Values (µg/g DW of Chaga) represents means ± standard errors. Means that are within the same column and contain different letters indicate a significant difference between the treatments. LSD=0.05, n=3 per each replicate of the sample. 0.00=not detected. Macro-mineral Chlorine, Trace Minerals Fluoride, Iodine and Molybdenum and toxic mineral. The Chaga extracts are at various pH values (2.5, 7, 9.5 and 11.5) and at temperatures 60°C and 100°C. Fe, Co, Cd and Pb were not detected at any treatment. Highest values of Mn, P, K, B, Cr and As were detected in pH 2.5 at 60°C.

3.2.2. Vitamins (water- and fat-soluble):

Water is an interesting and practical solvent in extraction, and its physiochemical properties can change with varying temperatures. These conditions allow water at near-

critical conditions to dissolve hydrophobic compounds 32 . Furthermore, an increased movement/rotation of water molecules can also be observed at higher temperature which allows for dissolving less polar compounds, analogous to that of methanol and ethanol (slightly polar solvents) which in turn may enable the extraction of nonpolar components [32,33]. In this context, we observed that when water was used as a solvent (pressurized fluid) in the modified Swiss method evaluated in this study both fat- and water-soluble vitamins were successfully extracted from chaga matrices. Vitamin E and K were the pre-dominant vitamins observed in the extracts with values with the average of 112460 and 2423 ng/g DW Chaga, respectively. The high alkaline and high-temperature treatment (pH11.5/100°C) was successful in extracting both classes of vitamins including vitamins D, and K (fat-soluble vitamins), as well as B-vitamins (water-soluble vitamins). On the other hand, total vitamin E (fat-soluble) including Beta-tocopherol analogue was found to be best extracted at the treatment with lower pH (2.5) and higher temperature (100°C). These findings suggest that high temperature strongly impacts the extraction of the fat and water-soluble vitamins (**Fig 3a. and b.**).

The PCA of fat-soluble vitamins (FSV) and water-soluble Vitamins (B-Vitamins) is shown in **Fig. 4a**. We observed that 57.43 % of the total variability in the data was repre-sented by PC1 and PC2. Delta-Tocopherol and Vitamin D2 are grouped in quadrant-1 (Q1), whereas Vitamin K2, total Vitamin D, total B-Vitamins, and total Vit. K are grouped in Q2 with higher temperature and alkaline pH (9.5 and 11.5) at 100°C. Alpha-Tocopherol and Vitamin K1 are grouped in Q-3 with pH7 and temperature of 100°C. On the other hand, quadrant-4, contained Vitamin A and Beta-Tocopherol grouped with pH 2.5/60°C, pH 2.5/100°C and pH 9.5/60°C extraction conditions.

Despite the large volume of research on Chaga in the scientific literature, to the best of our knowledge there is no work describing the vitamin content in the literature. Vita-min D2 was mentioned and evaluated in different mushroom genotypes and its quantity ranged from 0.3 to 5.3 µg/100g. However, our results are in the range of 0.02 to 0.04 µg/100g DW Chaga. Despite the low content of vitamin D in mushrooms, high levels of sterols could be found in mushrooms like ergosterol, as a potential source and precursor of vitamin D2 [34].

The transformation of ergosterol to vitamin D2 can be achieved by exposure to ultra-violet (UV) irradiation35.   Sunlight exposure is low in the boreal ecosystem compared to other regions, and this, as well as the extraction conditions employed in this study, may account for the low vitamin D levels observed in Chaga extracts.

The effect of solvent pH and temperatures on vitamin A, vitamin D, vitamin K1, vit-amin K2, and Vitamin E which includes Alpha, tocopherol is shown in **Fig. 4b**. Vitamin A content in samples extracted with pH 2.5 and 9.5 at 60°C are not significantly different from each other. No significant difference was found among the different extraction sys-tems for Vitamin D2 B5.

Vitamin B1 (Thiamin) was reported to be in the range of 0.02–1.6 µg/g DW, while vitamin B3 (Niacin) was found in the range of 1.2–6 µg/g DW in different kinds of mush-room [36]. In Chaga, the best extract contained 0.17 ng/100g of B3 at pH 9.5 and a temper-ature of 100°C.

a.

