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Investigation of Anti-Inflammatory and Antioxidants Properties of Phenolic Compounds from Inonotus obliquus Using Different Extraction Methods

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INVESTIGATION OF ANTI-INFLAMMATORY AND ANTIOXIDANTS PROPERTIES OF PHENOLIC COMPOUNDS FROM *INONOTUS OBLIQUUS* USING DIFFERENT EXTRACTION METHODS

By

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A DISSERTATION

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Doctor of Philosophy of Science

(in Food Science and Human Nutrition/Food Chemistry)

The Graduate School

The University of Maine

May 2020

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By Weaam Abdulwahid Abdulnabi Alhallaf

Dissertation Advisor: Dr. L. Brian Perkins

An Abstract of the Dissertation Presented
in Partial Fulfillment of the Requirements for the
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Inonotus obliquus, commonly known as Chaga, is a fungal pathogen of birch trees, known to synthesize a range of phenolic compounds with remarkable health benefits. These presumed medicinal properties have generated increased interest in Chaga consumption. Prior research has demonstrated the diverse chemical composition of Chaga sourced from a variety of geographical locations. However, to our knowledge, there is currently no available literature regarding the extraction of bioactive compounds from Chaga grown in the United States. Additionally, the effect of the extraction method on the antioxidant and anti-inflammation properties specifically, has yet to be validated. Therefore, the present study was developed to examine the effects of extraction conditions on phenolic compounds in Maine sourced Chaga and correlate these findings to anti-inflammatory benefits.

A high-performance liquid chromatography–diode array detection (HPLC–DAD) method was developed to determine the phenolic acids content in Chaga. The method

demonstrated good linearity (0.994-0.999) and precision within (RSD ≤ 3) and between (RSD ≤ 4.2) -day precisions. The procedure also produced good recovery within (≥ 90.1) and between (≥88.5) -day precisions, as well. The majority of phenolic acids were extracted from the base hydrolysis fraction (2794.91 μg/g).

The response surface methodology (RSM) was also applied to establish optimum extraction conditions to obtain phenolic-rich extracts. Results indicate that an extraction temperature of 170°C and ethanol concentration of 66% were optimal for recovering phenolic compounds, with a total phenolic content (TPC) value of 39.32 mg GAL/g DW and 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity of 76.59%. The extractions that produced the highest yields of TPC and DPPH were then assessed for the ability to remediate inflammation using lipopolysaccharide (LPS) activated RAW 264.7 macrophages. The results showed various Chaga extracts have significant anti-inflammatory activity on LPS-stimulated RAW 264.7 cells. The inhibitory effect was evident through a decrease in the production of nitric oxide (NO) and down-regulation of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-β (IL-1β) in RAW 264.7 macrophages. Therefore, findings confirm that Maine harvested Chaga demonstrates anti-inflammatory properties. However, the phenolic yields (total phenolic acids and TPC) and antioxidant activity are highly dependent upon the extraction methodology.

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INTRODUCTION

Inonotus obliquus or Chaga is an unusual polypore fungus that infects living trunks of birch trees (*Betulaceae*) often resulting in white heart rot. Historically, Chaga extracts have been utilized in medicine to prevent and treat gastrointestinal diseases as well as certain cancers (Lee et al., 2008). More recently, researchers have scientifically validated these practices, isolating substances from Chaga which demonstrate a range of biological health benefits including antioxidant, anticancer, antiviral, immune modulation, and hepatoprotective activities (Chung, Chung, Jeong, & Ham, 2010;Ham et al., 2009;Lu, Chen, Dong, Fu, & Zhang, 2010). Chemical investigations have further supported these findings, as I. obliquus has been shown to produce a diverse range of bioactive compounds such as triterpenoids, polyphenols, melanin pigments, and polysaccharides (Burmasova et al., 2019; J. H. Lee & Hyun, 2014; Chung, Chung, Jeong, & Ham, 2010; Lu et al., 2010).

These health mediated effects have steadily increased interest in Chaga consumption among United States consumers. As a result, there are growing research initiatives surrounding optimization of extraction procedures defined by maximum target compound yields. Currently, there are few investigations on United States - harvested Chaga, or its bioactive content composition (such as phenolics) (Brydon-William, 2019). Unsurprisingly, the extraction method(s) effect on total phenolic content, antioxidant, and anti-inflammatory activity has yet to be confirmed. Thus, the primary objectives of this study were to (1) develop and validate a High Performance Liquid Chromatography (HPLC) method to assess the phenolic acids content in Chaga in both free and bound forms, (2) apply the response surface methodology (RSM) approach to obtain the most phenolic rich extracts from Chaga and (3) screen the anti-inflammatory activity of Chaga extracts obtained by different extraction methods.

CHAPTER 1

LITERATURE REVIEW

1.1 Naming Origins of Chaga

Inonotus obliquus, commonly known as Chaga, is a fungal pathogen that belongs to the Hymenochaetaceae family (Hawksworth et al., 1995). The English name "Chaga" is derived from the Siberian word "Czaga." which originated from the Komi Permyak language, spoken by the indigenous people of the Kama River Basin, in the West Uralian region of the country (Shashkina et al., 2006). The fungus was first identified and described by Persoon (1801), who named it *Boletus obliquus*, then it was later renamed *Polyporus obliquus* by Fries (1830), followed by Quélet (1888) who called it *Poria obliqua* (under the bark of dry Fagus). In 1927, Bourdot and Galzin termed the fungus *Xanthochrous obliquus*, and its current name, *Inonotus obliquus*, was given by Pilàt (1936 and 1942), who studied it thoroughly (Lee et al., 2008). The genus name, *Inonotus*, directly translates to "black fiber" while the specific epithet, *obliquus*, refers to its geometric shape. Specifically, the pores of the reproductive body have sloping direction to the horizon. *Inonotus obliquus* is also known by other common names, such as true tinder fungus, clinker polypore, sterile conk, cinder conk, and cancer polypore (Lee et al., 2008).

1.2 Distribution and Ecology

Chaga is best characterized as a "circumboreal" organism, as it is found throughout the Northern Hemisphere, mainly within dense birch forests in characteristically temperate to subarctic climates. As a result, significant Chaga proliferation has been reported in forests throughout Russia (Western Siberia, partial regions in the Far East, Kamchatka peninsula), Poland, France, China (Heilongjiang province, Chinghai

mountain area of Jilin province), Japan (Hokkaido), Korea, and Canada. In the United States, Chaga has been harvested domestically in the Northeast (Maine through Pennsylvania), the Great Lakes region (Michigan, Wisconsin, and Minnesota), Alaska, and high-altitude areas of the southern Appalachian Mountains, including western North Carolina, where there is extensive yellow birch growth (Sinclair et. al, 2005; Brydon-Williams, 2019).

Chaga has been isolated from a variety of tree species including hardwoods such as red alder (*Alnus rubra*), American and European beech (*Fagus grandifolia and sylvatica*), oak (*Quercus* spp.), red maple (*Acer rubrum*), hophornbeam (*Ostrya virginiana*), and poplar (*Populus* spp.) However, the fungus is primarily grown on the living trunks of mature white birch (*B. papyrifera, B. pendula, B. pubescens*) yellow birch (*B. alleghaniensis*) black birch (B. lenta), water birch (*B. occidentalis*), and grey birch (*B. populifolia*) species. Additionally, the characteristic sterile conk has only been isolated from birch tree varietals (Brydon-Williams, 2019; Lee et al., 2008).

A 2015 study conducted by Balandaykin and Zmitrovich, determined the preferred growth environments of the fungal pathogen in birch stands within the Ulyanovsk region of Russia (mainly comprised of *B. pendula* and *B. pubescens*). Particularly, the researchers determined that Chaga prefers sprout coppiced trees to those with seedling coppice, mature stands to a younger counterpart, and oligotrophic soils to eutrophic soils. Furthermore, anthropogenic disturbance in the birch stands have demonstrated a positive correlation with incidences of Chaga infestation (Balandaykin & Zmitrovich, 2015). In a similar study conducted in seven regional forests throughout Poland between 1995-2011, incidences of Chaga growth were higher among stands aged 60 years or older. Additional Chaga prevalence increased in mixed birch-coniferous forest and bog forest

compared to wet broadleaved forests, with an estimated total Chaga volume of 46 metric tons (Szczepkowski et al., 2013). However, these studies are only applicable to European South Boreal forests, and thus have a limited relevance to Chaga distribution in North America (Balandaykin & Zmitrovich, 2015).

To better illustrate the relationship between tree species and Chaga fungal growth in the United States, a recent survey conducted in the White Mountain National Forest of New Hampshire studied Chaga growth on white birch and yellow birch trees. Among the examined species, yellow birch was determined to be a more suitable host for the Chaga fungus. Characteristics including comparative hardiness as well as enhanced ability to survive through both damage and growing conditions (in particular, larger diameters and greater heights than other birch species) contributed to these findings (Brydon-Williams, 2019).

1.3 Morphological Features of Chaga

Chaga is a perennial sclerotium, or resting body, which appears as a massive black charcoal structure with a rust-colored woody texture containing internal interwoven mycelia. Although Chaga is often referred to as a "mushroom", scientists are skeptical of this designation. As mentioned, it is classified as a member of the Hymenochaetaceae family, which includes a few other dark, woody botanicals that grow on bark and decaying trees. Unlike the sclerotium, the fruiting body occurs once during the infection cycle. The structure of the fruiting body resembles a tumor and appears as a crust-like layer of pores with a light-yellow inner portion surrounded by irregularly cracked charcoal grey on the exterior. This external feature varies in size between 25-40 cm in diameter. Chaga fungus grows wild on decaying logs and wound sites of tree stumps. The invaded tree will utilize various defense mechanisms to resist fungal invasion; however,

Chaga can persist for up to 80 years, producing 1–3 sclerotia on the main stem and branches (Lee et al., 2008). It is unclear whether Chaga invasion leads to the eventual death of the hosted tree, or if mortality of the tree is caused by infection related to other opportunistic organisms (Niemelä et al., 1995). Various methods such as infected tree felling, girdling, fungicide, and cutting the trunk into bolts have been tested to control Chaga infections. None of these methods have successfully prevented the eventual formation of Chaga conks (Sinclair et al., 2005). However, some infected trees have been found to reject Chaga infection, ultimately resulting in the falling off of the conk, though the decay column is left intact (Spahr, 2009).

1.4 History of Medicinal Use

Chaga has been widely recognized as a medicinal source traditionally used to treat stomach ailments and cancer. Russian legend suggests the famous 12th century prince, Vladimir Monomakh, was cured from a lip tumor by regularly ingesting Chaga tea. The Russian novelist, Solzhenitsy similarly mentioned Chaga in his book "The Cancer Ward", in which the author describes cancer patients in rural villages, who used habitual drinking of a hot-water Chaga as a natural remedy to cure aliments. There are also reports of the Khanty people, an ethnic group from western Siberia, using Chaga to prevent and treat digestive disorders, cardiac illnesses, and hepatic disorders. They also utilized the fungus as an antiseptic agent used to clean body wounds (Saar, 1991). The Ainu people, a tribe indigenous to northern Japan, have also utilized Chaga as a natural treatment for stomach pain and inflammation. In addition to the perceived health effects of Chaga consumption, the Ainu people also regarded the fungus as sacred. The act of inhaling Chaga smoke, known as "eating the smoke" was common practice in particular religious ceremonies. The Skolt Sami people of Northern Scandinavia, similarly, used Chaga to

address certain illnesses including cold, flu, and stomach ailments. Additionally, the fungus was also utilized as a recreational tea in place of other tea or coffee beverages (Magnani Natalia, 2016). Today the fungus is still regarded for its medical properties. In 1955, following several clinical investigations, the Russian Medical Research Council approved Chaga preparation, which is listed in the Soviet Pharmacopeia under the name of "Befunginum", for several forms of cancers such as genital and breast (Balandaykin & Zmitorvich, 2015).

1.5 Chemical Analysis of Chaga

Chemical analysis of Chaga reveals that the fungus contains more than 200 bioactive components. These compounds have been associated with a wide array of biological activities attributed to several health-mediated effects (Diao et al., 2014; Olennikov et al., 2012; Y. M. Park et al., 2005). The predominant bioactive constituents in Chaga include polysaccharides (Wold et al., 2018); triterpenoids (Nakata et al., 2007); steroids (Nikitina et al., 2016), melanin (Burmasova et al., 2019), and phenolic compounds (Nakajima et al., 2007). Dragendorff first examined the chemical composition of Chaga in 1864. In addition to polysaccharides, (Ludwiczak & Wrecino, 1962) first detected and identified lanostane triterpene compounds (lanosterol-3β- hydroxyl-lanosta-8,24-diene and its derivative inotodiol) in this mushroom as well. Kahlos and his group also isolated β-hydroxylanosta-8,24-dien-21-oic acid (trametenolic acid), 3β-hydroxylanosta-8,24-dien-21-al, 3β,22,25-trihydroxylanosta-8,23-diene, and d 3β,22-dihydroxylanosta-8,24-dien-7-one (Kahlos & Hiltunen, 1983; Kahlos et al.,1984). Recently, chagabusone, a lanostane-type triterpenoid, was also isolated following the fractionation of methanolic extracts of this fungus (Baek et al., 2018). Careful examination of the sclerotia has led to the isolation of additional constituents including 3 β-hydroxylanosta-8,24-diene-21,23-

lactone, 21,24-cyclopentalanost-8-ene-3β,21,25-triol, and lanost-8-ene-3β,22,25-triol (Shin et al., 2000; Shin et al., 2001) . In the available literature, approximately 40 triterpene compounds from the lanostane-type structure have been identified in Chaga, including trace amounts of pentacyclic-type structure of triterpenes, such as betulin, lupeol, and lupenon (Gao et al., 2009). Further, steroids and alkaloid-like chemical compounds have also been extracted (Nikitina et al., 2016). Melanin; a high-molecular weight polyphenol pigment that is synthesized by Chaga as a result of oxidative polymerization of phenols, has similarly been isolated following physicochemical examination (Kukulyanskaya et al., 2002). This is a rather noteworthy discovery, as melanin extracts widely contributed to the varied medicinal properties of this fungus, due to their high antioxidant properties (Burmasova et al., 2019).

Different solvents have been used in the extraction of bioactive compounds from Chaga. Previous work has demonstrated that the solvent utilized for extraction may affect the bioactivity (Kallithraka et al., 2007; Zheng et al., 2010). Chemical profiles of some bioactive compounds of Chaga are presented in Table 1-1.

Table 1-1 some of the bioactive compounds extracted from Chaga using different solvents

Compound	Biological activities	Extraction solvent	Reference
Melanin	Hypoglycemic; antioxidant	Water	Burmasova et al., 2019; Lee & Hyun, 2014).
3β□Hydroxylanosta□8, 24□diene□21□al	Antimutagenic and antioxidative; anticarcinogenic, hypoglycemic	Ethyl acetate; Methanol	(Chung et al., 2010; Ham et al., 2009; Lu et al., 2010).
Lanosta□24□ene□3β,21□ diol	Antitumor activity	Chloroform	(Taji et al., 2008).
Ergosterol peroxide	Hypoglycemic	Ethyl acetate	(Lu et al., 2010).
Lignin	Inhibits HIV-1protease activity	Water	(Ichimura et al., 1999).
Lignin	Antioxidant, immunostimulants	Water	(Niu et al., 2016)
Betulinic acid	Antiproliferative effect against human lung adenocarcinoma cells (A549)	Water	(Géry et al., 2018).
Ergosterol; ergosterol peroxide, trametenolic acid	Anti-inflammatory activity	Ethanol	(Ma et al., 2013).
Ergosterol peroxide, trametenolic acid	Anticancer activity	Ethanol	(Ma et al., 2013).
4-hydroxy-3,5-dimethoxy benzoic acid 2-hydroxy-1-hydroxymethyl ethyl ester, protocatechic acid, caffeic acid, 3,4-dihybenzaladehyde, 2,5-dihydroxyterephtalic acid, syringic acid, 3,4-dihydroxybenzalacetone	Antioxidant	Methanol	(Nakajima et al., 2007)

Table 1-1 continued

Compound	Biological activities	Extraction solvent	Reference
3,4-dihydroxybenzalacetone	Prevents hydrogen peroxide-induced oxidative stress in PC12 cells	Methanol	(Nakajima et al., 2009)
Inotodiol	Inhibits Cell Proliferation	Chloroform	(Nomura et al., 2008)
Polysaccharides	Antioxidant; antihyperglycaemic effects	Water	(Liu et al., 2018; Mu et al., 2012)

1.5.1 Polysaccharides in Chaga

Within the last decade, there has been steadily growing interest in Chaga functionality due to its abundant polysaccharide composition. These compounds have been associated with several therapeutic functions including antitumor, antioxidant, hypoglycemic and nontoxigenic effects (Mu et al., 2012; Diao et al., 2014). More specifically, Fan et al reported that the water-soluble polysaccharides within Chaga exert in vivo antitumor activity and enhance immune defense via lymphocyte proliferation in addition to increased tumor necrosis factor-α TNF production (Fan et al., 2012). Similarly, Hu et al found that a purified fraction of Chaga had a therapeutic effect against chronic pancreatitis in mice via multiple pathways including antioxidative effects (Hu et al., 2016). Additionally, Diao et al determined that Chaga derived polysaccharides possess antihyperglycemic effects in mice, which could be a potential therapeutic option for diabetes (Diao et al., 2016).

There are several previously published reports regarding Chaga polysaccharide extraction. Some of this available literature is focused on polysaccharide extraction by hot water, alkaline-treated hot water, or more novel methodologies. Hu et al, for example, examined the effects of different temperatures on polysaccharide extraction efficiency. The study determined the total polysaccharide yield was highest when an 80°C hot water method, compared to water heated to 50°C and 70°C, was used. In addition to compound yield, the ratio of polysaccharide to protein was also highest using this procedure (Hue et al., 2009). This finding indicates that more substances may be extracted by water using an elevated temperature. It has also been suggested that application of ultrasonic/microwave will assist in extraction yield and purity of crude polysaccharides, which are advantages over the traditional hot water methodology (Chen et al., 2010).

The monosaccharide composition of aqueous Chaga extracts consists primarily of glucose and mannose. Kim et al previously reported that purified endo-polysaccharide from cultivated Chaga mycelia was an α-linked fucoglucomannan, composed primarily of mannose and glucose, with small amounts of galactose, fucose, and glucosamine as well (Kim et al., 2006a). This is in accordance with the results of Huang et al, in which glucose and mannose were the major monosaccharides isolated from Chaga polysaccharides along with galactose and rhamnose, which were found in smaller ratios (Huang et al., 2012)

Formation of polysaccharide complexed minerals has been proposed to alter both the physiological properties and biological activity of the polysaccharides, but also enhance the bioavailability of the minerals. Wang et al investigated the effect of an interaction between Chaga polysaccharides and iron (III) on the antioxidant activity and bioavailability of the final product. Data from this work suggested that the

polysaccharide-iron (III) complex may be a suitable candidate for a new iron supplement due to its high antioxidant capacity and increased bioavailability (Wang et al.,2015). On the other hand, Selenizing modification polysaccharides have been successfully prepared by a HNO3- Na2SeO3 method. Following this modification, polysaccharides were found to have significantly increased antioxidative capacity in vivo, as well as in vitro (Hu et al., 2017).

Polysaccharides are usually conjugated with other molecules such as proteins exhibiting various bioactive properties. The protein content within Chaga polysaccharides is typically determined using the Bradford method, which uses bovine serum albumin (BSA) as a standard (Bradford M., 1976). Some studies indicate that the protein content in polysaccharides may exist as polysaccharide-protein complexes. This hypothesis is supported by evidence that after several deproteinization processes using the Sevage method, a small amount of protein remained attached to the isolated polysaccharides (Mu et al., 2012).

Uronic acid, a polysaccharide constituent, is mainly determined by the m-hydroxydiphenyl method that utilizes galacturonic acid as a standard. The compound was found in less than 5% Chaga polysaccharides derived from both water-soluble and alkali-soluble samples. However, contrary to this finding, there have been several literature reports linking high uronic acid content and protein content to increased bioactivity of polysaccharides. Huang et al obtained five polysaccharide fractions (IOP1b, IOP2a, IOP2c, IOP3a and IOP4) from aqueous Chaga extracts and found the higher content of uronic acid and proteinous substances resulted in stronger antioxidant activities of polysaccharides (Huang et al., 2012). The effects of three different drying methods (freeze drying, hot air drying and vacuum drying) on bioactivities and chemical

compositions of polysaccharides were also comparatively investigated. Despite its high polysaccharide yield and nutrient content after drying application, results demonstrated that the higher activity of the freeze-dried polysaccharides might be related to its higher uronic acid and protein contents (Ma et al., 2013).

1.5.2 Phenolic Compounds in Chaga

Phenolic compounds are a group of secondary metabolites that are synthesized in plants as a response to environmental stress, such as pathogens, insect attack, UV radiation, and wounding. Chaga fungus synthesizes a range of phenolic compounds (PC), which possess remarkable potential for free radicals scavenging (Babitskaia et al., 2000; Nakajima et al., 2007). This property has been associated with reduced incidences of oxidative stress-induced diseases including cancer, hypertension, neurodegenerative, and autoimmune diseases (Zheng et al., 2010). The common structure of phenolic compounds is characterized by the presence of aromatic ring(s) bearing one or more hydroxyl groups. PCs are further classified into different groups based on the number of phenolic units and other functional attributes that link these rings (Dai & Mumper, 2010). As a result, different phenolic classes have been formed, as shown in Figure 1-2.

Different classes of phenolic comounds have been reported in Chaga from both wild sclerotia and liquid cultures. Zheng et al determined that the production of flavonoids from cultures of Chaga was enhanced in response to oxidative stress induced by hydrogen peroxide H_2O_2 (1mM H_2O_2 at a rate of 1.6 mL/h) (Zheng et al 2009b). Similarly, a high yield of flavonoids, i.e., epicatechin-3-gallate (ECG), epigallocatechin-3-gallate (EGCG), and naringin, was also obtained from Chaga under fermentated conditions (Xu et al., 2016). Low molecular weight phenolic ingredients, i.e., 3,4-dihydroxybenzalacetone (DBL), and high-molecular-weight phenolic pigments, i.e.

melanin, have also been identified in Chaga (Nakajima et al., 2007; Olennikov et al., 2012).

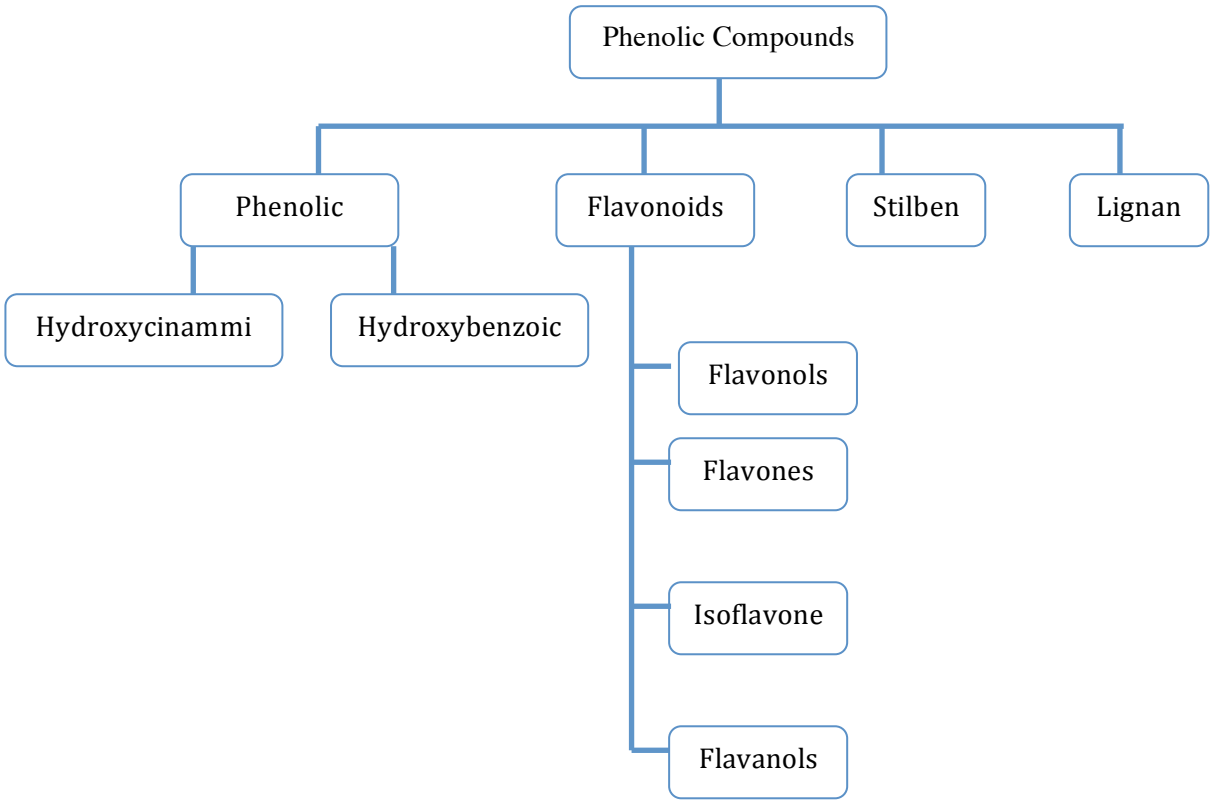
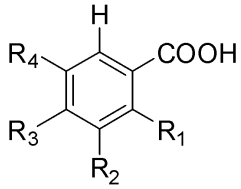


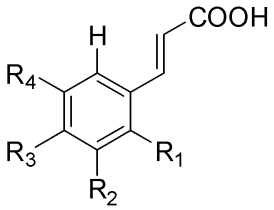
Figure 1-1 Classification of the main polyphenols (Dai & Mupper, 2010)

Among phenolic compound constituents, phenolic acids have previously been reported in Chaga, including gallic acid, protocatechuic acid, protocatechuic aldehyde, caffeic acid, vanillic acid, ferulic acid, and syringic acid (Ju et al., 2010; Nakajima et al., 2007). Structurally, phenolic acids are composed of one aromatic ring, a carboxylic acid group and one or more hydroxyl groups. Despite these structural commonalities, phenolic acids can be further differentiated into two parent structures: hydroxycinnamic acid and

hydroxybenzoic acid (Khoddami et al., 2013). The distinction within these groups is related to the number and position of hydroxyl groups in the molecule (Pereira et al., 2009) (Figure 2). Often, aldehyde analogues such as vanillin and protocatechuic aldehyde are also referred to as phenolic acids (Robbins, 2003).

Table 1-2 Structures of the important naturally occurring phenolic acids





Hydroxybenzoic Acids Hydroxycinnamic Acids

Phenolic acids	Hydroxybenzoic Acids				Hydroxycinnamic Acids			
	R_1	R_2	R_3	R_4	R_1	R_2	R_3	R_4
Protocatechuic acid	H	OH	OH	H				
Gallic acid	H	OH	OH	OH				
Vanillic acid	H	OCH_3	OH	H				
Syringic acid	H	OCH_3	OH	OCH_3				
Caffeic acid					H	OH	OH	H
Cinnamic acid					H	H	H	H

Table 1-2 continued

Phenolic acids	Hydroxybenzoic Acids				Hydroxycinnamic Acids			
	R_1	R_2	R_3	R_4	R_1	R_2	R_3	R_4
Ferulic acid					H	OCH_3	OH	H
Sinapic acid					H	OCH_3	OH	OCH_3

1.6 Extraction of Phenolic Compounds

In general, the extraction of phenolic compounds from natural sources is challenging due to several factors including chemical diversity of the compounds, uneven distribution within the cell matrix, and interference from other components within the plant. However, determining optimal extraction conditions is key in the recovery of phenolic compounds of interest (Dai & Mumper, 2010). In Chaga, the structural diversity of its components and the presence of interfering substances such as terpenes and carbohydrates, results in large variability of the chemical properties including different biomolecules polarity and hydrogen bonding, which can greatly influence the extraction yields and biological activities of the phenolic extracts. Prior work has indicated that the efficiency of an extraction method and resulting bioactivity of phenolic compounds is strongly dependent on specific extraction conditions such as methodology, solvent type, and extraction temperature. Published data has shown varying levels of total phenolic content from Chaga depending on the extraction method and conditions as well. For example, Zheng et al. investigated solvent effects on phenolic metabolites and antioxidant activities of Chaga extracts using a heated reflux system. The study showed that polar solvents (ethyl acetate, acetone, ethanol, and water) had a higher extraction rate

for PC compared to extractions using non-polar solvents. The authors also reported enhanced antioxidant activities following the increase in solvent polarity. For instance, the scavenging for superoxide anion, DPPH, and hydroxyl radicals reached 71.48, 357.73 and 52.18%/mg, respectively, via ethyl acetate extraction (Zheng et al., 2011a). However, under ultrasonic extraction conditions, an additional report deemed aqueous acetone (70% v/v) as the most efficient solvent for extracting total phenolic compounds in Chaga (Zhao et al., 2013). In addition to solvent type, temperature of the extraction protocol needs to be considered when extracting phenolic compounds. Relatively high temperatures are reported to increase extraction efficiency by decreasing viscosity of the solvent. This leads to better diffusion of the solvent through the cell walls which ultimately increases phenolic compounds solubility in the solvent (Ju et al., 2010; Seo & Lee, 2010). However, there are upper limits to this observation, with extremely high temperatures causing degradation of phenolic compounds (Lindquist & Yang, 2011).

1.6.1 Conventional Extraction and Advanced Extraction Methods

Different extraction methods have been used to recover phenolic compounds from Chaga. Conventionally, a large amount of organic solvent such as methanol, ethanol, acetone, ethyl acetate, or their mixture with water, either at room temperature, or at the boiling point of the chosen solvent(s) is used. However, the major concern with these methods are long extraction times, the large amount of solvent required to execute the procedure, potential negative environmental impacts, as well as decomposition of thermo-labile compounds. Therefore, advanced "green" extraction methods have been designed to reduce energy consumption and allow the use of alternative solvents and/or reduce organic solvent consumption, while producing safe and high-quality extracts (Axelsson et al., 2012; Chemat et al., 2012; Cvjetko et al., 2018). Some of these

technologies such as supercritical fluid extraction (SWE), microwave-assisted extraction (MAE), ultrasound extraction (UAE) and accelerated solvent extraction (ASE) have previously been applied to the extraction of phenolic compounds from a variety of natural sources (Andrade et al., 2012;Şahin & Şamli, 2013; Tripodo et al., 2018; Švarc-Gajić et al., 2013). Zhao et al has applied aqueous two-phase extraction (ATPE) associated with ultrasonic extraction to extract these compounds from Chaga, specifically (Zhao et al., 2013). ATPE technique depends on the selective separation of the compounds of interest from the sample matrix into two separate phases. Ultrasonic extraction on the other hand, breaks the cell wall structure, which allows for a better diffusion rate, improving the solubility of the analyte in the extraction media. Applying the combined methods to Chaga derived phenolic compound extraction, previous literature has reported increased yields in phenolics (37.8 mg gallic /g dw compared to 29.0 mg gallic/ g) obtained by traditional ultrasonic extraction. Additionally, the anti-oxidative activities of the extract obtained by the combined methods have been shown to enhance scavenging superoxide anions SOD, hydroxyl radicals, and DPPH radical isolation. Microwave assisted extraction (MAE) compared to the traditional aqueous Chaga extraction (maceration) methods, also increased melanin output and extracted antioxidative properties. It was found that the melanin extraction efficiency and antioxidative properties were equal to or higher than those of the melanin obtained by the classic extraction method. Further, the MAE reduced the extraction time by 2–3.5 times that of the traditional method (Nitriles 1975; Parfenov et al., 2019). Seo & Lee highlighted that the use of a high temperature during Subcritical Water Extraction (SWE) of Chaga-derived phenolic compounds resulted in 30 times more phenolic content than that obtained using a lower extraction temperature. The researchers found the highest total phenolic content (TPC) (10.72 mg/mL) was obtained in SWE extracts produced at 250°C for 30 minutes,

compared to TPC (0.61 mg/mL) produced at 50°C for 10 minutes. The authors also suggested utilizing SWE as a tool to increase the antioxidant activities of Chaga extracts (Seo & Lee 2010). As suggested, phenolic levels within these fungal extracts are largely dependent on the extraction methods.

1.6.2 Acid- Base Hydrolysis

In general, phenolic acids occur as free, soluble conjugates (e.g., glycosides and esters of fatty acids) and insoluble-bound forms (Naczk & Shahidi, 2004). These different forms of phenolic acids require specific extraction conditions considering the chemical nature of these acids in terms of stability and susceptibility to degradation (Mattila & Kumpulainen, 2002). Aqueous organic solvents such as methanol, acetone, and/or water, sometimes with a small portion of acetic acid, have been used to extract soluble phenolic acids (free, soluble esters, and soluble glycosides) (Escarpa & Gonzlez, 2001; Mattila & Kumpulainen, 2002). However, organic solvents cannot extract the bound forms of phenolic acids. These phenolics are coupled to the cell wall components through ester, ether, and glycosidic bonds (Escarpa & Gonzlez, 2001; Luthria, 2012; Mattila & Kumpulainen, 2002; Nardini & Ghiselli, 2004). Base and/or acid hydrolysis are typically utilized to cleave the ester linkage to the cell walls and release the bound phenolic acids (Mattila & Kumpulainen, 2002). Enzymatic treatments (mainly pectinases, amylases, and cellulases) have been reported as being a less prevalent technique to release phenolic acids from their corresponding conjugates (Robbins & Bean, 2004) Generally, acid hydrolysis involves treating the sample with HCl ranging from 1 to 4N, either at room temperature or at high extraction temperatures; while in base hydrolysis procedures, samples are treated with NaOH ranging from 2 to 10N for a few hours or overnight at room temperature (Nardini & Ghiselli, 2004). Some studies have adopted a sequential

extraction regime to systematically release the phenolic acids from their respective forms. In this extraction system, the sample is first subjected to organic solvent or acidified organic solvent to extract soluble phenolic acids. The base hydrolysis is then utilized to release the bound phenolic acids, followed by acid hydrolysis to liberate the bound phenolics that have not been hydrolyzed during the base hydrolysis process (Li et al., 2020; Ross et al., 2009).

1.6.3 Accelerated Solvent Extraction (ASE)

Many modern techniques such as accelerated solvent extraction (ASE) have been developed to overcome the previously discussed phenolic extraction challenges. The most notable benefits of ASE include increases in extraction efficiency, extract selectivity, and procedure reproducibility. Specifically, using elevated pressure and temperatures for a relatively short duration, results in successful degradation of the cell membranes, which considerably reduces the extraction time and increases product yields. The application of high pressure (>1000 psi) allows solvents to be heated to temperatures higher than their boiling point, which disrupts the cell wall structure, decreases liquid solvent viscosity, and accelerates diffusion through membranes; thus, allowing better penetration into the matrix and further improving the extraction procedure (Ameer et al., 2017; Mustafa & Turner, 2011).

Figure 1-2 Accelerated solvent extraction unit

In the literature, accelerated solvent extraction ASE has been described to improve the extraction of phenolic compounds in cereals and grains. The phenolic compounds within these mediums are typically bound to the cell wall components, presenting considerable challenges in their extraction. Barros et al evaluated the extraction efficiency of phenolic compounds from sorghum bran and found that using ASE compared to conventional methods, significantly improved extraction of phenolic compounds from the product (Barros et al., 2013). Gomes et al optimized these phenolic compound extraction conditions (including temperature, solvents, extraction time, and number of extractions) to primarily isolate flavonoids, from species of Passiflora. The

researchers' methodology employed ASE with a Box-Behnken design. Compared to traditional extraction approaches, ASE results in substantial decreases in solvent consumption and extraction time Gomes et al. (2017. This is because the technique can be automated in an inert atmosphere, which results in minimal phytochemical degradation due to limited extraction times. Further, ASE allows the operator to better control the extraction parameters including temperature, static extraction time, and the number of extraction cycles, further enhancing the method efficiency (Ameer et al., 2017; Mustafa & Turner, 2011).

ASE relies on the use of elevated pressure (500-2000 psi) and temperature (40-200 °C) for a relatively short time to accelerate the rate of extraction. Pressure allows the extraction cell to be filled faster, forcing liquid into the pores and maintaining the liquid state of extraction solvents, even at high temperatures. This elevated temperature results in an increase in the solvation ability of the solvent due to a decrease in both its' viscosity and surface tension. The result is an accelerated diffusion rate and mass transfer of the analyte into the solvent; thereby improving the recovery of compounds of interest (Ameer et al., 2017; Carabias-Martínez et al., 2005; Wang & Weller, 2006).

1.6.3.1 Response Surface Methodology RSM

The response surface methodology (RSM) has been at the forefront of recent extraction optimization research. RSM has been used to develop and improve process parameters among various food, biology, and chemistry applications (Ferreira et al., 2007). RSM is a combined mathematical and statistical tool that facilitates a multifaceted evaluation of the impact of several parameters and their interactions relative to the desired response. This technique simultaneously optimizes experimental conditions, while reducing the required number of experimental trials (Bezerra et al., 2008; Gunst,

Myers, & Montgomery, 1996). This methodology is widely used to overcome the limitations of a single factor approach, in which only one variable can be analyzed with respect to all other factors remaining constant. As suggested, the single factor approach is time-consuming, labor-intensive, and lacks critical data on factor interaction outcomes. Thus, employment of RSM can aid in ensuring optimal processing or extraction conditions (Bezerra et al., 2008; Liyana-Pathirana & Shahidi, 2005).

1.7 Identification and Quantification of Phenolic Compounds

Several techniques have been used for the identification and quantification of Chaga-derived phenolic compounds. The Folin-Ciocalteu (FC) assay and high performance liquid chromatography (HPLC) combined with various detectors are the most frequently applied methods (Table 3 and Table 4). Other chromatographic approaches such as gas chromatography (GC) and ultra-fast liquid chromatography (UFLC) have also been reported. Ju et al employed GC in combination with a mass spectrometer detector (MS) to identify and quantify 2,5-dihydroxyterephthalic acid, a low-level phenolic compound, previously reported to be present in Chaga (Ju et al., 2010). This proposed procedure boasts higher sensitivity and selectivity to identify compounds present in Chaga at low concentrations. Similarly, Hwang et al utilized the same GC-MS method to quantify minor levels of vanillic acid and 2,5-dihydroxyterephthalic acid in Chaga under various extraction conditions (Hwang et al., 2019). In addition to these compounds, hispin analogs and melanins have also been detected in the diverse range of phenolic metabolites that are present in the complex Chaga matrix (Zheng et al., 2009b). Such compounds are difficult to identify using chromatographic approaches; establishing suitable extraction conditions and/or subsequent metabolite fractionation is required prior to chromatographic analysis.

Further, the need of pure reference standards, many of which are unstable and/or not available, is essential for accurate quantification of these compounds (Olmo-Cunillera et al., 2020).

Unlike the previously described procedures, nuclear magnetic resonance (NMR) spectroscopy is a potent tool used for both qualitative and quantitative analysis of phenolic compounds in Chaga without extraction or derivatization. NMR spectroscopy is known to be non-destructive, non-selective, and capable of acquiring a comprehensive range of organic metabolite profiles. Previous studies have examined the NMR profiles of phenolic compounds from cultured mycelia of Chaga grown under different light conditions (Olmo-Cunillera et al., 2020; Zheng et al., 2009a; Zheng et al., 2009b), conducted NMR- spectroscopy analysis to compare the phenolic profiles of mycelia exposed to fungal elicitor from cultures grown under normal physiological conditions.

1.7.1 Folin-Ciocalteu Assay

Folin–Ciocalteu assay is widely used to quantify the total phenolic content from different naturally derived extracts. The FC is a calorimetric method based on the oxidation/reduction reaction. In this assay, the phenolic group becomes oxidized while the metal ion is reduced. When conducted in an alkaline medium, electrons from the phenolic compounds are transferred to the FC reagent, forming a blue chromophore that can be read spectrophotometrically. This newly formed blue coloration absorbs UV-vis radiation in wavelength range of 700- 760 nm(Cicco et al., 2009). Generally, gallic acid is used as the reference standard compound and results are expressed as gallic acid equivalents (mg/mL). For many years, the FC method was commonly used to measure the phenolic content in natural products because it is simple, standardized, and the reagent is commercially available(Magalhães et al., 2010;Ramirez-Sanchez et al., 2010).

Nevertheless, there are limitations to this assay, including lack of specificity. This is because other products of oxidation can interfere, causing over-estimation of the polyphenol content within the sample. Substances such as sugars, aromatic amines, organic acids and bases can react with the FC reagent (Ramirez-Sanchez et al., 2010).

1.7.2 High Performance Liquid Chromatography (HPLC) Method Development

A combination of water and organic solvents such as methanol and acetonitrile are typically used for the reverse phase HPLC separation of phenolic compounds. For satisfactory chromatographic resolution and peak shapes of phenolics, the pH of the mobile phase should be maintained at the range (2-4) to avoid the ionization of phenolic compounds (Michalkiewicz et al., 2008). Formic acid, acetic acid, and phosphoric acid have been utilized most often for MP acidification. Generally, acetonitrile and acidified water are the dominant mobile phases utilized in HPLC quantification of phenolic compounds extracted from Chaga. Previous studies have shown that the increase in the pH of the mobile phase dramatically reduces the retention of phenolic acids. This is because phenolic acids are weak acids and exist primarily in the protonated form at a pH below their pKa, so in order to increase the retention capacity of phenolic acids, the pH of the eluent should be lower than their pKa (Michalkiewicz et al.,2008 ;Joseph & Palasota, 2001). The choice of a stationary phase is key for phenolic compound separation. Typically, C18 or reversed phase (RP-C18) column 1.8-4.6mm ID and 2.0- 5 μm particle size has been used for phenolics separation in Chaga. It has been reported that most HPLC assays of phenolic compounds are carried out at ambient column temperature and recently, however, higher temperatures have also been reported (Khoddami et al., 2013). HPLC elution time to detect phenolic compounds in Chaga have ranged from 35 to 75 min. phenolic compounds in Chaga are detected using UV-VIS, PDA coupled with

fluorescence, Mass spectrometric (MS), and electrospray ionization mass spectrometry (ESI-MS). Table 1- 3 presents some of the HPLC procedures that have been used to identify and quantify phenolic compounds in Chaga.