Vitamins (water- and fat-soluble) and treatments
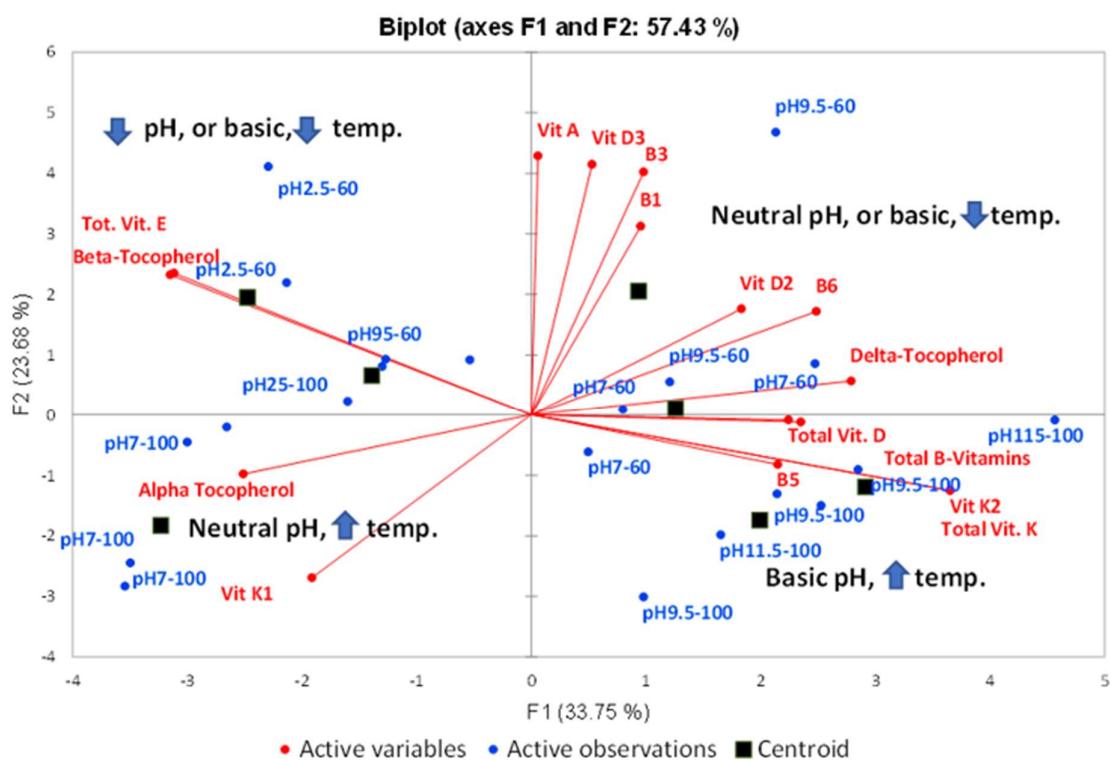
b.

## Bar chart of vitamins from Chaga and treatments
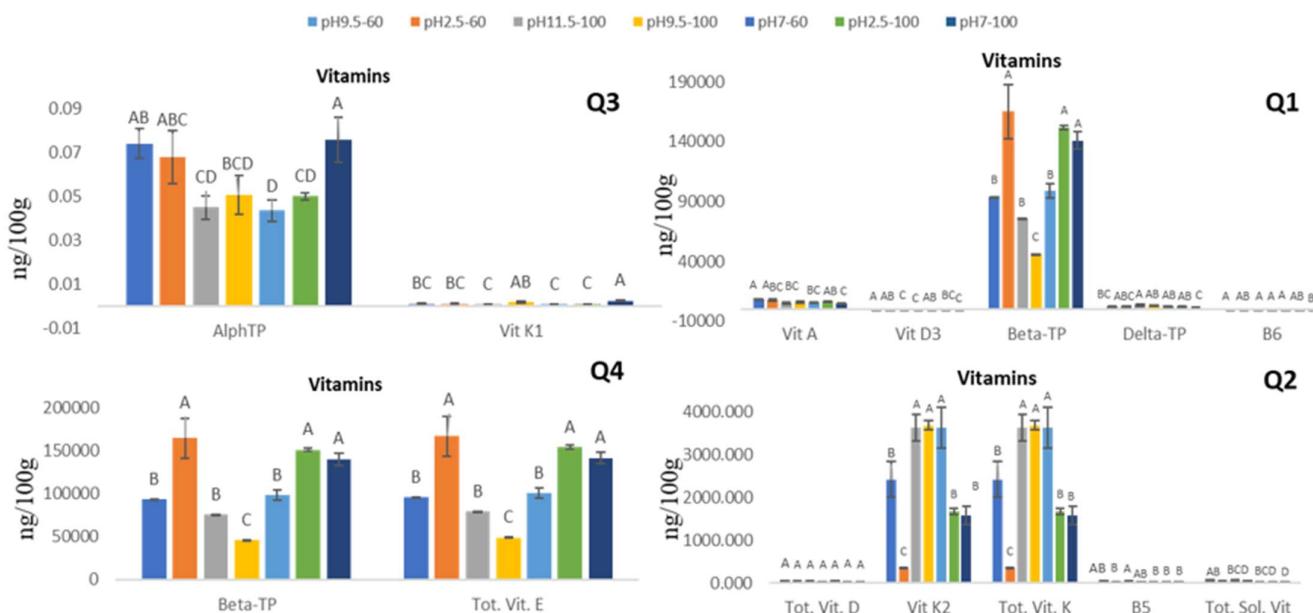
Fig. 4 a. Principal Component Analysis (PCA) illustrating the relationships between vitamin contents in Chaga samples as affected by solvent pH (2.5, 7, 9.5, 11.5) and extraction temperatures (60°C and 100°C). **b.** Bar charts showing the difference in vitamins and following ANOVA for the components segregated per quadrant in the PCA biplot. Means that have the same subscripts indicate no

significant difference between extraction conditions. LSD=0.05, n=4 per experimental replicate. The antioxidant activities are reported as μmol/g of Trolox equivalents. The minerals content is represented as μg/g of Chaga DW.

Vitamin B5 content of Chaga samples extracted at pH 2.5, 7, 9.5 at both 60°C and 100°C are not significantly different from each other, whereas the samples extracted at pH 11.5/100°C is significantly different from samples at pH 2.5 and pH 7 and results in a high content of vitamin B5. Extraction temperature had no significant impact on vitamin B5 content. The lowest vitamin B6 content was found in the mushroom sample extracted at pH7/100°C. The highest and the lowest vitamin B3 content were extracted at pH 9.5/60°C, and pH 7/100°C, respectively, and these values were significantly different from each other. Vitamin B1 content is highly elevated in samples extracted at pH 9.5/60°C and shows the least value at pH 7/100°C. Vitamin B2 and Vitamin B12 were undetectable. Vitamin B3 was found in the range from 3.25 to 11.62 ng/100g in Chaga extracts, with the highest level observed at pH 9.5 and temperature of 100°C. Although the levels for some of the vitamins recovered using this method are lower than that for vitamins in other mushroom varieties. This work demonstrates for the first time the acquisition of both fat- and water-soluble vitamins simultaneously in chaga extracts obtained using water as pressurized extraction fluids at varying pH and temperature in the modified Swiss method proposed.

3.2.3. Phenolics Compounds

The phenolic compounds identified and quantified in the extracts obtained using the modified Swiss method is illustrated in **Table 2**. The PCA of the phenolic compounds is shown in **Fig. 5a**. Analysis of the phenolic composition of the different extractions/treatments resulted in the identification and quantification of twenty-four phenolic compounds in Chaga mushroom extracts following LC-HESI-HRAM-MS/MS analysis (**Fig. 5**). The results for the composition at the best extraction conditions at pH11.5 and a temperature of 100C is presented in **Table 2**. The identification was based on the molecular formula, retention time, and exact molecular mass (**Table 2**). The compounds: 3,4-dihydroxybenzaldehyde, 4-Methoxycinnamic acid, dihydroxyterephtalic acid, Protocatechuic acid, and Caffeic acid were found as the highest phenolics in the Chaga extracts produced using the modified Swiss water method (**Fig. 5b**). It was suggested that phenolics in Chaga are the main components responsible for the antioxidant activity 37 and this is consistent with the high correlations observed between these components in our study.