Table 1-3 HPLC conditions to identify phenolic compounds in Chaga

Chaga	Phenolic Compounds	Column/Detector	Solvent/flow rate	Temperature C/Time min	Reference
Wild	gallic acid, protocatechuic acid, protocatechuic aldehyde, caffeic acid, syringic acid	Zorbax Eclipse Plus C18 (250 × 4.6mm × 5 μm) column/DAD	A: water containing 0.1% formic acid; B: acetonitrile containing 0.1% formic acid; Elution profile: 0–6 min, 8–12% B; 6–15 min, 12–17.4% B; 15–27 min, 17.4–27% B; 27–27.1 min, 27-8% B; and 27.1–35 min, 8% B/Flow rate:1 mL/min: Injection volume: 20μL	NM/35	(Hwang et al., 2019)
Wild	2,5-Dihydroxyterephthalic and vanillic acids	U-VDSpher PUR C18-E (100 × 2.0 mm × 1.8 μm) column/ ESI-MS	A: water containing 0.1% formic acid; B: acetonitrile containing 0.1% formic acid; Elution profile: 0–6 min, 8–12% B; 6–15 min, 12–17.4% B; 15–27 min, 17.4–27% B; 27–27.1 min, 27-8% B; and 27.1–35 min, 8% B/Flow rate:1 mL/min: Injection volume: 20μL	NM/35	(Hwang et al., 2019)

Table 1-3 continued

Chaga	Phenolic Compounds	Column/Detector	Solvent/flow rate	Temperature C/Time min	Reference
Wild	Protocatechuic acid, vanillic acid, syringic acid	Synergi Hydro-RP (250 × 4.60 mm × 4 μm) column/PDA	A: water containing 0.1% formic acid; B: acetonitrile; Elution profile: 0-20 min,5-15%B, 20-50 min, 15-30%B, 50-65 min, 30-50%B, 65-75 min, 50-95% B; Flow rate: 1.0 mL/min; Injection volume:10 μL	NM/75	(Ju et al., 2010)
Wild	2,5-dihydroxyterephthalic acid, Protocatechuic acid, vanillic acid, syringic acid	Synergi Hydro-RP (250 × 4.60 mm × 4 μm) column/ESI-MS	A: water containing 0.1% formic acid; B: acetonitrile; Elution profile: 0-20 min,5-15%B, 20-50 min, 15-30%B, 50-65 min, 30-50%B, 65-75 min, 50-95% B; Flow rate: 1.0 mL/min; Injection volume:10 μL	NM/75	(Ju et al., 2010)

Table 1-3 continued

Chaga	Phenolic Compounds	Column/Detector	Solvent/flow rate	Temperature C/Time min	Reference
Fermented	ferulic acid, gallic acid, epicatechin-3-gallate (ECG), epigallocatechin-3-gallate (EGCG), naringin.	NM/DAD–ESI–MS/MS	A: water containing 0.1% formic acid; B: acetonitrile; Elution profile: 0-20 min, 95-90%A, 20-40 min, 90-5%A	NM/40	(Xu, Zhao, & Shen, 2016b).
Fermented	gallic acid, ferulic acid, epicatechin-3-gallate (ECG), epigallocatechin-3-gallate (EGCG), phelligridin G, davallialactone, inoscavin B	Synergi Hydro-RP (250 × 4.60 mm × 4 μm) column/ DAD–ESI–MS/MS	A: water containing 0.1% formic acid; B: acetonitrile; Elution profile: 0-20 min, 5-10% B, 20-45 min, 10-95%B	NM/45	(Zhu & Xu, 2013)
Wild	gallic acid, protocatechuic acid, p-hydroxybenzoic acids, cinnamic acid	NM/PDA	NM	NM	(Glamočlija et al., 2015)

1.8 Biological Activity of Chaga

1.8.1 Antioxidant Activity

The DPPH assay is based on the reduction of the radical DPPH• by receiving a hydrogen atom from the antioxidant species. The DPPH• is a stable radical, which has an unpaired valence electron at one bridge nitrogen atom.

As an antioxidant source, Chaga has been previously reported to have the strongest antioxidant activity among other fungi in both superoxide and hydroxyl radical

scavenging activities (Nakajima et al., 2007). Chaga contains an abundance of polyphenols and natural black pigments known as melanin, which are responsible for this antioxidant action. Babitskaia et al investigated the melanin complex production of *Inonotus obliquus* in fermented conditions. The researchers found that copper ions (0.008%), pyrocatechol (1.0 mM), and tyrosine (20.0) stimulated the formation of this complex. As a result of its formation, melanin in *Inonotus obliquus* demonstrates both high antioxidant and genoprotective activities for the fungus (Babitskaia et al., 2000). Hu et al similarly tested the antioxidant activity of ethanol crude and hot water (50°C, 70 °C, and 80 °C) extracts of *Inonotus obliquus*. The total antioxidant capacity was measured by superoxide anion scavenging activity (SOD) and DPPH assays. The results from this work showed that the ethanol extract exhibited the maximum SOD-like activity, whereas the hot water extract (70 °C) exhibited the maximum DPPH radical-scavenging activity (Hu et al., 2009). Cui et al demonstrated that the polyphenolic extract, although dose-dependent, was most effective in scavenging superoxide radicals, followed by the extracts containing triterpenoids and steroids (Cui et al., 2005). The polysaccharide extracts on the other hand were entirely inactive. Further, the data from this study suggest that there are several direct human health benefits attributed to Chaga extract consumption. More specifically, the polyphenolic extract efficiently may protect human keratinocytes from oxidative stress. Yun et al also showed that the antioxidant properties of Chaga could have a tremendous benefit on the human aging process. Particularly, conditions such as enhanced apoptosis and elevated levels of Reactive Oxygen Species (ROS), which are caused by direct cell exposure (several types) to various oxidants (i.e. hydrogen peroxide or ultraviolet (UV) radiation) play a significant role in oxidative stress and the aging process. This study determined that a pretreatment with Chaga extract scavenged intracellular ROS and prevented lipid peroxidation in H_2O_2-treated human

fibroblast. An additional finding was that MMP-1 and MMP-9 activities in the fibroblasts were inhibited, leading to increases in collagen synthesis. A third benefit that the researchers observed was that the deleterious effects of UV exposure, such as skin thickening, and wrinkle formation was impeded on a hairless mice model. The oxidative stress in PC12 cells after H2O2 toxicity was suppressed by Chaga extract as well (Yun et al., 2011).

1.8.2 Anti-Inflammatory

Inflammation is a complex biological response of body tissues elicited by harmful physical, chemical, and biological stimuli such as chemical toxins and pathogens (Bak et al., 2013). The inflammatory response is regulated by a cascade of inflammatory mediators and growth factors that are produced by activated macrophages. Macrophages are important cells that play a key role in the immune system and are associated with inflammatory diseases. Activated macrophages produce various cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), as well as other inflammatory mediators including the nuclear factor kappa-B, nitric oxide (NO), and prostaglandin which primarily serves to protect the host. However, excessive uncontrolled production of these defense molecules can lead to severe inflammation and tissue damage (Dai et al., 2019; Robbins et al., 2016).

The production of pro-inflammatory cytokines such as the nuclear factor kappa-B NF- κB is an indication of the inflammation reaction. NF- κB is a protein complex that is implicated in the inflammation process of both macrophages and lymphocytes. It also regulates the expression of genes involved in cellular proliferation and adhesion. The activation of NF-κB has been reported to play a key role in the induction of the transcriptions of multiple pro-inflammatory mediators, including tumor necrosis factor α

(TNF-α) and interleukin-1β (IL-1β). During normal physiological conditions, NF-KB is sequestered in the cytosol of the unstimulated cell as an inactive complex bound to inhibitor kappa B (I κB). Exposure to one of several possible inflammatory agents activates the cell and initiates the inflammation reaction. These stimuli agents can be exogenous (such as lipopolysaccharides, LPS) or endogenous (TFN- α or IFN-) (Mendis et al., 2008). The stimulation of this reaction then causes the enzyme IjB-kinase (IjK) to phosphorylate the inactive complex, and release NF-KB molecules to move freely into the nucleus. This movement then leads to the induction of multiple inflammatory mediators, such as iNOS, COX-2, TNF-α, interleukins (IL)-6 and -8, and others (Cheung et al., 2013).

Nitric oxide (NO) is an important inter and intracellular messenger participating in cellular signaling and a wide range of physiological and pathological functions within many cells and tissues, including the brain, the cardiovascular system, and certain host immune cells (Paige & Jaffrey, 2006). NO is synthesized enzymatically, through the conversion of L-arginine to L-citrulline by nitric oxide synthase (NOS) in the presence of molecular oxygen and NADPH (Alderton et al., 2001). Nitric oxide synthase enzymes are present in three main isoforms which exist in various cell types. nNOS is the first type of NOS that localizes in the central nervous system (neurons). Those which localize in the vascular endothelial cells are termed eNOS, and NOS that are induced in hepatocytes and macrophages after infection are called iNOS. All three isoforms have similar molecular structures; however, each show tissue specific variations in their nitric oxide expression and pathway regulations (Choudhury & Saha, 2012). Both nNOS and eNOS are constitutive (cNOS), and capable of producing suitable amounts of NO that plays a key role in various biological functions in the human body. In contrast, the inducible isoform

iNOS is expressed in response to pro-inflammatory cytokines and bacterial LPS. It has previously been reported that the overproduction of NO due the action of iNOS is a major contributor in inflammatory processes is indicative of the degree of the inflammation (Alderton et al., 2001).

The anti-inflammatory effects of various Chaga extracts which are characterized by high phenolic content have been reported in both LPS induced macrophages and dextran sulfate sodium_(DSS)_induced colitis mice models. Methanol–based Chaga extracts have also been shown to significantly inhibit the productions of NO, PGE_2, and TNF-. This suppresses the expressions of both inducible nitric oxide (NO) synthase (iNOS) and cyclooxygenase-2 (COX-2) via the down-regulation in the binding activity of nuclear factor B (NF-B) in LPS induced macrophage cell systems 2005. Kim et al investigated the effect of the addition of different portions of water to organic solvent on both antioxidant and anti-inflammatory activities of the extract (Kim et al., 2007). Seventy percent aqueous ethanol extracts of chaga performed better than the 100% ethanol extract alternative for both antioxidant and anti-inflammatory activity in macrophage cells. This finding could be attributed to the addition of water to polar solvent, leading to an increase in the efficiency of phenolic compound extraction by increasing their solubility. Van et al reported that all four fractions (triterpenoids, water-soluble polysaccharides, polyphenolic, and low molecular weight polysaccharides) separated from methanol extract were found to significantly reduce NO production in the murine macrophage cell system as well as reduce the level of other inflammatory cytokines depending on dosage (Van et al., 2009). In addition, water Chaga extracts have demonstrated significant anti-inflammatory activity when used against a DSS- induced colitis mice model, due to down-regulation of the expression of inflammatory mediators

(Mishra et al., 2012). Debnath et al similarly demonstrated the in vivo anti-inflammatory activity of ethanol extracts from Chaga grown on germinated brown rice against DSS -induced colitis mice model (Debnath et al., 2012).

CHAPTER 2

HPLC METHOD DEVELOPMENT TO IDENTIFY AND QUANTIFY PHENOLIC ACIDS IN CHAGA

2.1 INTRODUCTION

Epidemiological studies indicate that the consumption of chaga extracts is related to reduction in oxidative DNA damage in human lymphocytes, as well as to diabetes and cancer incidence rates (Geng et al., 2013; Kim et al., 2006; Park et al., 2004). These health benefits have been partially attributed to a wide variety of potential chemopreventive compounds present in Chaga, called phytochemicals, and include antioxidants such as phenolic compounds (Cui et al., 2005; Nakajima et al., 2007). There are large number of scientific investigations in the literature based on the extraction and analysis of different classes of phenolic compounds including phenolic acids (Dai & Mumper, 2010; Luthria & Mukhopadhyay, 2006; Luthria & Pastor-Corrales, 2006; Mattila & Kumpulainen, 2002).

In general, phenolic acids occur as free, soluble conjugates (e.g., glycosides and esters of fatty acids) and insoluble-bound forms (Naczk & Shahidi, 2004). These different forms of phenolic acids require specific extraction conditions considering the chemical nature of these acids in terms of stability and susceptibility to degradation (Mattila & Kumpulainen, 2002). Aqueous organic solvents such as methanol, acetone, and/or water, sometimes with a small portion of acetic acid, have been used to extract soluble phenolic acids (free, soluble esters, and soluble glycosides) (Escarpa & Gonzlez, 2001; Mattila & Kumpulainen, 2002). However, organic solvents cannot extract the bound forms of phenolic acids. These phenolics are coupled to the cell wall components through ester, ether, and glycosidic bonds (Luthria, 2012; Escarpa & Gonzlez, 2001; Mattila &

Kumpulainen, 2002; Nardini & Ghiselli, 2004). Base and/or acid hydrolysis are typically utilized to cleave the ester linkage to the cell walls and release the bound phenolic acids (Mattila & Kumpulainen, 2002). Enzymatic treatments (mainly pectinases, amylases, and cellulases) have been reported as being a less prevalent technique to release phenolic acids from their corresponding conjugates (Robbins & Bean, 2004). Generally, acid hydrolysis involves treating the sample with HCl ranging from 1 to 4N, either at room temperature or at high extraction temperatures, while in base hydrolysis procedures, samples are treated with NaOH ranging from 2 to 10N for a few hours or overnight at room temperature (Nardini et al., 2002). Some studies have adopted a sequential extraction regime to systematically release the phenolic acids from their respective forms. In this extraction system, the sample is first subjected to organic solvent or acidified organic solvent to extract soluble phenolic acids. The base hydrolysis is then utilized to release the bound phenolic acids, followed by acid hydrolysis to liberate the bound phenolics that have not been hydrolyzed during the base hydrolysis process (Li et al., 2020; Ross et al., 2009).

Reported extraction methods of phenolic acids from Chaga are still incomplete. One reason for this may be the lack of information regarding the total content of phenolic acids in Chaga. Most of the extraction procedures described in the literature describe the content of the free forms of phenolic acids of these extracts. Specific knowledge pertaining to the content of bound forms is scarce or missing. Common extraction solvents used to separate free phenolic acids from Chaga include methanol, ethanol, acetone, ethyl acetate and/or water (Nakajima et al., 2007; Van et al., 2009). Previous work has employed a high temperature during the extraction of phenolic compounds from Chaga as an attempt to liberate these compounds from the cell wall components (Seo &

Lee, 2010). Phenolic extracts produced by utilizing thermal processes such as steam treatment or high pressure and temperature have been found to contain a higher content of phenolic acids than those extracts produced without such treatments (Ju et al., 2010; Hawng et al, 2019). From what is stated above, it can be hypothesized that some phenolic acids in Chaga are bound to the cell wall structure, and thus, a hydrolysis procedure is required in order to liberate these bound acids from their respective forms.

A major problem associated with the hydrolysis procedures is the loss of several phenolic acids, particularly dihydroxy-cinnamic acids such as caffeic acid due to the oxidation of phenolic acids (Krygier et al., 1982). However, previous work of Nardini et al has introduced the addition of ascorbic acid AA, a powerful antioxidant, and ethylenediaminetetraacetic acid (EDTA), a metal chelator, to prevent the degradation of phenolic acids during the hydrolysis procedures (Nardini et al.,2002). No work has examined the addition of AA and EDTA during the extraction of phenolic acids from Chaga.

Several methods have been developed for the quantification of phenolic acids from different sources, including the Folin–Ciocalteu assay and chromatographic approaches (HPLC-UV, GC-MS, LC-MS) (Acosta-Estrada et al., 2014; Robbins, 2003; Shahidi and Yeo, 2016). The Folin–Ciocalteu assay, which provides an estimate of total phenolic content by quantifying the antioxidant capacity, is not specific to phenolic groups and suffers from interference by ascorbic acid and reducing sugars (Stalikas, 2007). Chromatographic methods occupy a leading position in the separation and quantification of individual phenolic acids (Stalikas, 2007). Therefore, there is a need for development of a chromatographic HPLC method that can separate and simultaneously measure the prominent phenolic acids in Chaga in a single run. The aims of this study

were thus (1) to examine the effect of acid and base hydrolysis on the extractability of total phenolic acids from Chaga and (2) to develop a suitable HPLC method for simultaneous identification and quantification of five phenolic acids in Chaga.

2.2 MATERIALS AND METHODS

2.2.1 Fungal Material

Chaga sclerotia were collected from a yellow birch (Betula alleghaniensis) from a forest in Maine, USA. Samples were freeze-dried (Model 7754511, Labconco Corporation, Kansas City, Missouri, USA), then ground using an electrical grinder (Nutribullet, model-NBR-1201M, Los Angeles, USA). The ground powder was then passed through a 20-mesh (0.84 mm) sieve, and only particles with a diameter smaller than 0.84 mm (20-mesh) were collected and pooled. All material was stored in a -20°C freezer until subsequent extraction preparation.

2.2.2 Chemicals

Phenolic acid standards—3,4-dihydroxybenzoic acid (PA), caffeic acid (CA), syringic acid (SA), and 3,4-dihydroxybenzaldehyde (PCA)—were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol, ethyl acetate, petroleum ether, ethylenediaminetetraacetic acid (EDTA), ascorbic acid (AA), ethyl acetate, sodium carbonate, vanillic acid (VA), hydrochloric acid, acetic acid, and formic acid were provided by Fisher Chemicals (Fair Lawn, NJ, USA). Ultrapure water was obtained from a Millipore water system (EMD Millipore, Billerica, MA, USA). All reagents and organic solvents were HPLC or analytical grade.

2.2.3 Instrumentation

Chromatography of phenolic acids was carried out on an HPLC system (Hewlett Packard model 1100) consisting of a pump, a vacuum degasser and Diode-Array Detector (Agilent Technologies, Paolo Alto, CA, USA). Chromatograms were recorded and evaluated by the software Agilent 3D Chemstation, version B.04.01 (Agilent Technologies).

2.2.4 Chromatographic Conditions

The chromatographic separation of the examined analytes was carried out on a reverse-phase Hypersil GOLD aQ C18 column (150 mm × 4.6 mm i.d., 3 μm particle size) (Thermo Scientific, USA) preceded by a Hypersil GOLD guard column (Thermo 4 × 3.0 mm). The column and guard column were held at 30°C, and the flow rate was set at 1 mL/min. The mobile phase of the HPLC system consisted of (A) methanol and (B) a 0.1% V/V formic acid/ultrapure-water, pH = 2.8. The gradient was linear at a flow rate of 1 mL/min The solvent gradient in volume ratios was as follows: 95% to 50% B for 16 min; the solvent gradient was reduced to 95% B at 18 min, and maintained at 95% for 2 min; the latter was followed by washing with methanol and re-equilibration of the column for 10 min. Total acquisition time was 30 min. The wavelength used for the quantification of the phenolic acids with the diode array detector DAD was 280 nm for all the phenolic acids except for caffeic acid, which was monitored at 329 nm. Identification of the phenolic acids was achieved by comparing retention times and UV spectra of the unknowns with the standards.

2.2.5 Preparation of Standards

All the identified phenolic acids were quantified with external standards.

Stock solutions of the phenolic acids were initially prepared at 1 mg/mL in an appropriate volume of methanol/water (75:25, v/v). Working standard solutions were obtained by appropriate dilution of the stock solutions with methanol/water (75:25, v/v) in the concentration range of 0.1–100 µg/mL. All solutions were kept at -20°C for further analysis. The standard solutions were stable for at least three months (Nnane & Damani, 2002).

2.2.6 Methods of extraction and hydrolysis

Following the general procedure of Ross et al. (2009), three different methods of extractions combined with hydrolysis regimes were tested.Method 1 employed acidified methanol to extract free phenolic acids; Method 2 used a pure base hydrolysis with NaOH at different concentrations with and without AA (1%) and EDTA (10 mM); and Method 3 was accomplished with a pure acid hydrolysis, with HCl at different concentrations - with and without the presence of AA (1%) and EDTA (10 mM). Experimental steps after hydrolysis were performed as prescribed by Luthria & Pastor-Corrales (2006) and Mattila & Kumpulainen (2002).

2.2.6.1 Extraction of free phenolic acids

Extraction was achieved by sonication of 0.5 g of ground Chaga sample in 10 mL methanol containing 10% acetic acid for 15 min. After centrifugation at 6,700 rpm (2,000 x g) for 10 min, the supernatant was removed, and extraction was repeated once more in a similar way. The combined extracts were evaporated to dryness under a gentle flow of nitrogen, and the residues were re-dissolved in 200 µL of methanol: water (75:25, v/v). The samples were filtered through a 0.45 µm filter (PTFE) before being analyzed by HPLC.

2.2.6.2 Base hydrolysis

The ground samples of Chaga 0.5 g were hydrolyzed by adding 10 mL of NaOH at each of the following concentrations: 2, 3, 6, and 10N (with or without 10 mM EDTA and 1% AA). The mixture was flushed with nitrogen and allowed to hydrolyze for 30 min at 40–45°C. After 30 min, the sample allowed to cool and 1.4 mL of 6N HCl was added to acidify the reaction mixture. The liberated phenolic acids were extracted with 20 mL of DE/EA (1:1, v/v). The mixture was vortexed for 45 s and centrifuged (eppendrof 5430, Hauppauge, NY) for 10 min at 6,700 g. Centrifuging caused the DE/EA organic phase to separate from the aqueous phase. The DE/EA organic phase containing the phenolic acids liberated from the base hydrolysis was collected by removing the upper organic (supernatant) phase from the bottom aqueous residue phase using a pipette. The DE/EA organic supernatants were combined, and the combined DE/EA was evaporated to dryness under a rotary vacuum. The residue was resuspended in 5 mL methanol: water (75:25, v/v). The samples were then filtered through a 0.45μm filter (PTFE), and were analyzed with HPLC.

2.2.6.3 Acid hydrolysis

The phenolics were extracted as previously described (Krygier et al., 1982), with some modifications. The ground samples of Chaga 0.5 g were hydrolyzed by adding 10 mL of HCl at one of the following concentrations: 1, 2, 3, and 4N (with or without 10 mM EDTA and 1% ascorbic acid). The mixture was incubated for 30 min at room temperature. The experimental steps after hydrolysis were performed as described in section 2.6.1.