a.   Individual phenolic compounds in Chaga
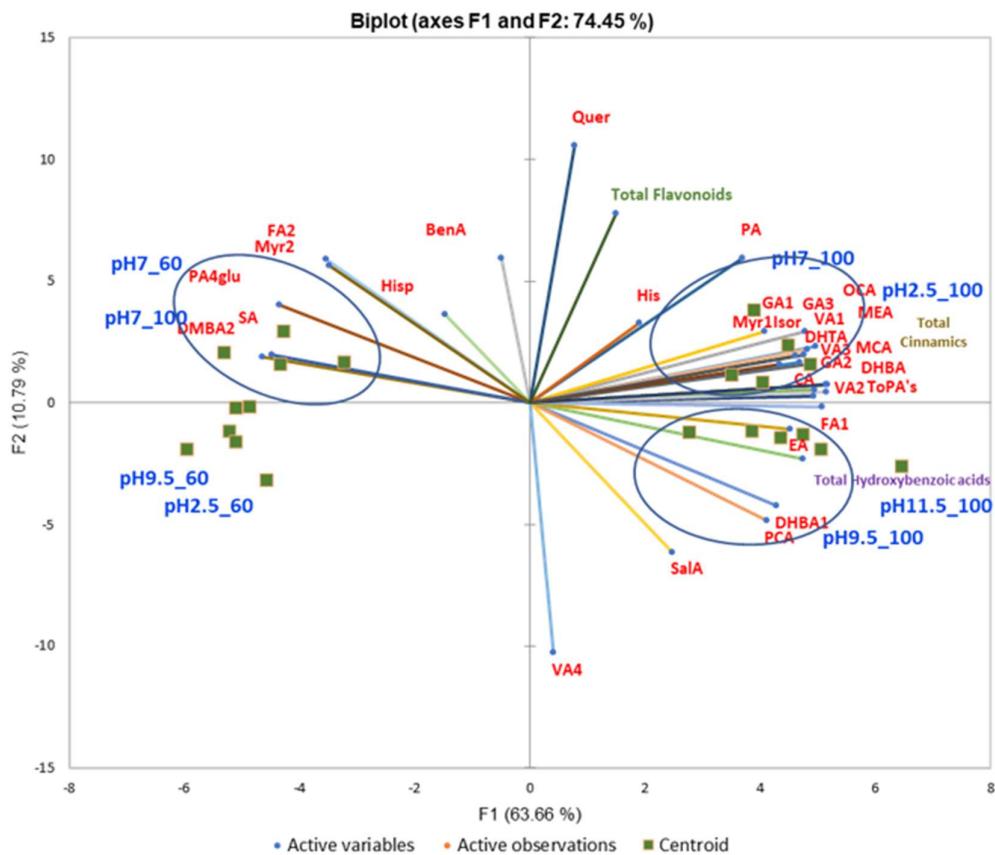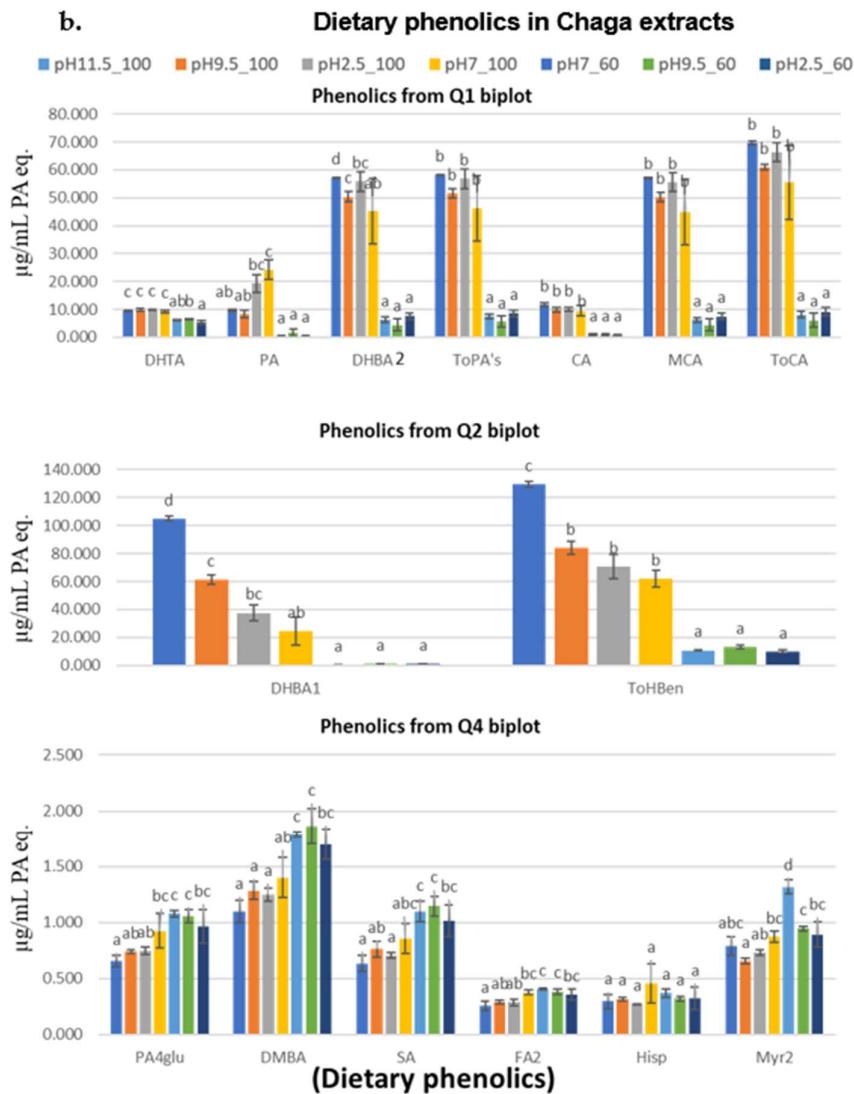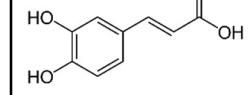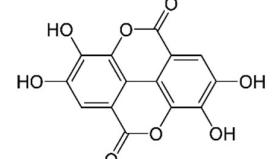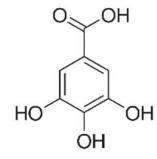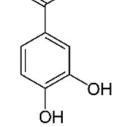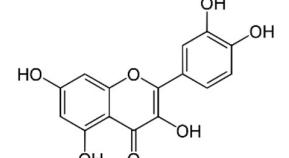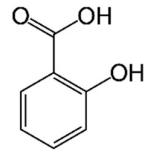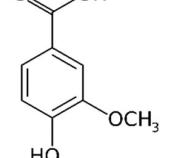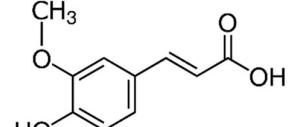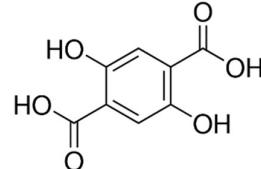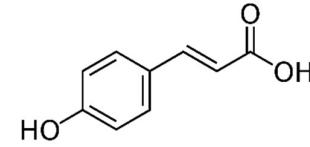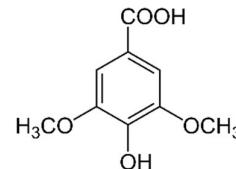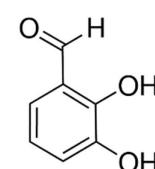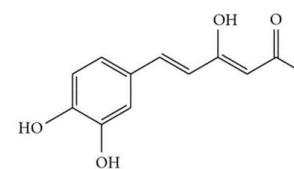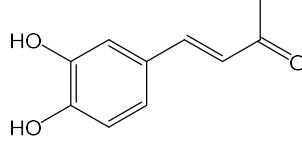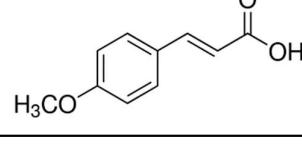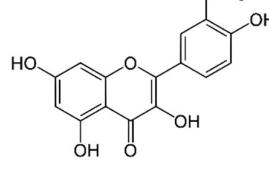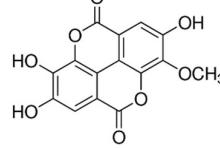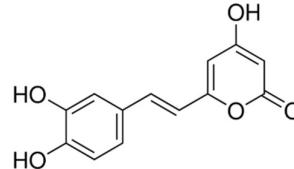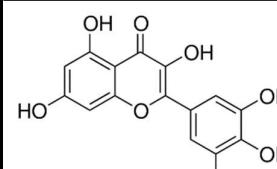
Figure 5 a. Biplot PCA showing relationships observed between phenolic compounds (GA: Gallic acid, VA: Vanillic acid, EA: Ellagic acid, Isor: Isorhamnic acid, PA4glu: Protocatechuic acid 4-glucoside, Myrs: Myristin, FA: Ferulic acid, MEA: Methyl-ellagic acid, extracted under different pH (2.5, 7, 9.5, 11.5) and Temp. (60 and 100 °C treatments. **b.** Bar charts showing the major phenolics, Total phenolic acids (ToPA's), Protocatechuic acid (ProAc), 3,4-dihydroxybenzaldehyde (DHBAd), Caffeic acid (CA), detected in Chaga mushroom extracts. Means that have the same subscripts indicate no significant difference between extraction conditions. LSD=0.05, n=4 per experimental replicate.

Table 2. Composition of phenolics present in the best Chaga extract as determined by pH and Temperature

Compounds	Formula	MW (g/mol)	m/z [M-H]-	RT (min)	Amount recovered* (μg/mL PA eq.)	Structure
Caffeic acid	$C_9H_8O_4$	180.16	179.0349	19.99	11.72	
Ellagic acid	$C_{14}H_6O_8$	302.197	300.9989	22.61	0.25	
Gallic acid_1	$C_7H_6O_5$	170.12	169.0142	11.12	0.26	
Gallic acid_2				13.35	0.20	
Gallic acid_3				14.74	0.11	
Protocatechuic acid	$C_7H_6O_4$	154.12	153.0193	16.90	32.21	
Quercetin	$C_{15}H_{10}O_7$	302.236	301.0354	30.27	0.54	
Salicylic acid	C7H6O3	138.122	137.0244	29.71	1.44	
Vanillic acid_1	C8H8O4	168.148	167.0350	19.48	0.47	
Vanillic acid_2					0.45	
Vanillic acid_3					0.33	
Vanillic acid_4					0.18	
Ferrulic acid (trans)	C10H10O4	194.18	193.0506	21.90	0.40	
Ferrulic acid_1 (cis)				17.10	0.18	

Terephtalic acid (2,5-Dihydroxyterephthalic acid)	C8H6O6	198.13	197.0092	16.00	9.83	
o-Coumaric acid p-Coumaric acid	C9H8O3	164.16	163.0401	18.11 22.91	0.22 0.24	
Syringic acid	C9H10O5	198.174	197.0455	17.30	1.14	
2,3-Dihydroxybenzaldehyde	C7H6O3	138.12	137.0244	18.28	104.85	
Hispolon	C12H12O4	220.224	219.0663	18.72	0.03	
3,4-Dihydroxybenzalacetone (DBL)	C10H10O3	178.187	177.0557	19.03	57.17	
4-methoxycinnamic acid	C10H10O3	178.187	177.0557	21.11	0.57	
Isorhamnetin	C16H12O7	316.26	315.0510	23.34	0.47	
3-O-Methylellagic acid	C15H8O8	316.22	315.0146	25.55	0.25	

Hispidin	C13H10O5	246.22	245.0455	18.81	0.45	
Myricetin_1 Myricetin_2	C15H10O8	318.24	317.0303	22.41 30.10	0.30 1.32	
Total cinnamates					69.65	
Total Benzoate					129.20	
Total flavonoids					1.80	
Total other phenolics					58.24	