2.2.7 Method validation

The method was validated in terms of linearity, accuracy, within-day and between-day precision, and sensitivity based on the limit of detection (LOD) and limit of quantification (LOQ). Linearity was evaluated using linear regression analysis of six-point calibration curves, which were obtained by plotting the peak area versus the concentration of each standard. Linear regression analysis was used to calculate the slope, intercept, and correlation coefficient of each calibration line. The within-day and between-day precisions were evaluated by analyzing Chaga samples fortified at three concentration levels (1, 10, and 25 μg/g). Each solution was measured three times in the same day for within-day precision and three times over four consecutive days for between-day precision. Both precision estimates are expressed as relative standard deviations (RSD), i.e. standard deviation of repeated measurements as a percentage of the mean value. Recovery was used to evaluate the accuracy. There were three different concentrations of standards added to the sample extract method (3N NaOH with AA and EDTA protection) at three concentration levels (1, 10, and 25 μg/g) for each compound examined. The recovery was calculated as follows:

$$Recovery = 100\% \times \left(\frac{amount\ found - original\ amount}{amount\ spiked}\right)$$

The calculations for the (LOD) were based on the standard deviation of y-intercepts of the regression lines (σ) and the slope (S), using the following equation LOD = 3.3 σ/S. (LOQ) were calculated using the equation LOQ = 10 σ/S.

2.2.8 Statistical analysis

All experiments were repeated three times. Data are expressed as mean of the replicates ± standard deviation (SD). The software IBM SPSS Statistics (version 25) was

used to perform statistical analysis of experimental results. A one-way analysis of variance (ANOVA) test was applied to compare means followed by Tukey's test. P values <0.05 were considered statistically significant.

2.3 RESULTS

2.3.1 Extraction of Phenolic Acids

Three different methods of extraction combined with hydrolysis regimes were employed to extract phenolic acids from Chaga. The free phenolic acids were extracted using acidified methanol, while bound phenolic acids were liberated by base hydrolysis and acid hydrolysis, respectively. Figure 2-1 represents the effect of base hydrolysis with NaOH at 1, 2, 3, and 4N with and without AA and EDTA on the extractability of total phenolic acids from chaga. Up to 47.42% higher extractability of total phenolic acids was observed after incubation of chaga with 3N NaOH compared to incubation with 1N NaOH. However, degradation of CA initiated at 2N NaOH and resulted in about 65% and 81% lower CA at 2N and 3N of NaOH, respectively. The results also showed a protective effect for AA and EDTA; higher extractability of total phenolics was achieved after extraction using NaOH at all concentrations with protection (AA and EDTA), in comparison to extracts obtained without protection. For example, the base hydrolysis (3N NaOH with AA and EDTA) resulted in 37% higher total phenolic acids content compared to 3N NaOH without AA and EDTA, and the same trend was observed at the other examined NaOH concentrations (Figure 2-1A).

For the acid hydrolysis extraction, it was observed that increasing the concentration of HCl up to 4N resulted in an increase of total phenolic acids by 31%, but the stability of CA was negatively affected, and extraction resulted in 65% lower CA. The results also showed a protective effect of AA and EDTA when added to HCl

hydrolysis, at all concentrations. The highest extractability of total phenolic acids was achieved after acid hydrolysis (4N HCl with protection) and resulted in 38% higher total phenolic acids content compared to the same concentration without protection (Figure 2-1 B).

The results in Figure 2-1C indicate that the majority of phenolic acids in chaga were extracted from the base hydrolysis (3N NaOH with EDTA and AA) followed by the acid hydrolysis (4N HCl with AA and EDTA), whereas the acidified methanol resulted in extracting the lowest total phenolic acids content. Base hydrolysis liberated nearly 3.5 and 10 times the amount of phenolics as acid hydrolysis and acidified methanol, respectively.

Qualitative and quantitative analyses of individual phenolic compounds of Chaga extracts at different extraction conditions were executed using high-performance liquid chromatography HPLC. Figure 2-2A and Figure.2-2B depict the HPLC chromatograms of the five phenolic acids (PA, PCA, CA, VA, CA, and SA with elution times of 6.2, 7.7, 11.5, 12.7, and 13.7 min, respectively.

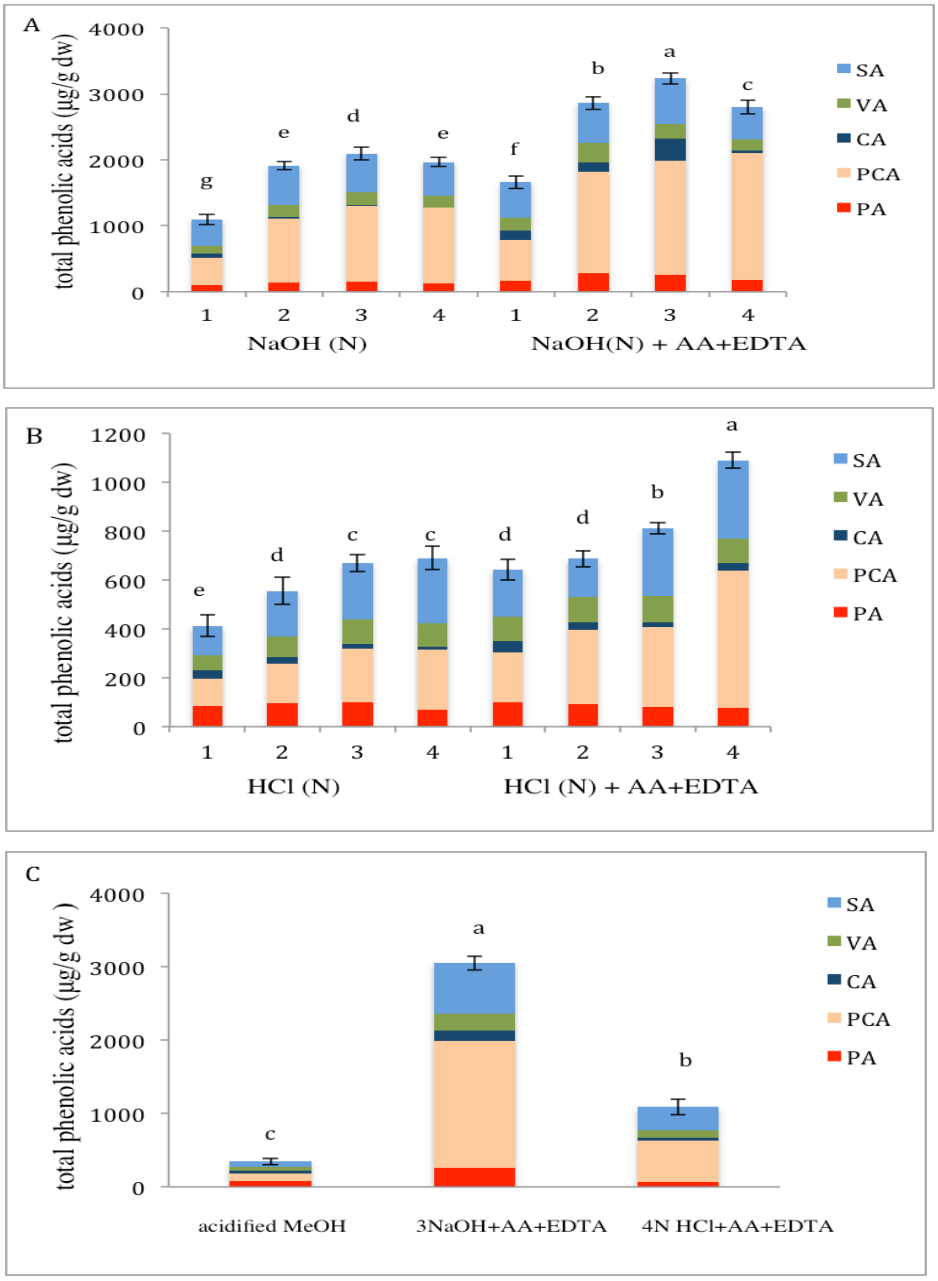


Figure 2-1 Effect of different extraction procedures: (A) base hydrolysis, (B) acid hydrolysis, and (C) acidified methanol and base and acid hydrolysis on the extractability of phenolic acids (µg/gdw) in Chaga. Columns represent means of duplicate samples (n = 3). Different superscripts represent significant differences ($P < 0.05$).

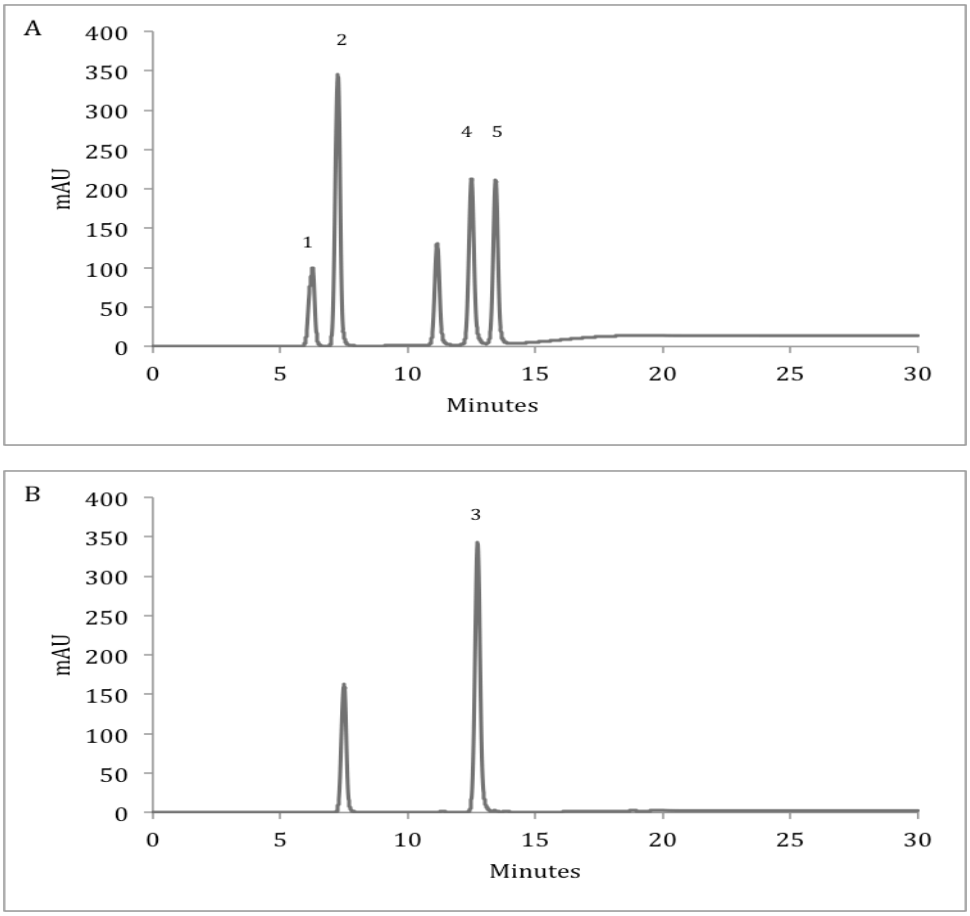


Figure 2-2 Representative chromatograms of phenolic acid standards recorded at (A) 280 nm and (B) 329 nm. 1 = protocateuchuic acid (6.2 min); 2 = protocatechuic aldehyde (7.7 min); 3 = caffeic acid (11.5 min); 4 = vanillic acid (12.7 min), and 5 = syringic acid (13.7 min).

Figure2-3A and Figure2-3B show reprehensive HPLC chromatograms of chaga treated with 0.1 acetic acid 70% (methanol: water) at 280 and 329 nm, respectively. All the examined phenolic compounds were detected, and the UV spectra of these peaks matched the UV spectra of their respective authentic standards. For this condition, all the examined compounds were detected, and the UV spectra of these peaks matched the UV spectra of their respective authentic standards.

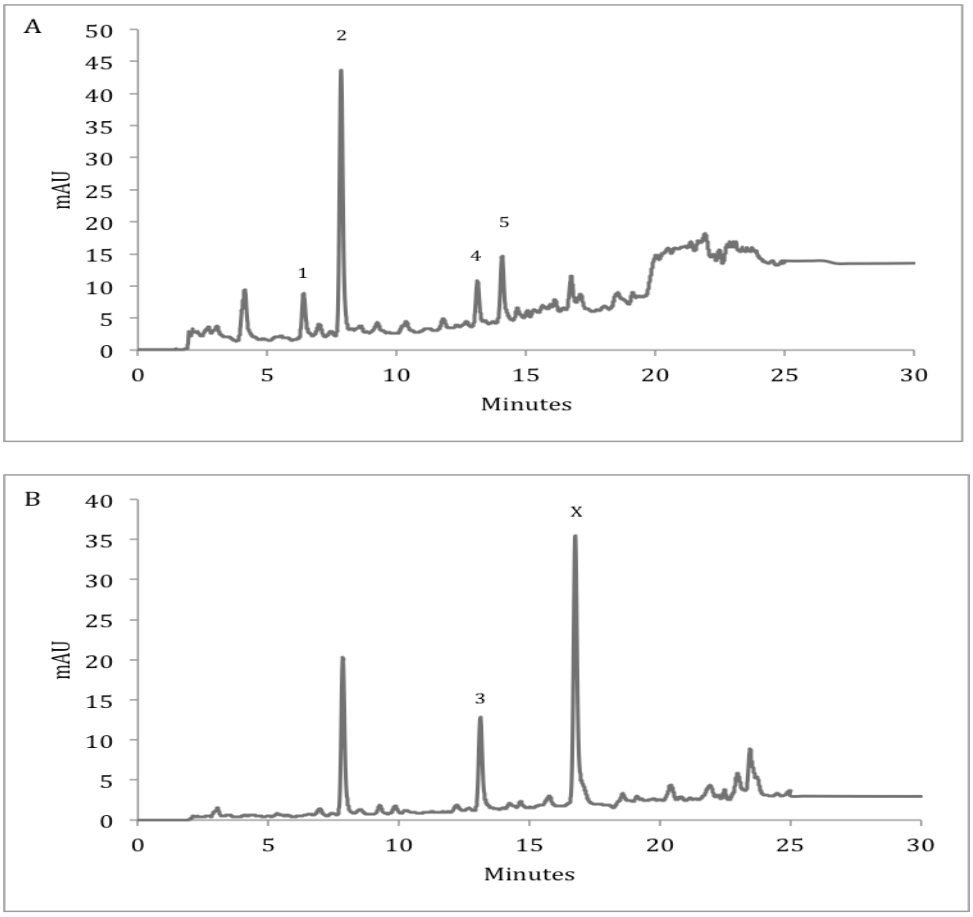


Figure 2-3 Representative chromatogram of extract obtained from Chaga using acidified methanol recorded at (A) 280 nm and (B) 329 nm. 1 = protocatechuic acid (6.2 min); 2 = protocatechuic aldehyde (7.7 min); 3 = caffeic acid (11.5 min); 4 = vanillic acid (12.7 min); and 5 = syringic acid (13.7 min).

Figure 2-4A and Figure 2- 4B show representative chromatograms of the Chaga sclerotia hydrolyzed with 3N NaOH without protectors monitored at 280 and 329 nm, respectively. For this condition, all the examined compounds were detected, except for CA, which was not detected at 329 nm. The UV spectra of these peaks matched the UV spectra of their respective authentic standards. Figure 2-4C and Figure 2-4D show representative chromatograms of the chaga sclerotia hydrolyzed with 3N NaOH with

13.4 mM EDTA and 2% AA monitored at 280 and 329 nm, respectively. For this condition, all the examined phenolic compounds were detected, and the UV spectra of these peaks matched the UV spectra of their respective authentic standards.

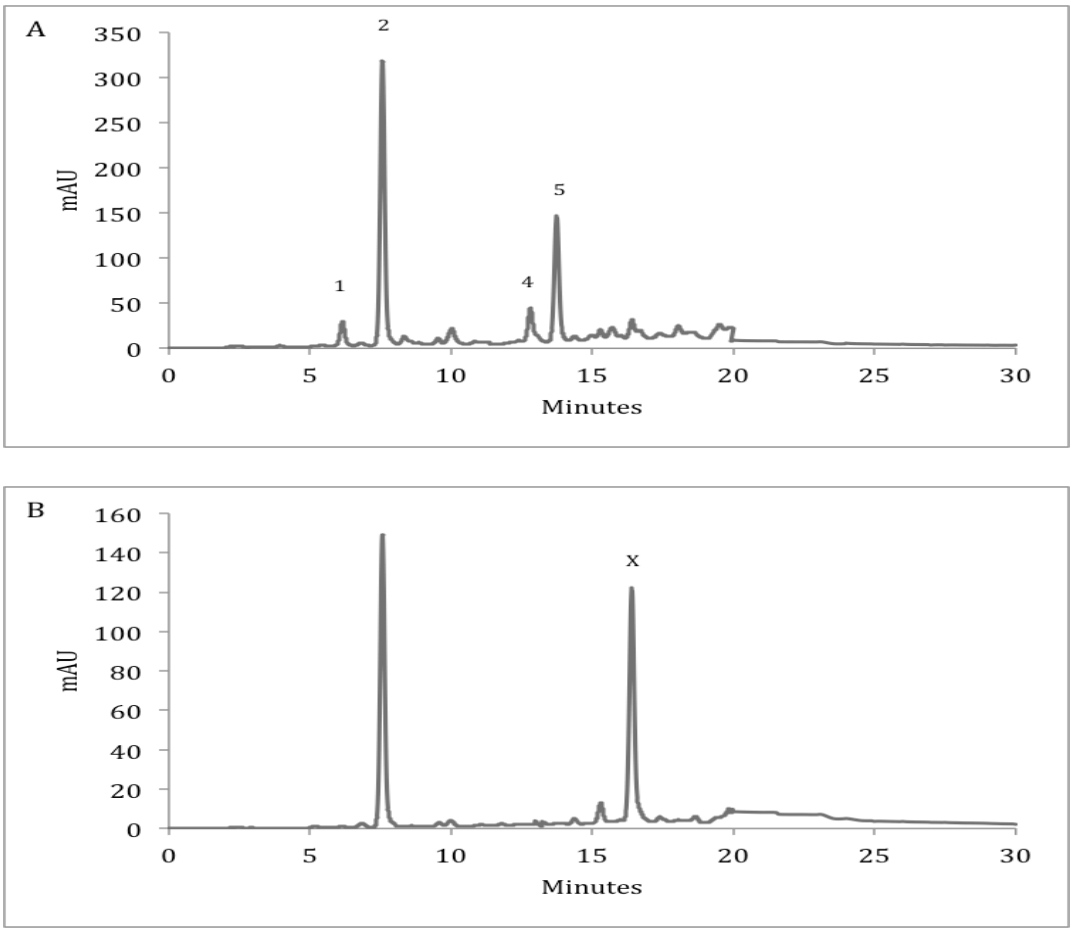


Figure 2-4 Representative chromatogram of extract obtained from Chaga using 3N NaOH hydrolysis recorded at (A) 280 nm and (B) 329 nm. . 1 = protocatuchuic acid (6.2 min); 2 = protocatechuic aldehyde (7.7 min); 3 = caffeic acid (11.5 min); 4 = vanillic acid (12.7 min), and 5 = syringic acid (13.7 min).

Figure 2-5A and Figure 2-5B show representative chromatograms of the Chaga sclerotia hydrolyzed with 6N HCl without protectors monitored at 280 and 329 nm, respectively. For this condition, all the examined compounds and the UV spectra of these peaks matched the UV spectra of their respective authentic standards. Figure2-5C and Figure2-5D representative chromatogram of the Chaga sclerotia hydrolyzed with 3N HCl with 13.4 mM EDTA and 2% AA monitored at 280 and 329 nm, respectively. For this condition, all the examined phenolic compounds were detected, and the UV spectra of these peaks matched the UV spectra of their respective authentic standards.

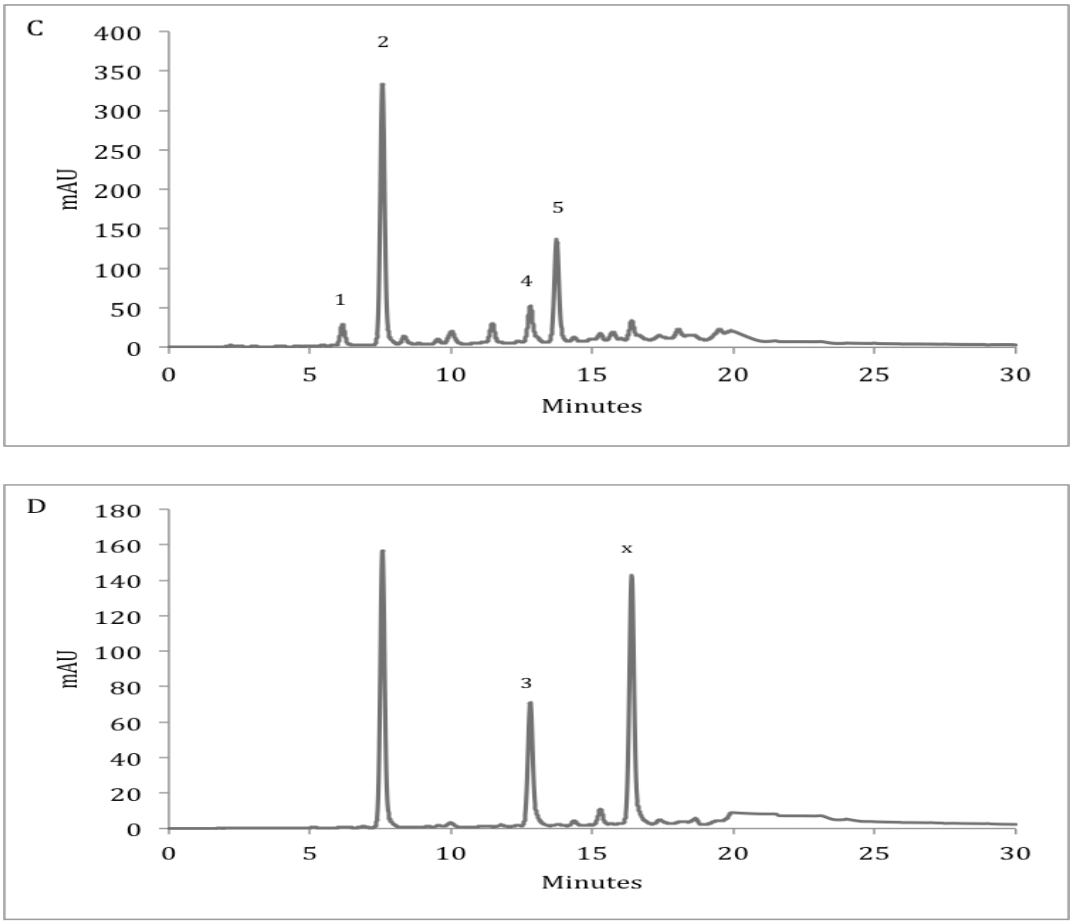


Figure 2-4 Representative chromatogram of extract obtained from Chaga using 3N NaOH hydrolysis recorded at 3N NaOH hydrolysis with 10 mM EDTA and 1% AA recorded at (C) 280 nm and (D) 329 nm. 1 = protocatuchuic acid (6.2 min); 2 = protocatechuic aldehyde (7.7 min); 3 = caffeic acid (11.5 min); 4 = vanillic acid (12.7 min), and 5 = syringic acid (13.7 min).