According to the results obtained, 3,4-dihydroxybenzaldehyde was significantly higher (104.851 ug/mL) when extracted at pH 11.5 and a temperature of 100°C compared to other treatments. The content found in our research are consistent with previous reports in the literature as this compound was a major phenolic compound observed, but the levels obtained in our work are much higher. Interestingly, 3,4-dihydroxybenzaldehyde has been reported to have significant biological activities such as antioxidative, antibacterial, anti-aging, and anti-inflammatory [38]. The present study also revealed other phenolics like flavonoid aglycones such as myricetin, quercetin, isorhamnetin, and phenolic acids such as gallic, vanillic, protocatechuic, ferulic, ellagic acid, methyl ellagic, syringic, and salicylic acids to be major compounds in the Chaga extracts obtained. Total hydroxycinnamic acids (69.6μg/mL), total hydroxybenzoic acids (129.20μg/mL), and total phenolics (58.22μg/mL) were found at higher levels in the extracts obtained using pH11.5-100°C. Phenolic acids like 4-Methoxycinnamic_acid (57 μg/mL), 3,4-dihydroxybenzaldehyde (104.85 μg/mL), 3,4-dihydroxybenzalacetone (57.17 μg/mL), and Caffeic acid (11.73 μg/mL) were observed in the extracts. Protocatechuic acid 4-glucoside is the only glycosylated phenolic acid detected along with Syringic acid, which was significantly higher at pH 7 and pH 9.5, and temperature of 60°C compared with the other treatments. Ferulic acid showed similar trend, though the amounts were lower. Myricetin was significantly (p<0.05) higher in pH7-60°C than other pH-temperature treatments. Vanillic acid, ferulic acid, isorhamnetin, and salicylic acid were detected in moderate amounts between 0.5-0.6 μg/mL. It is worth mentioning that the flavonoids (isorhamnetin, myricetin), salicylic acid, and protocatechuic acid 4-glucoside are identified in Chaga mushroom for the first time in our study to the best of our knowledge.   These phenolics were detected as aglycones, which might be due to the heat /and or pH impact on the aglyconic linkage with sugars' moieties. Salicylic acid was significantly (p< 0.05) higher at pH 11.5 and temperature of 100°C compared to the other treatments. One other major phenolic compound detected in Chaga mushroom using the extraction conditions was 3,4-dihydroxybenzaldehyde (104.851 μg/mL). Furthermore, 4-methoxycinnamic acid (57.23 μg/mL) is being reported in Chaga for the first time. This compound was found to have antioxidant, antihyperglycemic, hypolipidemic activities [39,40,41].   Other new compounds that were identified and quantified in for the first time in Chaga include Methyl-ellagic acid and ellagic acid. Both these compounds were found to exist at higher amounts in extracts obtained using

pH11.5/100°C treatment. These compounds were reported to have anti-radical and strong neuroprotective and antioxidants properties [42]. Quercetin has previously been reported in Chaga and it was also detected in our present work but at lower amounts [25].

3.2.4. Association of the myconutrient composition and antioxidant activities the chaga extracts recovered

To get a better understanding of the antioxidant potential of the Chaga extracts obtained as a function of its chemical composition, we assessed the correlations between the TAA and total antioxidant minerals, TPC, vitamins, and dietary phenolics (**Fig. 7**). As previously mentioned, a high correlation between antioxidant capacity and TPC was found, which indicates that phenolic compounds could be the foremost contributors to the antioxidant activity of the Chaga extracts obtained. This is consistent with several reports in the literature demonstrating high association between phenolics and the antioxidant activity [43].
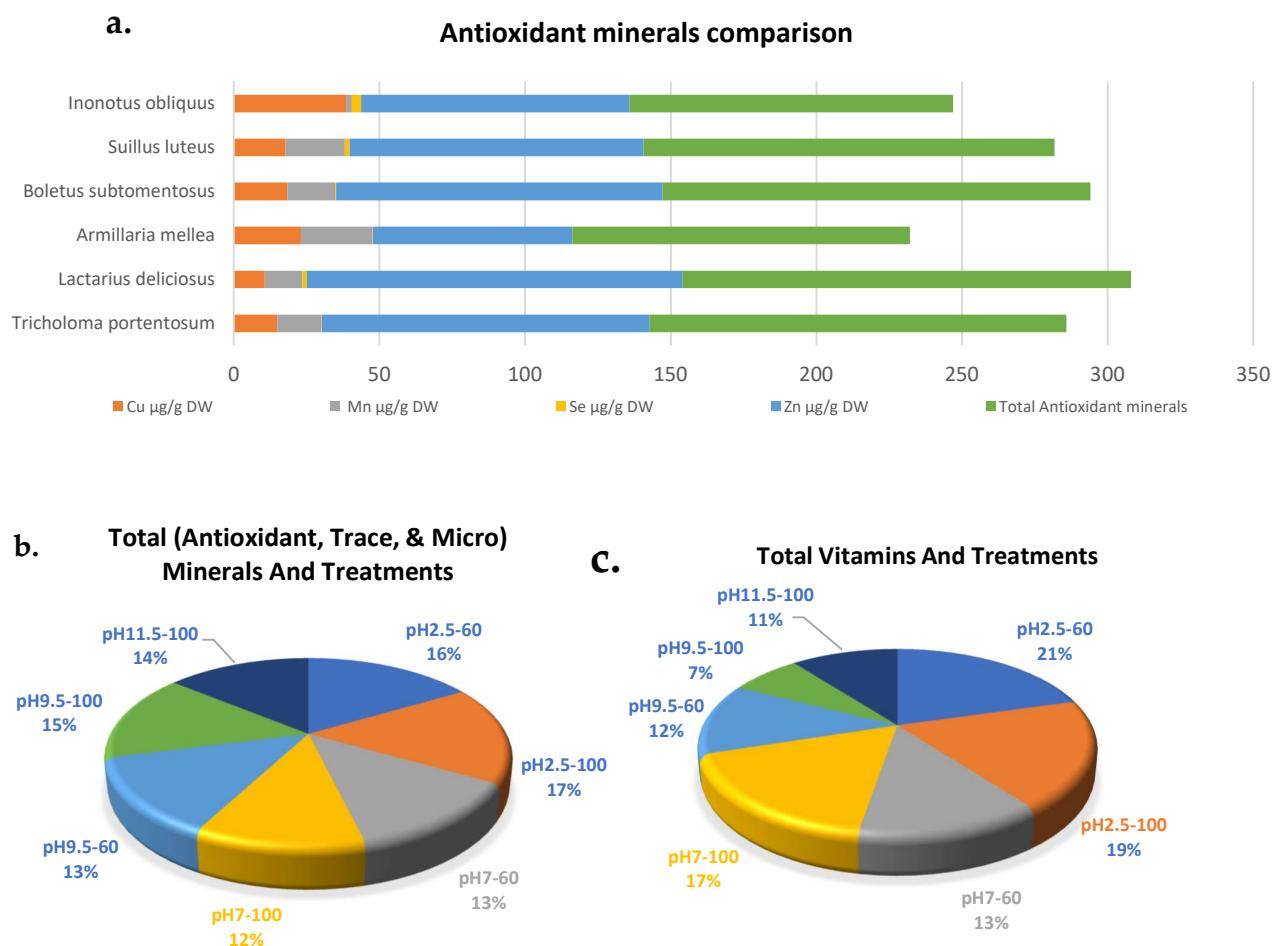