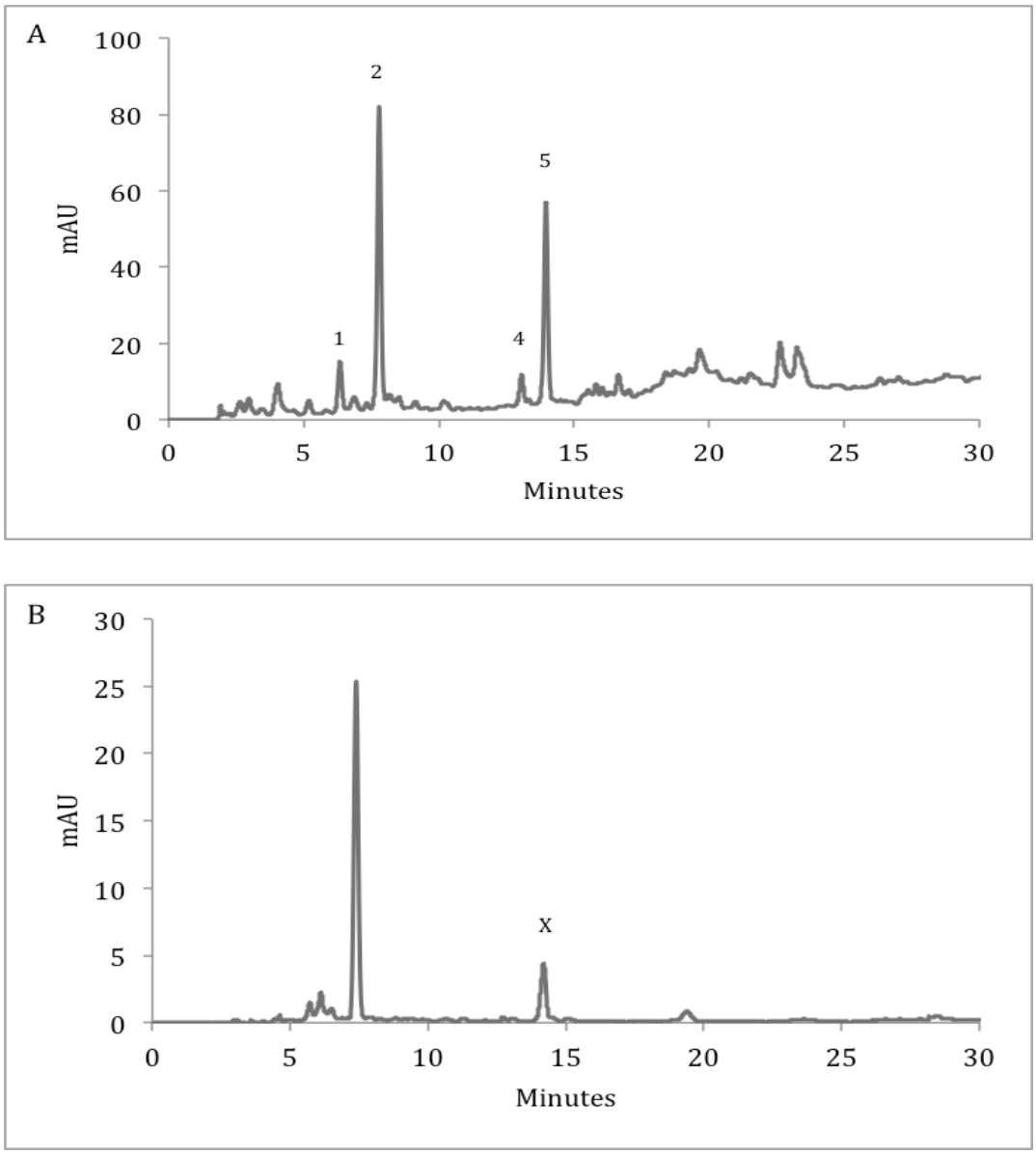
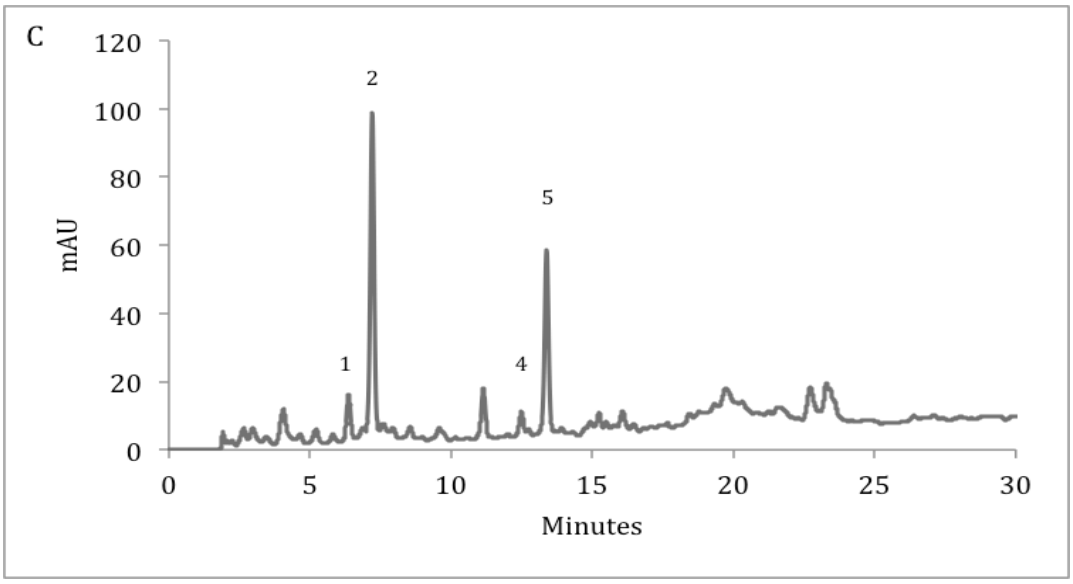


Figure 2-5 Representative chromatogram of extract obtained from Chaga using 4N HCl hydrolysis recorded at (A) 280 nm and (B) 329 nm. 1 = protocatuchuic acid (6.2 min); 2 = protocatechuic aldehyde (7.7 min); 3 = caffeic acid (11.5 min); 4 = vanillic acid (12.7 min), and 5 = syringic acid (13.7 min).



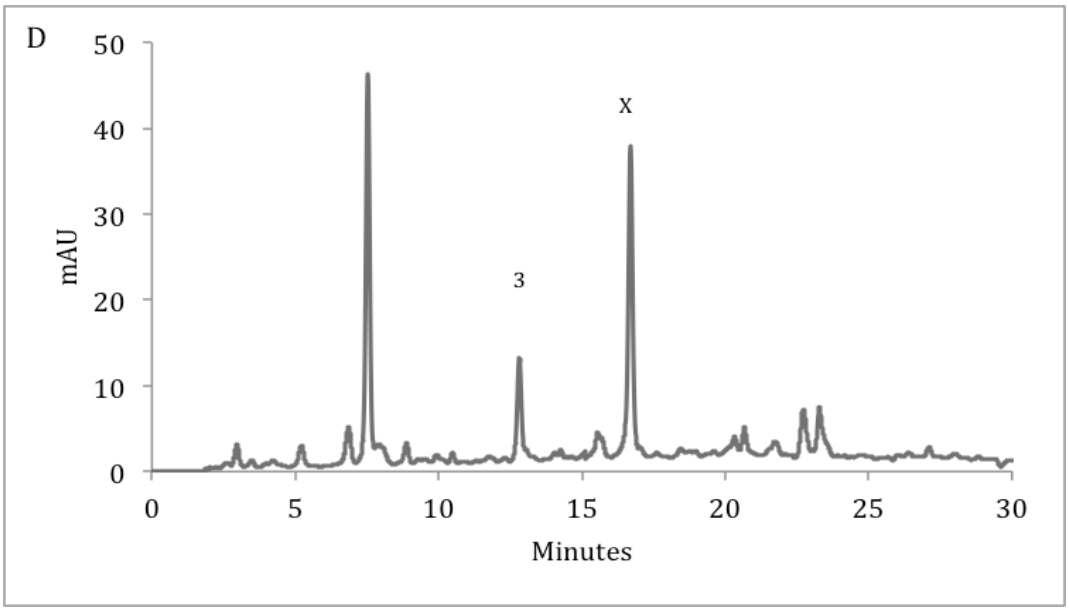


Figure 2.5 Representative chromatogram of extract obtained from Chaga using 4N HCl hydrolysis with 10 mM EDTA and 1% AA recorded at (C) 280 nm and (D) 329 nm. 1 = protocatuchuic acid (6.2 min); 2 = protocatechuic aldehyde (7.7 min); 3 = caffeic acid (11.5 min); 4 = vanillic acid (12.7 min), and 5 = syringic acid (13.7 min).

2.3.2. Method Validation

The method was validated in terms of linearity, accuracy, within-day and between-day precision, and sensitivity based on LOD and LOQ according to the International Conference on Harmonization (ICH,1996/2005) and the Commission Decision (2002/657/EC) guidelines. The calibration curves were constructed with six concentrations, in triplicate, for each phenolic compound, using the external standard method. The results in Table 2-1 show good linear correlation and high sensitivity under these conditions as confirmed by correlation coefficients (R2, 0.994–0.999). The LODs and LOQs determined in the chaga sample ranged between 0.13–0.17 μg/g and 0.39–0.52 μg/g, respectively. Detailed information of these compounds regarding calibration curves, linear ranges, LODs, and LOQs is summarized in Table 2-1.

Table 2-1 Calibration curve, limit of detection (LOD), and limit of quantitation (LOQ) for determination of phenolic acids in a spiked Chaga sample.

Compound	Regression equation	R^2	LOD (μg/g)	LOQ (μg/g)
PA	y = 32,149x - 5.9164	0.994	0.17	0.51
PCA	y = 48,482x - 19.389	0996	0.16	0.48
CA	y = 50,114x - 21.822	0.999	0.13	0.39
VA	y = 32,505x - 16.607	0.999	0.15	0.45
SA	y = 28,432x - 10.508	0.999	0.17	0.52

PA, protocatechuic acid; PCA, protocatechuic aldehyde; CA, caffeic acid; VA, vanillic; and SA, syringic acid.

Precision of the method was assessed by performing within-day precision and between-day precision experiments. As shown in Table 2, the results showed satisfactory within- and between-day precision for the analytical method, with RSD lower than 4.2% for all compounds tested. Accuracy, expressed as % recovery of the five standards, ranged with the base hydrolysis method (3N NaOH with AA and EDTA protection) from 89.1% to 103.3% for within-day analysis and from 85.5% to 103.5% for between-day analysis.

Table 2-2 Within-day and between-day precisions and accuracy data at three concentration levels (1, 10, and 25 μg/g) in Chaga samples.

Compound	Within-day precision (n=3)						Between-day precision (n=3 replicates ×4 days)					
	1μg/g		10μg/g		25μg/g		1μg/g		10μg/g		25μg/g	
	RSD	R	RSD	R	RSD	R	RSD	R	RSD	R	RSD	R
PA	2.6	98.1	2.1	97.7	0.9	98.2	3.8	98.3	3.5	97.2	4.1	96.5
PCA	2.4	99.2	2.5	98.4	1.5	99.2	2.3	97.3	3.2	98.1	3.0	99.1
CA	1.2	89.1	1.3	91.4	0.5	91.1	2.7	91.7	4.0	85.5	3.7	93.1
VA	1.5	95.8	2.9	98.8	3.0	99.8	4.2	99.2	0.8	97.3	4.1	101.3
SA	2.1	101.9	2.3	99.2	2.3	103.3	0.9	103.5	3.3	98.5	4.2	98.5

PA, protocatechuic acid; PCA, protocatechuic aldehyde; CA, caffeic acid; VA, vanillic; and SA, syringic acid. RSD: relative standard deviation, expressed as%, R: recovery rate, expressed as%.

2.4 DISCUSSION

The first aim of this study was to examine the effect of extraction method and hydrolysis on the extractability of total phenolic acids from Chaga, including free, bound, and conjugated forms. Many studies have established that phenolic acids in nature are rarely present in soluble free form and that they most often occur as esters, glycosides, and complexes bound to the cell wall. Organic solvents have been described to separate the soluble phenolic acids, while base and acid hydrolysis is known to liberate the insoluble phenolic acids through breaking their linkages to the cell wall components. Our results show that base hydrolysis (3NaOH with AA and EDTA) is the most effective system for the extraction of phenolic acids from the Chaga matrix followed by acid hydrolysis (4N HCl with AA and EDTA), while acidified aqueous methanol is much less effective. Previous investigations have revealed that base hydrolysis primarily breaks the ester bonds, while acid hydrolysis often breaks the glycosidic bonds (Acosta-Estrada et al., 2014; Ross et al., 2009; Nardini et al., 2002). This suggests that most phenolic acids in Chaga may be present in the bound form, mainly ester-bonded and to a lesser extent glycosidic-bonded to the cell wall of Chaga. It is difficult to compare the results obtained in this study to those of previous studies because of the lack of investigations concerning the forms of phenolic acids in chaga. However, one study has suggested a heat pretreatment as an attempt to liberate bound phenolic acids from Chaga (Ju et al., 2010). The authors revealed that the levels of the identified phenolic acids were significantly increased as a result of the thermal treatment. Other reports employed high temperature and pressure during the extraction of phenolic compounds from Chaga to release these phenolics from the cell wall components (Hawng et al, 2019; Seo & Lee, 2010). The results from our study and the previous studies support the idea that hydrolysis

procedures are required during the extraction of phenolic acids from Chaga to release these acids from their respective conjugates.

The highest total phenolic content was achieved after base hydrolysis (3N NaOH with AA and EDTA). At this concentration, PCA was the dominant phenolic acid in the acid hydrolysates and made up 68.36% of the total identifiable phenolic pool. An increase in the base concentration beyond 3N resulted in lower total phenolic acids content. For example, raising the concentration to 4N NaOH with AA and EDTA resulted in 13.22% lower extractability of total phenolic acids with 83.91% less CA compared to 3N NaOH with AA and EDTA. Ross et al reported higher extractability of phenolic acids after extraction using 10N NaOH compared to 2N NaOH (Ross et al., 2009). However, it should be noted that, in their study, the 10N NaOH was diluted with water before extraction, and therefore, the final concentration was 3.3N NaOH, which is similar to the concentration used in this study.

Although acid hydrolysis was found to increase the total content of phenolic acids, the concentration of CA decreased at a high level of HCl. For example, raising the concentration of HCl up to 4N, even with the presence of AA and EDTA, resulted in the extraction of less CA. This is consistent with previous studies that have reported lower stability of CA under strong acidic conditions (Krygier et al., 1982; Verma et al., 2009). Conversely, acidic conditions favored the extraction of SA, supporting Verma et al who suggested that acidic hydrolysis is the optimum method to liberate some phenolic acids, particularly SA, from wheat grains (Verma et al.,2009). At 4N HCl (with AA and EDTA), PCA was the dominant phenolic acid in the acid hydrolysates and made up 51.26% of the total identifiable phenolic pool. PCA is the predominant phenolic acid in both acid and base hydrolysis conditions.

In terms of the stability of phenolic acids, our study showed that the addition of AA and EDTA during acid and base hydrolysis improves the stability of phenolic acids. For example, CA, which was found in all concentrations of the base hydrolysis, with AA and EDTA protection, was not detected at a high level of NaOH without AA and EDTA protection, supporting the findings of Krygier et al who reported low stability of CA when hydrolyzed under alkaline conditions (Krygier et al.,1982). In contrast, Nardini & Ghiselli (2004) reported good stability of phenolic compounds during alkaline hydrolysis in the presence of AA and EDTA. Similarly, SA was detected in higher levels in the acid hydrolysis fractions with AA and EDTA protection than those without protection, indicating a protective effect of EDTA and AA when added to the acid hydrolysis methods. This is consistent with a previous study (Ross et al., 2009) where, in investigating the use of HCl with and without EDTA and AA protection, the authors reported higher total phenolic acids content with the use of AA as EDTA from dry beans.

The effectiveness of the HPLC method was tested using standard solutions of five phenolic acids. Generally, a combination of water and organic solvents such as methanol and acetonitrile are utilized for the separation of phenolic acids. For satisfactory chromatographic resolution and peak shapes of these acids, the use of acidified mobile phases is required (Michalkiewicz et al., 2008). Generally, formic acid, acetic acid, and phosphoric acid have been used. In this study, formic acid was selected. It has a sufficiently low pH to ensure better phenolic acids separation; methanol was selected as an organic modifier. Previous studies have shown that the increase in the pH of the mobile phase dramatically reduces the retention of phenolic acids. This is because phenolic acids are weak acids and exist primarily in the protonated form at a pH below their pKa, so in order to increase the retention capacity of phenolic acids, the pH of the

eluent should be lower than their pKa (Seema et al., 2001; Michalkiewicz et al., 2008). Therefore, following preliminary trials, a pH of 2.8 with 1% v/v formic acid (acidified water) was chosen as the optimum pH of the elution solvent. The analysis was completed in about 20 min, but elution of the rest of the material in the column took almost 30 min. Using this HPLC method, four phenolic acids and one phenolic acid aldehyde were identified in each extract and compared to the retention times and calibration curve of external standards. The elution times of PA, PCA, VA, and SA were 6.2, 7.7, 11.5, and 13.7 min, respectively, at 280 nm. CA was eluted at 12.7 min at a wavelength of 329 nm. In all HPLC chromatograms of Chaga extract, under all conditions, there was a peak eluting around 16.5 min at a wavelength of 329 nm, which was too early to be ferulic acid. Also, the UV spectra for this compound did not match the UV spectra of the ferulic acid standard. Therefore, the peaks were not identified as ferulic acid. Identification of these peaks was not pursued.

The method performance was validated by the determination of linearity, precision, and detection limits. The calibration curves, obtained by triplicate injections, were constructed from peak areas of the reference compounds versus their concentrations. The calibration curves showed good linearity with regression coefficients >0.994 (Table 2-1). The detection limit is the lowest amount of analyte in a sample that can be detected but not necessarily quantified. The LOD and LOQ were estimated based on the standard deviation of y-intercepts of the regression lines (σ) and the slope (S). The LOD for all the compounds was in the range of 0.13–0.17 μg/g (Table 2-2). The LOQ is defined as the lowest concentration that can be determined with acceptable accuracy and precision. The LOQ for all the compounds was in the range of 0.39–0.52 μg/g (Table 2-2). In this study, the limit of linearity in analytical ranges was studied, and the results

agreed with the required criteria (Irakli et al., 2012). The within- and between-day precisions of the analytical method were determined in terms of percent relative standard deviation (%RSD). The %RSD values for evaluated concentrations (1, 10, and 25) were 0.5–3.0% and 0.8–4.2% for the within- and between-day precisions, respectively. Recoveries of the experiment were performed in order to study the accuracy of the method. There were three different concentrations of standards (1, 10, and 25 μg/g) added to the sample extract (3N NaOH with AA and EDTA protection) for determination of percentage recovery of the five phenolics in Chaga extract. Recoveries ranged from 90.1% to 103.3% for within-day analysis and from 88.5% to 103.5% for between-day analysis.

2.5 CONCLUSION

A simple, sensitive, precise, and accurate HPLC method for the simultaneous quantification of five phenolic compounds from Chaga was developed and validated. Different hydrolysis conditions, with and without AA and EDTA protection, were explored in order to liberate phenolic acid from chaga. The majority of phenolic acids were extracted from the base hydrolysis (3N NaOH with AA and EDTA). This work shows that both base and acid hydrolysis procedures have a substantial effect on the amount of phenolic acids present in Chaga. For the first time, addition of AA and EDTA has shown a protective effect on degradation of phenolic acids in Chaga during the hydrolysis conditions.