Fig. 7.a. Antioxidant minerals comparison from different species (Mirończuk-Chodakowska, Socha, Zujko, Terlikowska, Borawska, Witkowska, 2019), **b.** Minerals and extraction percentages based on treatments **c.** Total vitamins and extraction percentages based on treatments

Likewise, the total antioxidant minerals (TAM) were moderately correlated with the TAA (r=0.52; p=0.016), so too was vitamin E with the LPC (r=0.64). Vitamin E exists in different forms called "Tocopherols" which are known antioxidants [34]. In our present work, we have determined three types of these antioxidant vitamins, namely, Alpha-, Beta-, and Delta-Tocopherols in the Chaga extracts using the modified Swiss method. These results demonstrate that phenolic acids are highly associated with the antioxidant activity present in the Chaga extracts obtained using the modified Swiss water method evaluated in this study. Collectively, pH11.5/100°C appears to be very useful for extracting individual dietary phenolics, TPC and TAA from Chaga matrices (**Fig. 6**). The correlation results between antioxidant activity, TPC, phenolics, vitamins, and antioxidant minerals suggest the Chaga extracts obtained appears to be a promising source of myconutrients with functional and antioxidant properties.
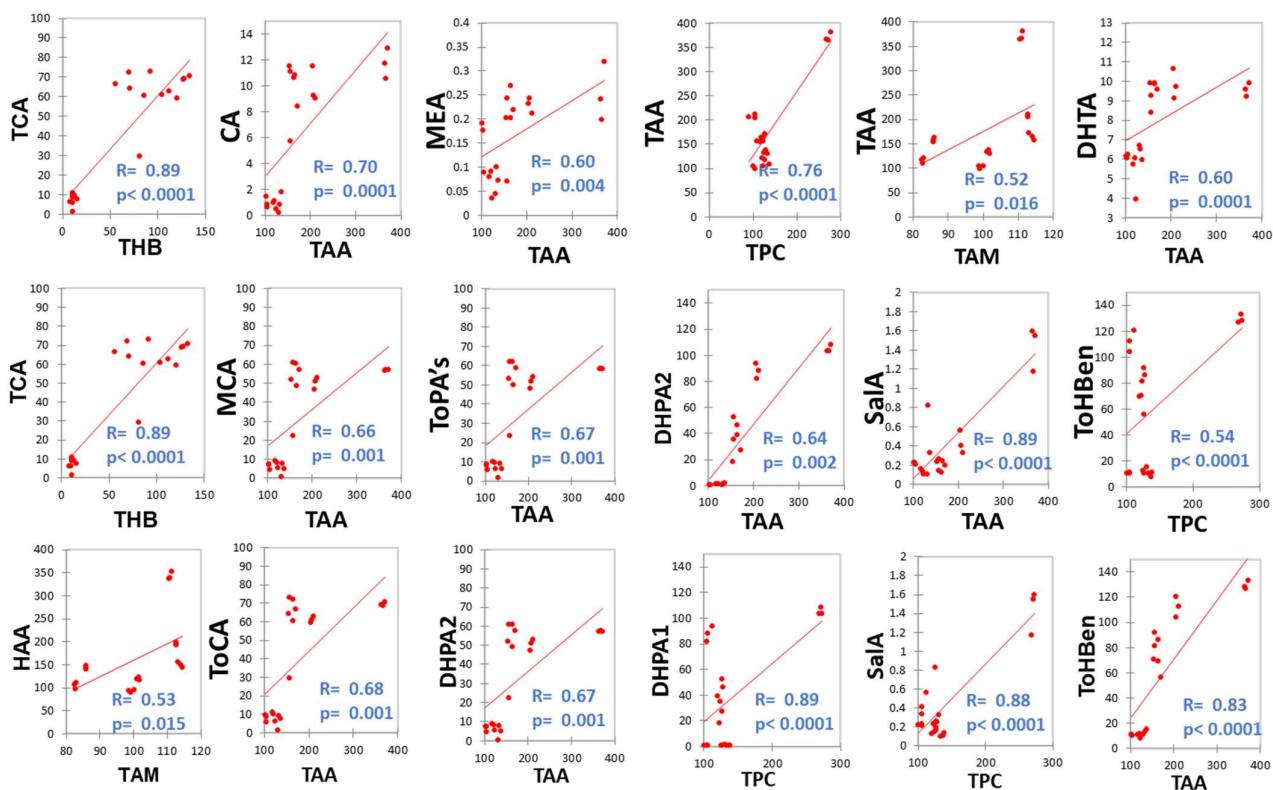