CHAPTER 3 OPTIMIZATION OF ACCELERATED SOLVENT EXTRACTION OF PHENOLIC COMPOUNDS FROM CHAGA USING RESPONSE SURFACE METHODOLOGY

3.1 INTRODUCTION

An effective extraction method is required to isolate phenolic compounds from the cellular matrix of various food substrates. The efficiency of the extraction process is determined by the composition of the natural source of phenolic compounds, the chemical structure of the compounds themselves, and the extraction condition and methodology that is utilized. In general, due to chemical diversity and uneven distribution of the individual phenolic compounds within the cell matrices, no universal approach is ideal for efficient extraction of all kinds of phenolic compounds. Moreover, the presence of other molecules in the cellular matrix such as sugars, pigments, and terpenes may complicate the extraction process of the compounds of interest due to interfering effects (Dai & Mumper, 2010; Luthria & Mukhopadhyay, 2006). Several extraction parameters such as solvent polarity, time and temperature, pH, solid-liquid proportion and particle size, also contribute to the efficiency of the extraction process (Dai & Mumper, 2010; Khoddami et al., 2013). Hence, it is of critical importance to optimize the extraction conditions and select an appropriate extraction technique for each phenolic source in order to achieve good extraction efficiency.

Response surface methodology (RSM) has been at the forefront of recent extraction optimization research. It has been used to develop, improve and optimize process parameters among various food, biology, and chemistry applications (Ferreira et al., 2007). RSM is a combined mathematical and statistical tool that facilitates a multifaceted evaluation of the impact of several parameters and their interactions, relative to the

desired response. This technology simultaneously optimizes experimental conditions while reducing the required number of experimental trials (Gunst et al., 1996). This methodology is widely used to overcome the limitations of a single factor approach, in which only one variable can be analyzed with respect to all other factors remaining constant. Traditional single factor methods are time-consuming, labor-intensive, and often lack data on factor interaction outcomes. Thus, establishing true optimum conditions may be inadvertently jeopardized (Liyana-Pathirana & Shahidi, 2005).

Conventional and modern extraction techniques have been reported in phenolic compound recovery among several different natural sources, including Chaga (Dai & Mumper, 2010; (Khoddami et al., 2013). Typically, conventional methods such as maceration, soxhlet, and reflux are used by employing large volumes of organic solvents such as methanol, ethanol, acetone, ethyl acetate, or their mixture with water, either at room temperature, or at the boiling point of the solvent (Espinoet al., 2018). However, these methods have many demonstrated drawbacks such as low selectivity, labor intensity and prolonged extraction time. This ultimately results in high-energy consumption in addition to targeted compound degradation, due to both internal and external factors such as light, air, high temperatures and enzymatic reactions (Palma et al., 2001). Moreover, environmental constraints associated with excessive organic solvent usage during the extraction process evoke serious concern (Khoddami et al., 2013; Liazid et al., 2007). To overcome these difficulties, “green” extraction techniques have been introduced with special emphasis on environmental and economic aspects. Green extraction technologies have been reviewed in several comprehensive studies. Briefly, the main goal of these green strategies is to design extraction processes that reduces energy consumption and allow the use of alternative solvents or reduces organic solvents consumption while

producing safe and high-quality extract (Chemat et al., 2012). Some of these technologies such as supercritical fluid extraction (SFE), microwave-assisted extraction (MAE), ultrasound extraction (UAE) and accelerated solvent extraction (ASE) have been applied to phenolic compound extraction method development from different plant materials (Andrade et al., 2012; Şahin & Şamli, 2013; Švarc-Gajić et al., 2013;Tripodo et al., 2018).

Among these extraction methods, accelerated solvent extraction (ASE) is a good alternative to conventional procedures. Compared with traditional approaches, there is a substantial decrease in the solvent consumption and extraction time required for ASE. This is because ASE can be automated in an inert atmosphere, which results in minimal phytochemical degradation due to long extraction time. Further, ASE allows better control over several extraction conditions such as temperature, static extraction time, and the number of extraction cycles, further enhancing method efficiency (Ameer et al., 2017; Mustafa & Turner, 2011).

ASE relies on the use of elevated pressure (500-2000 psi) and temperature (40-200 °C) for a relatively short time to accelerate the rate of extraction. Pressure allows the extraction cell to be filled faster, helps to force liquid into the sample pores and maintains the liquid state of extraction solvents, even at high temperatures. This elevated temperature results in an increase in the solvation ability of the solvent due to a decrease in both its' viscosity and surface tension. The result is an accelerated diffusion rate and mass transfer of the analyte into the solvent; thereby improving the recovery of compounds of interest (Ameer et al., 2017).

Although there are several reports that assess the different biological activities of *inonotus obliquus* and conventional methods used for phenolic content recovery, very

little attention has been given to green technology impacts on Chaga target compound extraction efficiency. In addition, a thorough investigation of several experimental factors that can significantly affect the efficiency of the extraction and the quality of the phenolic compounds is lacking. Therefore, this study aims to (i) apply the response surface methodology (RSM) approach to ASE extraction parameters (extraction temperature and ethanol concentration) in order to optimize maximum total phenolic and antioxidant activity of *inonotus obliquus*. This study also aims to (ii) evaluate the efficacy of accelerated solvent extraction (ASE) as an environmentally friendly (green) technique for phenolic compounds extraction in Chaga. The differences among the total phenolic content and the antioxidant activity of the extracts are discussed and compared with those obtained by conventional extraction methods. Finally, High-Performance Liquid Chromatography HPLC analysis was developed (see Chapter 2) to assess the effect of ASE extraction temperatures on individual phenolic compounds

3.2 MATERIALS AND METHODS

3.2.1 Fungal Material

Chaga sclerotia were collected from a yellow birch (Betula alleghaniensis) from forest in Maine, USA. Samples were freeze dried (Model 7754511, Labconco Corporation, Kansas City, Missouri, USA), then ground using an electrical grinder (Nutribullet, model-NBR-1201M, Los Angels, USA). The ground powder was then passed through a 20-mesh (0.84 mm) sieve and only particles with a diameter smaller than 0.84 mm (20-mesh) were collected and pooled. All material was stored in a -20 °C freezer until subsequent extraction preparation.

3.2.2 Chemicals

Folin–Ciocalteu (FC) reagent, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 3,4-dihydroxybenzoic acid (PA), caffeic acid (CA), syringic acid (CA), and 3,4-dihydroxybenzaldehyde (PCA) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Methanol, absolute ethanol, acetone, and ethyl acetate, sodium carbonate, vanillic acid (VA), diatomaceous earth, and Ottawa sand were provided by Fisher Chemicals (Fair Lawn, NJ, USA). Ultrapure water was obtained from a Millipore water system (EMD Millipore, Billerica, MA, USA). All reagents and solvents were HPLC or analytical grade.

3.2.3. Extraction of Phenolic Compounds

3.2.3.1. Green extraction, Accelerated solvent extraction (ASE)

Accelerated solvent extraction of chaga derived phenolic compounds was executed using a Dionex ASE 200 (Dionex Corp., Sunnyvale, CA) system. The dried samples (1 g) were mixed with diatomaceous earth (0.25 g), and the mixture was placed into a stainless-steel cell (11 mL). The cells were equipped with a stainless-steel frit and a cellulose filter at the bottom to avoid accumulation of suspended particles in the collection vial. The extraction cells were loaded in the cell tray and were extracted using single-factor and RSM guided-experimental design conditions.

More specifically, for all sample extractions, the cell containing the ground sclerotia sample was preheated for 2 minutes, filled with extraction solvent (methanol, ethanol, acetone, and ethyl acetate) or a varied concentration (40-100% v/v ethanol: water), depending on the desired solvent, up 1500 psi of pressure. Samples were then heated for a specific time depending at the desired extraction temperature. The ASE device automatically controlled the heating duration. The automatic settings for this equipment requires a 5 min heating period with extraction temperature set to 60, 80, 100 °C, 6 min

at 120 °C, 7 min at 140 °C, 8 min at 160 °C, and 9 min at 180 and 200 °C, respectively. This step was followed by a specific static period (1-20 min). These operations were repeated during (1-5 cycles). At the conclusion of the final static period, the cell was rinsed with fresh extraction solvent (100% of the extraction cell volume) and purged with nitrogen for 90 sec. Once the extraction was completed, the suspension obtained was centrifuged (10 min, 2000 x g) and the solvent was removed using a rotary evaporator (Rotavapor R3000, Buchi, Switzerland). The resulting residue was then dissolved in 10 mL of 75% methanol and filtered through a 0.45µm membrane filter for further analysis.

3.2.3.1.1. Experimental Design and Statistical Analyses

This analysis was performed in two stages. A set of preliminary single-factor testing was first developed to investigate the effect of extraction variables (such as solvent type, solvent concentration, extraction temperature, number of cycles and static time) in order to assess the effects of these conditions on the total phenolic content (TPC). The experimental results were analyzed using SPSS statistical software (Version 25.0). All data were expressed as means ± standard deviations, in triplicate measurements. One-way analysis of variance (ANOVA) with Tukey's test was used to determine significant differences ($P < 0.05$). Based on the results from the single-factor experiments, major variables influencing the extraction process were selected and optimal ranges for each were determined.

In the second stage, a response surface methodology was then employed to optimize phenolic compound extraction based on the dependent variables (Y_1, total phenolic content TPC (mg GAL/ g DW) and Y_2, DPPH), respectively. A Face Central Composite Design (FCD) was also used to optimize Chaga phenolic compound extraction. The two independent variables chosen for this study were extraction

temperature (X_1, °C) and ethanol concentration (X_2, %). Both factors had three equally spaced levels in the design, coded as follows: -1, 0, +1, corresponding to the low, middle and high level of Xi, respectively. The variables were coded according to the equation:

$$x_i = \frac{(X_i - X_0)}{\Delta X} \qquad (1)$$

Where xi the (dimensionless) coded value of the variable is Xi, X_0 represents X at the center point, and ΔX denotes the step change. This design required thirteen experiments, which were performed in a random order to avoid systematic errors. Experimental data from FCD were fitted to the following second-order polynomial model and regression coefficients were obtained.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X^2 + \sum_{i>j}^{k} \beta_{ij} X_i X_j \qquad (2)$$

Where Y is the predicted extraction yield of TPC; B0 is a constant; βi, βii, and βij are the coefficients of the linear, quadratic and interactive terms, of the model. Xi and Xj are the code values of extraction temperature and ethanol concentration. In order to determine optimum conditions and assess the relationship between the responses and experimental levels of each factor, the regression coefficients were used to generate 3D surface plots from the fitted polynomial equation. The Design Expert (Version 7.0.12, Stat-Ease Inc., Minneapolis) statistical software was used to design the FCD and to analyze the experimental data in RSM. The goodness of fit of the model was evaluated by the coefficient of determination (R^2) and the lack of fit obtained from the analysis of variance (ANOVA). Coefficients with a p-value lower than 0.05 were defined significant. To verify the adequacy of the model, additional experiments were performed at optimal conditions predicted with the RSM, and the experimental data were then compared to

values predicted by the model. ASE method at optimized condition was compared with ME; SE and RE based on the TPC, DPPH scavenging activity, and HPLC results.

3.2.3.2. Conventional Solvent Extraction (CSE)

3.2.3.2.1. Maceration extraction (ME)

Chaga powder (1g) was macerated with 25 mL of aqueous ethanol (70%) for 48 h at room temperature. After filtration through Whatman no. 1 filter paper, the solvent was removed using a rotary evaporator (Rotavapor R3000, Buchi, Switzerland). The resulting residue was then dissolved and filtered in accordance with the procedure defined in section 3.2.3.1. Extraction was carried out in triplicate.

3.2.3.2.2. Reflux extraction (RE)

Chaga powder (1 g) was mixed with 25 mL of aqueous ethanol (70%) in a round-bottom flask. The extraction mixture was then refluxed in a water bath at 70°C for 3 h. The resulting residue was dissolved and filtered in accordance with the procedure defined in section 3.2.3.1. Extraction was carried out in triplicate.

3.2.3.2.3. Soxhlet Extraction (SE)

One gram of Chaga powder was continuously extracted with 500 mL of aqueous ethanol (70%) for 48 h at 70 °C in a Soxhlet apparatus. The resulting residue was dissolved and filtered in accordance with the procedure defined in section 2.3.1. Extraction was carried out in triplicate.

3.2.4. Determination of Total Phenolic Content (TPC)

The total phenolic content (TPC) of each extract was determined by the Folin–Ciocalteu method described by (Jaramillo-Flores et al., 2003). Briefly, 20 µL of supernatant was mixed with 90 µL of a 10-fold diluted Folin–Ciocalteu reagent in a 96-

well microplate. After standing for 5 min at room temperature, 90 µL of 6% sodium carbonate (Na2CO3) solution was added and the mixture was incubated at room temperature for 90 min. The absorbance of the reaction mixtures was measured at 750 nm in a spectrophotometric microplate reader (Bio-Tek ELx808, Vermont, USA). The absorbance of the extract was compared with a gallic acid standard curve for estimating the concentration of TPC in the sample. The TPC was expressed as milligrams of gallic acid equivalent per gram of dry weight Chaga (mg GAL/g DW).

3.2.5 Determination of Antioxidant Activity (DPPH)

The antioxidant activity of the extract was measured with the DPPH method according to the procedure reported by (Brand-Williams et al., 1995) with some modifications. Briefly, 150 µL of DPPH• solution prepared in methanol (0.2 mM) was mixed with 150 µL of chaga extract and the mixture was incubated at 37 °C. The tests were performed on a micro-plate reader (Bio-Tek ELx808, Vermont, USA). Absorbance readings of the mixture were taken at 517 nm over a period of 20 min. The percentage inhibition of radicals was calculated using the following formula:

$$\%inhibition = \frac{(A_{control-} A_{sample})}{A_{control}} \times 100$$

where $A_{control}$ is the absorbance of DPPH solution without extract; and A_{sample} is the absorbance of the sample with DPPH solution. The half-maximal inhibitory concentration (IC50) was reported as the amount of antioxidant required to decrease the initial DPPH concentration by 50%. At minimum, all tests were performed in triplicate, and graphs were plotted using the average of three determinations.

Table 3-1 Experimental design with the observed responses for the recovery of the TPC from Chaga sclerotia samples using ASE. The codes (-1, 0, 1) and values used for X1 (130, 150, 170) and X2 (50,70, 90)

Run	Independent variables		Responses	
	Extraction temperature (°C)	Ethanol concentration (%)	TPC (mg GAL/ g DW)	DPPH%
1	0 (150)	0 (70)	29	69
2	1 (170)	1 (90)	30	70
3	0 (150)	0 (70)	28	69
4	0 (150)	-1 (50)	19	64
5	0 (150)	1 (90)	20	61
6	-1 (130)	-1 (50)	7	55
7	0 (150)	0 (70)	27	68
8	1 (170)	0 (70)	39	77
9	0 (150)	0 (70)	28	70
10	-1 (130)	1 (90)	13	51
11	1 (170)	-1 (50)	30	70
12	0 (150)	0 (70)	29	68
13	-1 (130)	0 (70)	18	59

3.2.6. HPLC Analysis of the Extracts

The chromatographic separation of the examined analytes was carried out on a reversed-phase Hypersil GOLD aQ C18 column (150 mm × 4.6 mm i.d., 3 μm particle size) (thermo scientific, USA) preceded by a Hypersil GOLD guard column (thermo 4 × 3.0 mm). The column and guard column were held at 30°C, and the flow rate was set at 1 ml/min. The mobile phase of the HPLC system consisted of (A) methanol and (B) a 0.1% V/V formic acid/ultrapure-water, pH = 2.8. The gradient was linear at a flow rate of 1 mL/min The solvent gradient in volume ratios was as follows: 95% to 50% B for 16 min; the solvent gradient was reduced to 95% B at 18 min, and it was maintained at

95% for 2 min; the latter was followed by washing with methanol and re-equilibration of the column for 10 min. Total acquisition time was 30 min. The wavelength used for the quantification of the phenolic acids with the diode array detector (DAD) was 280 nm for all the phenolic acids except for caffeic acid, which was monitored at 329 nm. Identification of the phenolic acids was achieved by comparing retention times and UV spectra of the unknowns with the standards.

3.3 RESULTS

3.3.1 Single- Factor Experiment

The results in Figure 3-1 represent the effect of different extraction parameters on the total content of phenolic compounds of Chaga extract. The TPC increased as the polarity of the extracting solvents also increased. Thus, the highest phenolic content was obtained in methanol (2.24 ± 0.04 mg GAL/g DW), followed by ethanol (1.05 ± 0.05 mg GAL/g DW), acetone (0.76 ± 0.01 mg GAL/g DW), with the least recovery in ethyl acetate (0.44 ± 0.01 mg GAL/g DW) (Figure 3-1A).

The effect of various concentrations of ethanol ranging from 40% to 100% (v/v) as an extraction solvent on the recovery of Chaga phenolic compounds was investigated. Data showed that TPC yield increased with increasing ethanol concentration, reaching a maximum (7.4 7.25 ± 0.14 mg GAL/g DW) at 70%. Beyond 70%, the amount of isolated TPC gradually decreased (Figure 3-1B).

In this study, the impact of temperature (ranging between 40 °C to 200 °C) on the TPC extraction level was investigated using 70% ethanol. The results showed that the extraction temperature significantly affected the total phenol value of the extracts. TPC

increased as the extraction temperature increased, reaching a maximum of (40 ± 0.1 mg GAL/ g DW) at 200 °C (Figure 3-1C).

The static cycle of ASE was determined by performing consecutive accelerated solvent extractions on the same samples (1-5) times at 130 °C for 1 min. The TPC value of the extract was found to increase significantly after two cycles, reaching a value of (16.53 ± 0.06 GAL/ g DW). Beyond two cycle repetitions, there was no significant increase in the TPC recovery (Figure 3- 1D)

The impact of the extraction duration of chaga phenolics was analyzed at six static periods (1, 5, 7, 10, 15, and 20 min) with 70% ethanol and an extraction temperature of 130 °C with 2 extraction cycles. The results showed that the extraction yields of the phenolic compounds significantly increased (16.53 ± 0.06 to 18.52 ± 0.42 mg GAL/g DW) as the time of extraction increased from 1 to 7 min. Maximum yield was obtained at 7 min. Further, an increase to 20 min resulted in a slight decrease in the extraction yield of phenolic compounds (Figure 3- 1E).

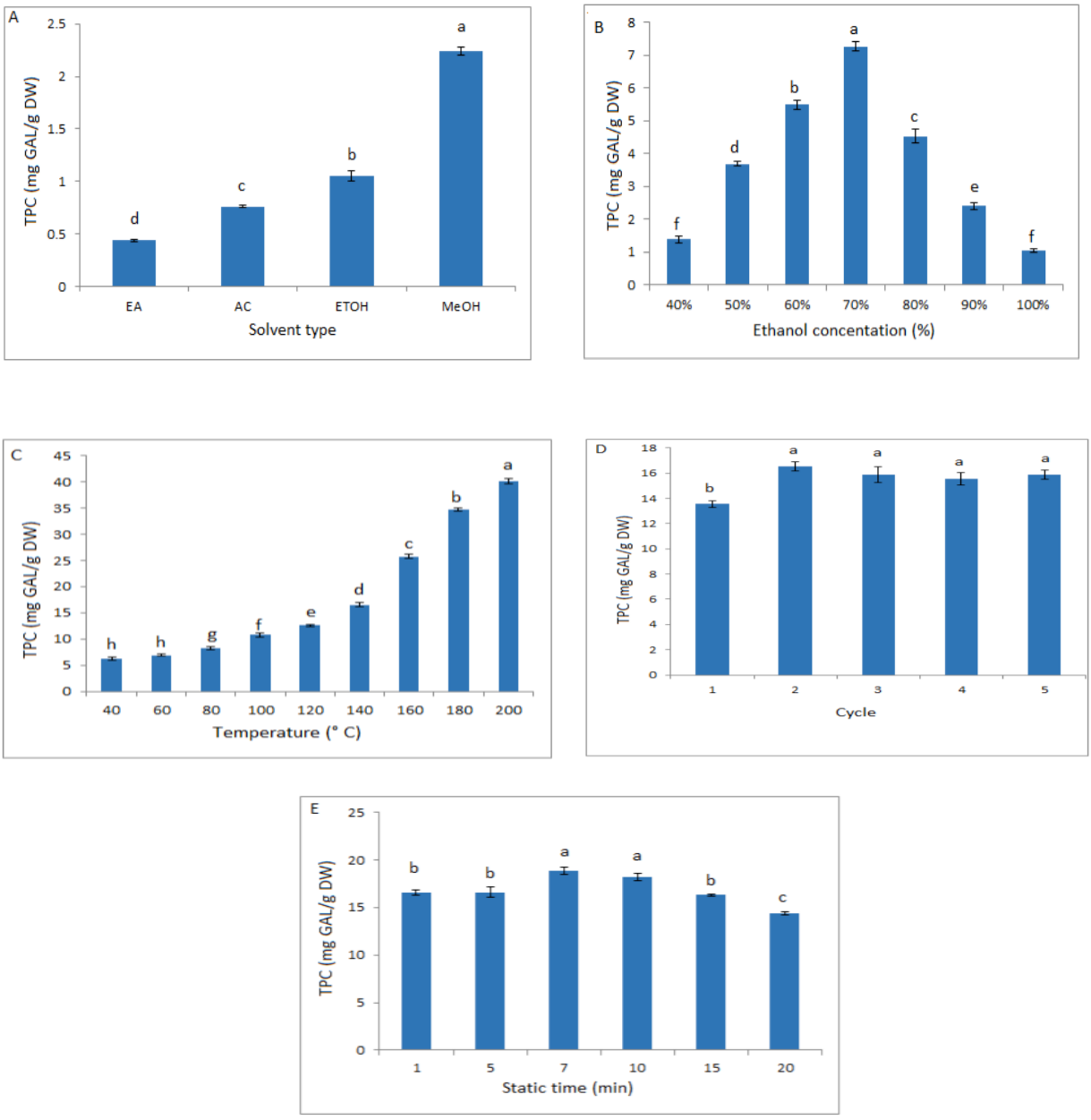


Figure 3-1 Single factor experiment for the total phenolic yield from Chaga with (A) accelerated solvent extraction with respect to solvent type, (B) solvent concentration, (C) extraction temperature, (D) number of cycles and (E) static time

3.3.2 Optimization of ASE by RSM

The results of the second-order response surface model fitting in the form of ANOVA are listed in Table 2. It can be seen that the two models were statistically significant and good predictors of the influence of the independent variables on their corresponding responses, as evidenced from the high values of F-test (270.57 and 245.1 for Y_1 and Y_2, respectively) and from the low p-values ($p < 0.0001$) for both (Y_1 and Y_2) (Yang et al., 2009).