Figure 6. Correlation between TAA and CA**,** TAA and MEA, TAA and TPC, TAA and TAM, DHTA and TAA, MCA and TAA, ToPA's and TAA, DHPA2 and TAA, SalA and TA, ToHBen and TPC, ToCA and TAA, DHPA2 and TAA, DHPA1 and TPC, SalA and TPC, ToHBen and TAA content. HAA= Hydrophilic antioxidant activity, LAA= Lipophilic antioxidant activity, TAA= total antioxidant activity, HPC= Hydrophilic phenolic content, LPC= lipophilic phenolic Content, TPC= total phenolic Content. TAM= Total antioxidant minerals, ToHBen=Total Hydroxybenzoic acids, ToPA's= Total other phenolic acids. ToCA=Total cinnamic acids. DHPA1= 3,4-dihydroxybenzaldehyde, DHPA2=3,4-dihydroxybenzalacetone, SalA=Salicylic acid, DHTA= Dihydroxyterephtalic acid   MEA= Methyl-Gallic acid, CA= Caffeic acid. Antioxidant activity was expressed as μmol Trolox equivalent/g extract of Chaga and phenolic content was expressed as μmol quercetin equivalent/g extract of Chaga. R-values represent Pearson Correlation Coefficients, p= 0.05, n=4 per replicate of each sample at various pH values and temperatures.

Additionally, the correlation between the TAA and TPC together with individual phenolics and total phenolic acids were also established. Notably, 3,4-dihydroxybenzaldehyde (DHBA) showed strong significant (p<0.0001) correlation with TAA (r=0.89) and TPC (r=0.61). Similarly, the salicylic acid (SalA) content in the extract was strongly correlated with the TAA (r=0.89) and TPC (r=0.88). The total hydroxybenzoic acids (ToHBen) was also significantly (p<0.0001) correlated with TAA (r=0.83) and TPC (0.54), respectively. That may explain the contributions of hydroxybenzoic acids to the total antioxidative capacity of the extract obtained using this solvent extraction system. On the contrary, the ferulic acid (FA) content in the extract was moderately, but significantly (p=0.0001) correlated with TPC (r=0.74) as was Caffeic acid (CA) with TAA (r=0.70). This trend in moderate association was consistent between the other phenolics and the TAA or TPC evaluated (**Fig. 7**).

5. Conclusions

The modified Swiss method of extraction using hot water with different pH values is very useful in obtaining high-quality food-grade and green extracts from Chaga enriched with myconutrients. The Chaga extracts obtained was good source of myconutrients containing phenolics, fat- & water-soluble vitamins, and minerals including those with antioxidant activities. The highest TPC and TAA levels were found in extracts obtained at pH 11.5 and a temperature of 100°C. This was the case also for most of the individual phenolics detected in Chaga. All together twenty-four phenolic compounds were detected in the extract with   3,4-dihydroxybenzaldehyde (104.851 ug/mL) the major component, which was highly correlated with the TAA and TPC. Vitamins are first analyzed in Chaga, being the Vitamin E and K to be with the highest amounts found. Furthermore, 4-methoxycinnamic acid, isorhamnetin, protocatechuic acid 4-glycoside, ellagic acid, methyl ellagic acid, and salicylic acid, and isorhamnetin were present in the extracts and are described for the first time in Chaga using the modified Swiss water method evaluated in this study.

The Chaga extracts contained elevated TPC content and antioxidant properties at higher pH and temperature under pressurized extraction conditions. Treatment using pH11.5 at 100°C was useful for recovering the phenolics (total hydroxycinnamic acids, hydroxybenzoic acids, total other phenolics) and total antioxidants. The antioxidant minerals were best extracted at pH11.5 at 100°C, while the macro and trace minerals extraction, was more efficient using either pH 2.5 or 9.5 at 100°C. These results suggest Chaga extracts obtained using the modified Swiss method could be promising sources of myconutrients or functional components that may have applications in producing nutraceuticals, or functional foods abundant with Zn, P, antioxidants, K, Mn, phenolic compounds, and vitamin E. This work demonstrates that water as pressurized fluid at high pH and temperature were very effective in extracting myconutrients from Chaga matrices and may also be useful for the recovery of bioactive phytonutrients from different plant-based matrices. This work presents a modified Swiss water method at high pH (11.5) and temperature (100°C) under pressurized conditions as a suitable green or food grade solvent for the extraction of myconutrients from Chaga matrices.

Conflicts of Interest: The authors declare no conflict of interest.

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