The values of (R^2) were 0.9949 and 0.9943 for Y_1 and Y_2, respectively, which indicates that more than 0.99 of the variability of the responses were accounted for. Also, the values of R^2 and the R^2adj (0.9912 and 0.9903 for Y_1 and Y_2, respectively) imply high degree of correlation between the observed and the predicted data from the regression models. ANOVA results also indicated non-significant lack of fit for both response surface models at a 95% confidence level (0.5522 and 0.8050 for Y_1 and Y_2, respectively), confirming the suitability of the models to accurately predict the variation. Additionally, the relatively low values of coefficients of variation (CV) 3.29 and 1.08 % for Y_1 and Y_2 respectively, suggest a high degree of precision and good reliability of the experimental values (Table 3-2).

Table 3-2 Analysis of variance (ANOVA) for the effects of extraction temperature and ethanol concentration on TPC of Chaga.

Parameter	TPC				DPPH			
	Estimated coefficient	std error	F-value	p-value	Estimated coefficient	std error	F-value	p-value
Model			270.57	< 0.0001			245.1	< 0.0001
Intercept	-174.6221	0.3327			-94.62787	0.2934		
Linear								
X1	0.4734	0.3271	965.94	< 0.0001	0.9307	0.2884	902.82	< 0.0001
X2	3.632	0.3271	53.25	< 0.0091	1.8054	0.2884	16.63	0.0049
Quadratic								
X^2_1	0.0009	0.4821	0.6765	0.4379	0.0022	0.4251	4.45	0.0729
X^2_2	-0.0215	0.4821	318.42	< 0.0001	-0.0159	0.4251	226.38	< 0.0001
Interaction								
X_1X_2	-0.0037	0.4006	14.02	0.0072	0.0025	0.3533	8.01	0.0254
Lack of fit	0.8068		0.5522		0.3306		0.805	
R^2	0.9949				0.9943			
R^2 Adj	0.9912				0.9903			
CV %	3.29				1.08			

TPC: total phenolic content; X_1: Extraction temperature (°C); X_2: Ethanol concentration.

The effect of extraction temperature and solvent concentration on TPC was significant in first-order linear effects (X_1) at $P < 0.001$ and (X_2) at $P < 0.01$, second-order quadratic effect (X^2_2) at $P < 0.001$, and an interactive effect (X_1X_2) at $p < 0.01$. The predicted model obtained for Y_1 is given below:

$$Y_1 = -174.6221 + 0.4734X_1 + 3.6320X_2 - 0.0037X_1X_2 + 0.0009X_1^2 - 0.0215X_2^2$$

The results also show that the effect of extraction temperature and solvent concentration on DPPH was significant in first-order linear effects (X_1) at $P < 0.001$ and (X_2) at $P < 0.01$, second-order quadratic effect (X^2_2) at $P < 0.001$, and an interactive effect (X_1X_2) at $p < 0.01$. The predicted model obtained for Y_2 is listed below:

$$Y_2 = -94.6278 + 0.9307X_1 + 1.8054\,X_2 + 0.0025X_1X_2 - 0.00221X_1^2 - 0.0159\,X_2^2$$

The effects of the independent variables and their mutual interactions on the TPC and DPPH values of Chaga extract can be visualized on the three-dimensional response surface plots and two dimensions contour plots shown in figure 3-2 and figure 3-3, respectively. The effect of extraction temperature and ethanol concentration on the recovery of TPC is presented in (figure 3-2A and figure 3-3A). The extraction temperature displayed a linear effect on the response TPC, while ethanol concentration demonstrated both linear and quadratic effects. TPC increased with a simultaneous increase in temperature (130-170 °C) and ethanol concentration up to 70%. The TPC then remained constant or slightly decreased along with the increase of ethanol concentration at a fixed extraction temperature.

(Figure 3-2B and figure 3-3B) presented similar linear and quadratic effect of extraction temperature and ethanol concentration on the response DPPH; raising the extraction temperature up to the highest level allow high recovery of TPC, while

increasing the concentration of ethanol to a moderate level show the highest DPPH scavenging activity at constant extraction temperature.

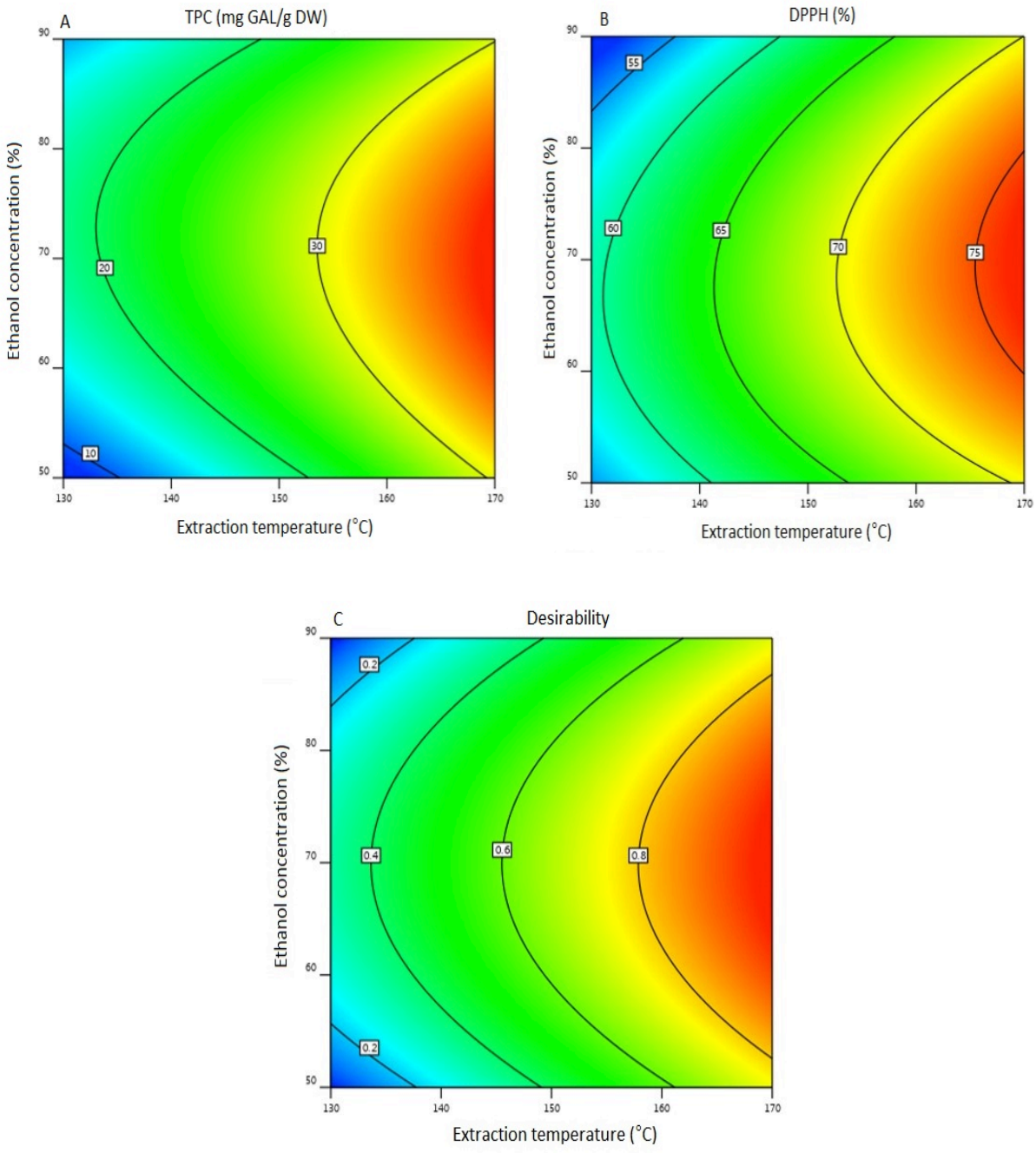


Figure 3-2 Counter plot analysis for the total phenolic yield from inonotus obliquus with accelerated solvent extraction with respect to total phenolic content (A); DPPH scavenging activity (B); desirability response (C).

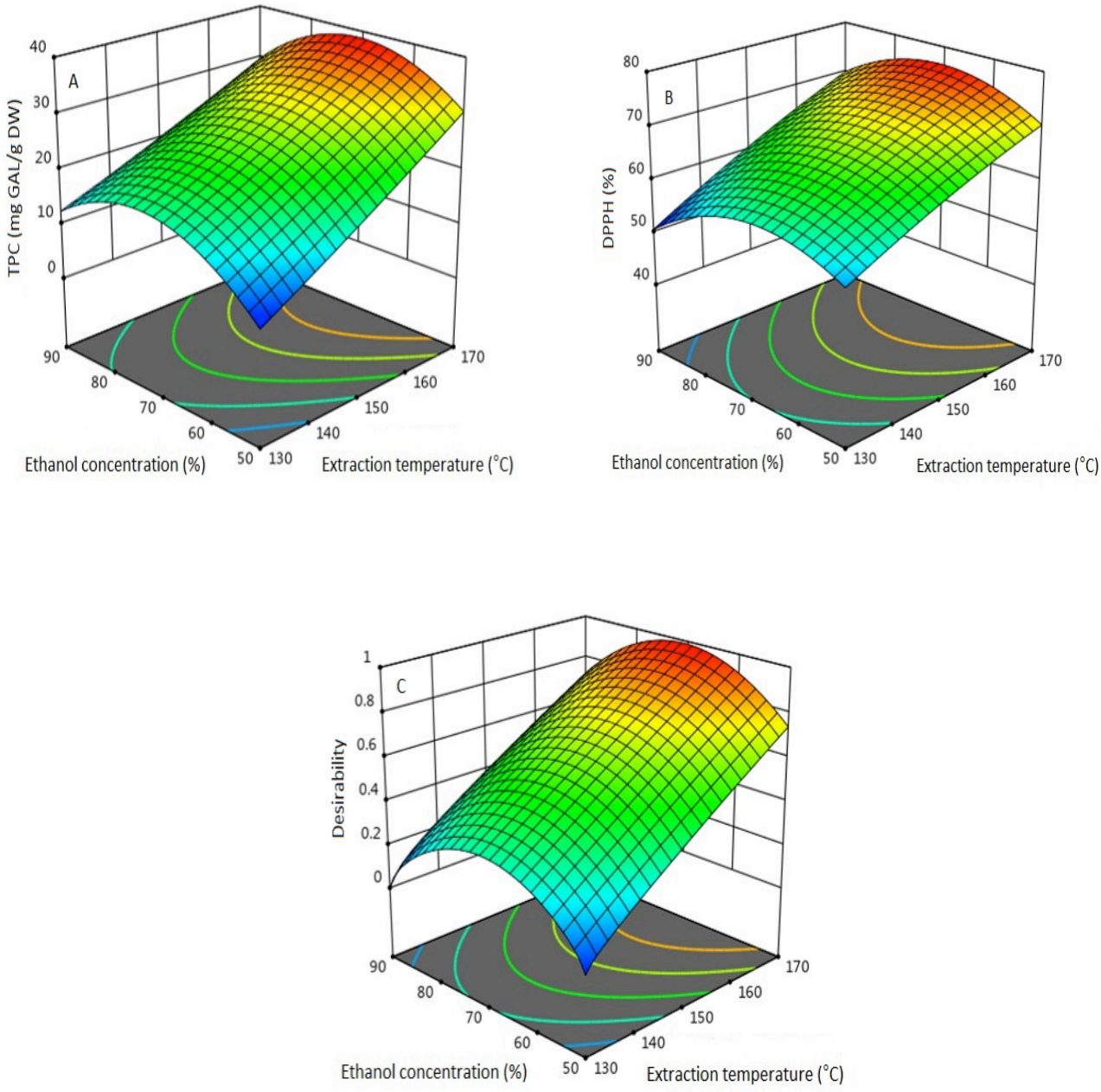


Figure 3-3 Response surface analysis for the total phenolic yield from inonotus obliquus with accelerated solvent extraction with respect to total phenolic content (A); DPPH scavenging activity (B); desirability response (C).

A comparison between the predicted value by the model and experimental values for TPC yield and DPPH from Chaga using ASE under optimized process conditions is presented in Table 3-3. The results indicate that there was no significant difference between the experimental values and the predicted values at ($p > 0.05$).

Table 3-3 Comparison of predicted and experimental values for TPC yield and DPPH from Chaga using ASE under optimized process conditions.

Optimum conditions		Responses			
Extraction Temperature (° C)	ETOH %	TPC (mg GAL/g DW)		DPPH%	
		Experimental value	Predicted value	Experimental value	Predicted value
170	66	39.32	38.45	76.59	76.37

3.3.3 Comparison of ASE and Conventional Methods

The results of comparing ASE to conventional methods of extraction in terms of total phenolic content, antioxidant activity, extraction time and solvent consumption are presented in Table 3-4. Data show that extract obtained using ASE at optimum conditions had significantly higher phenolic content and antioxidant activity than those obtained via conventional methods ($p < 0.05$) (Table 3-4). The data also show that the time of

extraction was reduced from 48 hours using SE to 30 minutes using ASE with solvent consumption being reduced from 500 mL to 25 mL (Table 3-4).

Table 3-4 Comparison of TPC and antioxidant activity (using DPPH radical scavenging assay) of Chaga sclerotia samples using ASE, RE, ME and SE extraction methods. Results are expressed as means ± standard deviation.

Extraction Method	TPC (mg GAL/ g DW)	Antioxidant (IC50 mg/mL)	Extraction time (hour)	Solvent Consumption (mL)
ASE	38.5 ± 0.87^{a}	0.18 ± 0.01^{a}	0.35	25
RE	8.61 ± 0.19^{b}	0.64 ± 0.05^{b}	3	30
SE	6.59 ± 0.58^{c}	0.78 ± 0.12^{b}	48	500
ME	3.11 ± 0.2^{d}	1.36 ± 0.32^{c}	48	30

Same letters in the same column denote non statistically different means according to ANOVA and Tukey's test; TPC (total phenolic content); GAL (gallic acid equivalents); DW (dry weight); ASE (accelerated solvent extraction); RE (reflux extraction); ME (maceration extraction); SE (soxhlet extraction).

3.3.4 High-Performance Liquid Chromatography (HPLC) Analysis of Phenolic Acids

The effect of different extraction temperatures using ASE on the extractability of phenolic acids is shown in Table 3-5. As the extraction temperature increased from (40-180 ˚C), a significant increase in the extractability of phenolic acids in the extracts is observed. For example, the total yield of phenolic acids was increased from (376.31 to 2407.87 µg/g). However, a further increase in the temperature (up to 200 °C) resulted in a reduction in the total yield to 1843.16 µg/g. Another example, the lowest quantity of SA (71.99± 0.74 µg/g) was observed when ASE temperature was 40 ˚C, whereas an extraction temperature of 180 ˚C provided the highest yield (585.03 ± 0.35 µg/g). Raising the temperature to 200 °C led to a significant reduction in the yield of SA (489.98 ± 0.08 µg/g). A similar trend was observed for all compounds except for CA, which undergoes degradation at a lower temperature.

Table 3-5 Quantity of individual phenolic acids (µg/ g DW) from Chaga extracts using ASE at different extraction temperatures

ASE temperature (°C)	PA	PCA	VA	CA	SA	Total
40	66.84± 0.83	120.79±0.74	44.36 ± 0.45	72.33 ± 0.68	71.99 ± 0.74	376.31[h]
60	65.75± 0.62	191.29 ± 0.96	74.09 ± 0.9	75.90 ± 0.58	73.45 ± 0.34	480.48[g]
80	85.92 ± 0.75	250.00 ± 0.27	75.48 ± 2.28	91.04 ± 0.54	126.00 ± 0.52	628.44[f]
100	97.36 ± 0.97	303.69 ± 0.65	102.44 ± 1.9	117.77 ± 0.4	181.04 ± 0.53	802.30[e]
120	118.58 ±1.68	470.90 ± 0.36	132.74 ± 1.21	193.69 ± 0.57	202.05 ± 1.01	1117.96[d]
140	153.52 ±1.03	623.53±1.95	154.19 ± 0.29	224.36 ±1.2	360.69 ± 0.28	1516.29[c]
160	218.99 ±1.61	798.90 ± 0.52	184.46 ± 0.1	199.87 ± 0.22	506.94 ± 1.5	1909.16[b]
180	256.07 ± 0.72	1097.86 ± 1.76	284.66 ± 1.17	184.25 ± 1.21	585.03 ± 0.35	2407.87[a]
200	189.61 ± 0.99	907.53 ± 1.78	191.08 ± 1.01	64. 96 ± 0.83	489.98 ± 0.08	1843.16[b]

PA, protocatechuic acid; PCA, protocatechuic aldehyde; CA, caffeic acid; VA, vanillic; and SA, syringic acid. Different letters in the same column denote statistically different means ($p < 0.05$) according to ANOVA and Tukey's test

The effect of different extraction methods on the extractability of phenolic acids from Chaga is presented in Table 3- 6. The yield of the phenolic acids obtained from extract made with ASE at optimized conditions contained higher concentrations of individual phenolic acids than the extracts produced from conventional extraction

methods. For example, the content of SA varied from 541.84 ± 0.4 μg/g in ASE at optimized conditions, to 115.66 ± 0.57 μg/g in RE, 200.55 ± 0.96 μg/g in SE and 47.43 ± 0.4 μg/g in ME, respectively. The results show that the content of SA 'extracted by ASE was over 3, 5, and 11 times higher than those extracted by RE, SE, and ME, respectively.

Table 3- 6 Quantity of individual phenolic acids (μg/ g DW) from Chaga extracts using ASE optimized conditions and conventional extraction methods

Extraction method	PA	PCA	VA	CA	SA	Total
ASE Optimized	250.39 ± 0.57	1121.58 ±0.94	276.25 ± 0.39	206 ± 0.83[a]	541.84 ± 0.4	2396.06[a]
RE	83.49 ± 0.66	230.19 ± 1.53	29.68 ± 0.54	82.67 ± 0.55	115.66 ± 0.57	514.69[b]
SE	80.31 ± 0.65	248.56 ± 3.02	39.7 ± 0.28	81.38 ± 0.8	200.55 ± 0.96	650.50[b]
ME	45.29 ± 0.95	90.65 ± 1.12	Nd	23.76 ± 0.98	47.43±0.4 0.4	216.87[c]

PA, protocatechuic acid; PCA, protocatechuic aldehyde; CA, caffeic acid; VA, vanillic; and SA, syringic acid. Different letters in the same column denote statistically different means ($p < 0.05$) according to ANOVA and Tukey's test. nd, not detected.

3.4 DISCUSSION

The first part of this research was to optimize the extraction of phenolic compounds from Chaga. Many studies have established that extraction of phenolic compounds from natural sources is governed by several parameters including extraction conditions such as solvent type, extraction temperature, and the applied extraction method (Dai & Mumper, 2010; Khoddami et al., 2013). Selection of extraction solvents plays a key role in the recovery of phenolic compounds from their sources. The polarity of solvent used as an extraction medium as well as the solubility of the phenolic compounds in the solvent, strongly influences the quality and the quantity of the phenolic compounds that are extracted. In this study, the effectiveness of four common solvents (Luthria & Mukhopadhyay, 2006) in extraction efficiency of Chaga sclerotia-derived phenolic compounds was evaluated. Methanol, ethanol, acetone, and ethyl acetate were used in this analysis, and the degree of effectiveness for each was determined by the total phenolic content (TPC) measured by Folin–Ciocalteu assay. As shown in Fig.3-1A, the highest yield of TPC obtained in methanol which could be attributed to better solvation of the phenolic compounds present in Chaga due to the high polarity of methanol. Although ethanol has similar polarity to methanol, the solvation efficiency of phenolic extracts was less. This could be due to the presence of the ethyl group in ethanol, which is longer than the methyl group in methanol. The elongated ethyl group may result in a lower solvation degree due to the larger hydrophobic characteristics of this structure (Boeing et al., 2014). Generally, methanol is more efficient for the extraction of low molecular weight polyphenols, as aqueous acetone presents a better yield for high molecular weight polyphenols. However, ethanol is a preferable solvent with respect to safety and

environmental considerations (Dai & Mumper, 2010). For these reasons, ethanol was selected as the extracting solvent for subsequent experiments.

The results in Figure 3-1B show TPC yield increased with increasing ethanol concentration to a certain level and then gradually decreased. The results from this experiment agree with previous reports, suggesting that the addition of water to organic solvents help to improve the relative polarity of the solvent. The addition of water results in swelling of the raw matrix, allowing the solvent surfaces to contact more phenolic compounds, leading to enhanced solubility of the phenolic compounds in the solvent (Luthria, 2012). Additionally, the high dielectric constant of water, leads to increased polarity indices of ethanol when in a water-based solution (Spigno & De Faveri, 2009). The structure of phenolic compounds themselves, also influences their solubility. More specifically, the presence of several hydroxyl groups makes these compounds hydrophilic, increasing solubility in hydroalcoholic mixtures compared to a mono-alcoholic solvent. However, a high concentration of organic solvent may lead to the dissolution of phenolic compounds as a result of a decline in the solvent polarity; thus, decreasing the solubility of phenolic compounds and the extracting rate (Yang et al., 2009). Based on these results, the concentration range 50–90% ethanol: water was selected for the RSM trials and 70% was fixed for the next single-factor experiments.

As a general rule, a relatively high extraction temperature of extraction assists in reducing the solvent viscosity, allowing better diffusion of the solvent into the solid matrix. This in turn, increases the solubility of the phenolic compounds, making the extraction process more efficient. Yet, there are upper limits to this applied temperature, as an excessive amount of heat can decrease the extraction efficiency due to the thermal degradation of phenolic compounds. Therefore, selecting an appropriate extraction

temperature is a necessary step in procedure optimization for phenolic compounds isolation. As it can be discerned from Figure 3-1C, raising the extraction temperature to the highest level resulted in increase the yield of TPC, However, severe degradation of phenolic compounds have been previously reported at 200 °C (Lindquist & Yang, 2011), Therefore, to prevent changes in the natural phenolic profile of the extracts, 130 °C was selected for subsequent single-factor trials and 130, 150 and 170 °C, were selected for the RSM study.

The number of extraction cycles represents the number of times the static heating and flushing steps are repeated. The number of extraction cycles can be manipulated to avoid prolonged heat exposure of the samples during the extraction process. For example, instead of conducting one extended cycle to extract specific compounds from their matrices, the extraction could be completed in multiple shorter cycles (Sarker, 2012). The results in Figure 3- 1D showed no significant increase in the TPC recovery after two cycle repetitions. Therefore, a total of two extraction cycles was selected as the optimum cycle number for phenolic extraction.

Extraction time is an additional factor which influences TPC recovery and extraction efficiency and was therefore further explored with a static cycle (Sarker 2012). A maximum yield in TPC was obtained at 7 min followed by reduction, which may be due to induced thermal degradation with the longer extraction time. Therefore, 7 min was fixed for RSM optimization (Figure3-1E).

In this study, the influence of extraction temperature (X_1), and ethanol concentration (X_2) on total phenolic content (Y_1) and the corresponding antioxidant activity (Y_2), was investigated. The ranges of these variables were determined as extraction temperature (X_1: 130, 150, and 170°C), and ethanol concentration (X_2: 50%,

70%, and 90%), based on the preliminary single-factor experiment determined described above.

The determination coefficient (R^2), adjusted determination coefficient (R^2adj), lack of fit, and coefficient of variation (CV) were estimated to check the models' adequacy (Erbay & Icier, 2009). R^2 measures the proportion of the total variation of the dependent variable attributed to each independent variable. This value falls between 0 and 1. Hence, a model with a higher R^2 value is a better model. However, R^2 is not always a representative indicator of model adequacy as this value either increases or never decreases, when more independent variables are introduced in the model. This may lead to an inflated R^2 value that does not explain the relationship between the independent variables and the response. Therefore, the R^2adj is used to compensate for the addition of more variables that do not improve the model, by considering both the number of independent variables and the sample size. With regard to this, the R^2adj will only increase if the added variables correlate with the dependent variables (Bonate, 2006). Therefore, a good statistical model, assesses the gap between R and the R^2adj, with these values reasonably close to 1 (R^2 value > 0.7 and R^2adj $\geq$ 7) representing an appropriate model.

The three-dimensional (D) response surface plots and two dimensions (D) contour plots explain the effects of the independent variables and their mutual interactions on the TPC and DPPH values of Chaga extracts. Each 3D plot represents the number of combinations of the two-test variables. 3D response surface and 2D contour plots are the graphical representations of the regression equation and are useful for assessing the relationship between independent and dependent variables. Different shapes of the contour plots indicate whether the mutual interactions between the variables are

significant. The circular contour plot indicates negligible interactions between the corresponding variables, while elliptical contour suggests significant interactions between the corresponding variables (Liu et al., 2013).

The results of the response surface plots confirmed the data of single factor experiments; raising the extraction temperature to allow linear increase of the yield of TPC, while raising ethanol concentration resulted in quadratic effect (Figure3- 2A and figure 3-3A). A rise in extraction temperature can disrupt the phenolic matrix bonds and weaken the cell wall structure, thus allowing an increase in phenolic compound release into the solvent. Higher temperatures can also improve the extractability of phenolic compounds through reducing both solvent viscosity and surface tension while increasing solute solubility and the diffusion coefficient, resulting in a higher extraction rate (Richter et al., 1996). These findings are in accordance with several other studies that reported a significant contribution of high extraction temperature towards phenolics extraction. Luthria (2012), recovered higher yields of total phenolic acids from vegetable waste when extractions were carried out between 100 and 160 °C, obtaining the optimum value at 160 °C. Tripodo et al. (2018) reported goji berry phenolic compound extraction was positively influenced by temperature. The researchers found that for all employed solvents, TPC increased with increasing extraction temperature, reaching the optimum value at 180 °C.

A similar quadratic effect of solvent concentration on the recovery of phenolic compounds was observed among different sources including blackcurrant (Cacace & Mazza, 2003) and rosemary extract (Hossain et al., 2011). This effect might be due to the change in solvent polarity with a change in ethanol concentration. It was reported that changes in ethanol concentration could enhance phenolic compound solubility by altering

the physical properties of the solvent such as density, dynamic viscosity, and dielectric constant (Cacace & Mazza, 2003). However, further increase of ethanol concentration might lead to dissolution of phenolic compounds due to a decline in the solvent polarity and the decrease solubility (Yang et al., 2009).

In parallel with phenolic content, extraction temperature and ethanol concentration displayed similar linear and quadratic effects on the antioxidant activity, reaching a peak at the highest temperature and a moderate ethanol concentration level (Figure 3-2B and figure 3-3B). These results are consistent with the significant increase in TPC, which was observed at the same extraction conditions. Our results agree with prior studies, which have reported the significant contribution of extraction temperature and ethanol concentration on the antioxidant activities from different sources in linear and quadratic effects, respectively (Karacabey & Mazza, 2010; Liyana-Pathirana & Shahidi, 2005).

Since both response variables are equally important, verification experiments were performed at optimal conditions derived for maximizing the desirability of the two responses (Figure3- 2C and figure 3-3C). Under these optimal conditions, the experimental values were found to be not significantly different from the predicted values at ($p > 0.05$), further confirming the validity and the adequacy of the predicted models (Table 3-3).

In previous work, a linear relationship was reported between the DPPH scavenging activity and the total phenolic content of Chaga extract (Debnath et al., 2013). Our results show a similar trend where the antioxidant activity of the extracts tends to increase with increasing phenolic content. A significant positive correlation of $R = 0.98$ ($p < 0.001$) between the phenolic content and the antioxidant activity, as determined by

the DPPH scavenging assay, was observed. Therefore, it is suggested that the phenolic compounds extracted from Chaga contribute strongly to the antioxidant capacity of the examined extracts. However, it should also be mentioned that the extracts were crude and may contain other components such as terpenoids and polysaccharides, which are also known radical scavengers (Handa et al., 2010). Significant correlations between the phenolic concentration in plants and the antioxidant activity displayed by the plants have been previously reported in literature. Piluzza & Bullitta investigated the relationship between phenolic content and the antioxidant capacity of several Mediterranean plant species, and obtained a good linear correlation using DPPH and ABST assays. They also suggested that phenolic content could be used as an indicator of the antioxidant properties of the examined plant species (Piluzza & Bullitta, 2011). Similarly, the high correlation coefficient between the antioxidant activity and the total phenolic content of grape cane extracts has been reported (Karacabey & Mazza, 2010).

In this study ASE were compared to conventional extraction methods in terms of total phenolic content, antioxidant activity, extraction time and solvent consumption. Data showed that extract obtained using ASE at optimum conditions had significantly higher phenolic content and antioxidant activity than those obtained via conventional methods ($p < 0.05$) (Table 3-4). The higher efficiency of the ASE technique could be explained by the combined application of high temperature and pressure leading to increased solubility and mass transfer rate between the phenolic analytes and the solvent. It also causes disruption in solute- matrix bonds (dipole-dipole, van der Waals, and H2-bonding) and weakens the cell wall structure. These combined effects allow the diffusion of targeted phenolic compounds to the outer surfaces of Chaga matrices, improving the phenolic recovery rate (Mustafa & Turner, 2011). In addition, ASE may have prevented

the degradation of phenolic compounds due to limited extract exposure to light and oxygen during processing. Moreover, the short extraction times possible (about 35 min) may have reduced the adverse effect of enzyme activity that could be produced during the extraction process (Khoddami et al., 2013; Palma et al., 2001).

The results from the present study agree with other reports, which indicated that ASE is an effective technique for extracting phenolics from different sources as compared to other extraction methods (Ameer et al., 2017; Ju & Howard, 2003; Tripodo et al., 2018). However, other studies have reported that high temperature in ASE resulted in reductions of both phenolic content and antioxidant activity of the extract (Nayak et al., 2015). Differences in the extraction efficiency of phenolic compounds using ASE may be related to the vast structure diversity of extracted phenolic compounds and their differences in reactivity stability at higher extraction temperatures. Ju & Howard reported that the highest recovery of anthocyanins, obtained from dried red grape skin using acidified methanol, occurred at 60 °C, whereas the highest recovery of total phenolics from the same source occurred at 120 °C. The researchers attributed the increased yield of total phenolics at higher temperatures to the improved extraction of more heat-stable procyanidins and phenolic acids (Ju & Howard 2003). Similarly, higher extraction temperatures lead to lower catechin and epicatechin recovery, whereas no decrease was observed in the recovery of the other assayed phenolics and their acid and aldehyde derivatives under the same conditions (Palma et al., 2001). Consequently, it is necessary to adjust the extraction method depending on the nature of the target compounds that are to be extracted.

Data has also shown that extracts obtained by RE and SE methods had higher TPC and DPPH values compared to extracts obtained by ME (Table3-4). Kähkönen et al

reported that the reflux extraction found superior to room temperature for phenolic extraction from apple, cowberry, and bilberry (Kähkönen et al., 2003). The above results support previous work which suggested that the absence of heat and long extraction time in maceration (few days) contribute to the low efficiency of the extraction (Brglez et al., 2016).

With consideration of environmental and practical aspects, our data shows prevalence in ASE over conventional methods with respect to a reduction in both extraction time and solvent consumption (Table 3- 4). Green extraction methods such as ASE, provides high-quality extract with less impact to the environment as a result of limited energy usage and solvent consumption (Chemat et al., 2012). The present study, therefore, proposes ASE as an efficient green technology to extract phenolic compounds from Chaga.

Qualitative and quantitative analyses of individual phenolic compounds of Chaga extracts at ASE and conventional conditions were executed using HPLC-DAD method. Many studies have reported different behavior of individual phenolic acids affected by extraction temperature and the applied extraction method. However, to our knowledge, no studies have analyzed phenolic acid profiles in Chaga. Firstly, to define the relationship between ASE extraction temperature and the phenolic acids profile, we evaluated extraction temperatures (40-200 °C) on the quantity of individual phenolic acids isolated from Chaga. The results showed that raising the extraction temperature resulted in higher phenolic acids extracted from Chaga. However, a decrease in the quantity of all phenolic acids is observed at 200°C. The results also showed that CA was more affected by raising temperature and less quantity of CA is obtained at 160 °C. The earlier decline in CA level at 160 °C could be attributed to its chemical structure. CA has

two hydroxyl groups in its aromatic ring which translates into reduced stability under MAE conditions (Liazid et al., 2007). Other studies have also reported the stability of phenolic acids at different elevated temperatures using ASE and MAE. These studies are in agreement with the result in the present study in which that the extraction temperature has a significant effect on the content of the individual phenolic compounds. Elevated extraction temperatures generate greater yields of those compounds, however, degradation at a certain temperature will result, depending on the chemical structures of the assayed compounds (Luthria, 2012; Palma et al., 2001).

Secondly, the yield of the selected individual phenolic compounds obtained using different extraction methods was compared. The results demonstrated that more phenolic acids are extracted using ASE at optimized conditions in comparison to the extracts produced from conventional extraction methods Table 3- 6. A similar trend was observed in the other assayed phenolic acids. The experimental results of phenolic acids agreed with a previous study, which presented ASE as the most efficient method in the extraction of chlorogenic acid from black eggplant compared to other conventional techniques (Luthria & Mukhopadhyay, 2006). The greater yield of individual phenolic acids obtained under ASE at optimized conditions may be linked to the presence of phenolic compounds (e.g. phenolic acids are rarely found in free form and are primarily conjugated to other components in the matrix (Luthria & Pastor-Corrales, 2006). Therefore, a pretreatment may be required to release them from their strong conjugate. The previous report showed that steam treatment of Chaga samples before extraction facilitated the release of more phenolic acids from Chaga matrices due to the disruption of the cell wall, as a result of thermal processing (Ju et al., 2010). Considering that the most important effect of ASE is related to the use of higher extraction temperatures, it

seems that the phenolic acids in Chaga are strongly bonded to the matrix and therefore higher temperatures are required to break these bonds and release the phenolic analytes. Based on the results obtained from this study, ASE is an efficient method to extract phenolic compounds from Chaga.

3.5 CONCLUSION

Isolation of bioactive compounds is only possible through determining an efficient extraction method. There has been growing interest in establishing environmentally conscious and green extraction methods, while also evaluating the economic feasibility of these procedures. In the present study, an ASE based green extraction method was developed for the first time to obtain phenolic compounds from Chaga. An experimental design was applied to establish optimum extraction conditions yielding both maximum total phenolic content as well as antioxidant activity within the extract. The optimal ASE conditions were achieved at 170°C and 66% ethanol in water as the solvent and it was found that ASE not only provided higher recovery of TPC, but also high quality phenolic compounds, with high levels of antioxidant capacity. The results of this study contribute to enhancement and utilization of ASE Chaga extract in food, pharmaceutical, and cosmetic industries while providing an environmentally method of isolating these healthful compounds.

CHAPTER 4 THE ANTI-INFLAMMATORY PROPERTIES OF CHAGA EXTRACTS OBTAINED BY DIFFERENT EXTRACTION METHOD AGAINST LPS INDUCED RAW 264.7

4.1 INTRODUCTION

Inflammation is a physiological immune response of the body to injury, characterized by fever, swelling, and pain. Inflammation is usually implicated in the pathogenesis of a variety of diseases including, asthma, heart disease, cancer, and diabetes. (Taofiq et al., 2016). During the inflammatory process, large amounts of pro-inflammatory mediators such as nitric oxide (NO), prostaglandin E2 (PGE2), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-1β (IL-1β) are generated, mostly for the primary protection of the host (Garlanda et al., 2007; Jeong & Jeong, 2010). However, excess uncontrolled production of these inflammatory products can lead to oxidative stress (Dai et al., 2019; Garlanda et al.,2007). Recently, there has been considerable interest in finding anti-inflammatory agents from natural sources without or with low toxic effect (Taofiq et al., 2016). Several compounds have shown anti-inflammatory activity; among them, phenolic compounds have attracted great attention due to their wide variety of biological activities.

Previous studies have indicated that Chaga contains various bioactive components with different chemical characteristics and polarities (Duru et al., 2019; Shashkina et al., 2006). Accordingly, extraction of bioactive compounds from Chaga using various solvents leads to separate extracts with different profiles. For instance, petroleum ether and chloroform were used to extract lanostane-type triterpenoids from Chaga; whereas water and aqueous alcohol were suitable solvents to separate polysaccharides, melanin

pigments, and phenolic compounds from Chaga (Mazurkiewicz, 2006; Zhang, et al., 2011). Aqueous preparations of Chaga have been used since the 12th century in Eastern Europe to treat a variety of ailments without toxic effect. The traditional way to prepare Chaga is that pieces of Chaga are either macerated or boiled in water for a few hours or a few days. The resultant extracts from these processes are readily consumed or saved at a suitable temperature until consumed (Géry et al., 2018). Currently, quick brewing is a more common way to consume Chaga as Chaga tea; Chaga in powder form or in tea bags are steeped in hot water for a short period of time, strained, and then consumed as a tea. Investigations into the chemical composition and biological properties of aqueous extracts from Chaga have revealed that aqueous extract of Chaga had therapeutic effects against diabetes via multiple pathways—including antioxidative effects (Diao et al., 2014). Research has found that orally administered aqueous extract of Chaga could ameliorate acute inflammation (Mishra et al., 2012). However, no investigation has been carried out to examine chemical composition of Chaga tea in powder and tea bag form.

Alcoholic extracts from Chaga are characterized by high phenolic content. It has been reported that phenolics are the main chemical compounds involved in Chaga biological effects—including antioxidant, anti-cancerous, antimicrobial, and anti-inflammatory activities (Glamočlija et al., 2015; Nakajima et al., 2007; Nakajima et al., 2009a; Park et al., 2004; Park et al., 2005;Van et al., 2009). Different conventional and advanced extraction methods have been used to separate phenolic compounds from Chaga (Hwang et al., 2019; Seo & Lee, 2010). Data from the second chapter showed that accelerated solvent extraction (ASE) increased the total phenolic content and enhanced the DPPH scavenging activity of Chaga extracts—compared to conventional extraction methods. In addition, the concentrations of various individual phenolic acids significantly

increased in relation to their concentrations in other extracts. Previous studies have demonstrated that alcoholic extracts from Chaga possess significant anti-inflammation effects *in vivo and in vitro*. However, no studies have investigated the effect of the extraction method on the anti-inflammatory properties of Chaga extracts.

This study investigated the effect of Chaga extracts obtained by different extraction methods on anti-inflammation in LPS-stimulated 264.7 cells. Our work is the first experimental approach on Chaga collected in Maine, USA. The study also aimed to investigate the chemical features of Chaga tea steeping in bag and powder form.

4.2 MATERIALS AND METHODS

4.2.1 Fungal Material

Chaga sclerotia were collected from Maine birch forests. Samples were lyophilized (Model 7754511, Labconco Corporation, Kansas City, Missouri, USA), then ground using an electrical grinder (Nutribullet, model-NBR-1201M, Los Angeles, USA). The ground powder was passed through a 20-mesh (0.84 mm) sieve and only particles with a diameter smaller than 0.84 mm (20-mesh) were collected. All particles were stored in a -20 °C freezer until subsequent extraction preparation.

4.2.2 Reagents

Folin–Ciocalteu (FC) reagent, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 3,4-dihydroxybenzoic acid, caffeic acid, syringic acid, 3,4-dihydroxybenzaldehyde, bovine serum albumin (BSA), galacturonic acid, Griess reagent, 3-(4,5- Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), *Escherichia coli* LPS, and were purchased from Sigma–Aldrich (St. Louis, MO, USA). Ethanol, sodium carbonate, vanillic acid, diatomaceous earth, and Ottawa sand were purchased from

Fisher Scientific (Fair Lawn, NJ, USA). Ultrapure water was obtained from a Millipore water system (EMD Millipore, Billerica, MA, USA). The murine macrophage (RAW 264.7) cell line, Dulbecco's modified media (DMEM), heat inactivated fetal bovine serum (FBS), and penicillin-streptomycin were obtained from Gibco Life Technologies. For the enzyme-linked immunosorbent assay (ELISA) the TNF-α, IL-6, and IL-1β ELISA kits were obtained from e-Bioscience, Inc. (Cincinnati, OH, USA). Cytokine ELISA kits were obtained from R&D Systems (Minneapolis, MN, USA). All reagents and solvents were HPLC or analytical grade.

4.2.3 Preparation of Polysaccharide Extracts

Chaga sample (1.5g) in the bagged form (B) or the powder form (P) was infused in 200 mL of boiled distilled water at 100 °C for (3, 6, and10 min). The infusions were filtered through sterilized gauze. Four volumes of cold 95% ethanol were added to the aqueous extract after concentrating to 30% of the original volume with rotary evaporator under reduced pressure at 60°C. The extracts were kept at 4°C overnight to isolate the crude polysaccharides. The precipitate was recovered by centrifugation at (20 min, 2000 x g) (Rotavapor R3000, Buchi, Switzerland), washed with absolute acetone to remove adherent sugar residue and other small molecules and dialyzed for two days with distilled water (cut-off Mw 8000 Da). The retained portion was concentrated; deproteinated with Sevag reagent ($CHCl_3$: BuOH = 4:1, v/v) for 30 min under the magnetic force stirring and the procedure was repeated two times. Finally, the extracts were centrifuged to remove insoluble material and the supernatant was lyophilized in the freeze–dry apparatus for 48 h to give the crude polysaccharide extracts from the powder form P3, P6, and P10 and from the bagged form B3, B6, and B10, depending on the brewing time.

4.2.4 Preparation of Phenolic Extracts

4.2.4.1 Green extraction, Accelerated solvent extraction ASE

ASE was performed with a Dionex (Sunnyvale, CA, USA) ASE 200 instrument with solvent controller. According to our previously optimized method, briefly, dried ground sample of Chaga (1 g) was placed in a stainless-steel extraction cell, preheated for 2 min, and extracted with 70% aqueous ethanol or 66% aqueous ethanol. The extractions were performed at three temperature ranges (130 °C, 150 °C, and 170 °C) for 30 min (two cycles for every sample) at a pressure of 1500 psi. Once the extraction was complete, the suspension obtained was centrifuged (10 min, 2000 x g) and the solvent was removed using a rotary evaporator (Rotavapor R3000, Buchi, Switzerland). The resulting powders were stored at −20 °C for further experiments.

Table 4-1 extraction conditions of Chaga using accelerated solvent extraction ASE

Extraction conditions		Extract
Temperature °C	ETOH %	
170	66	ASE1
150	70	ASE2
130	70	ASE3

4.2.4.2. Conventional Solvent Extraction (CSE)

4.2.4.2.1 Maceration Extraction (ME)

Chaga powder (1 g) was macerated with 25 mL of aqueous ethanol (70%) for 48 h at room temperature. After filtration through a Whatman no. 1 filter paper, the solvent was removed using a rotary evaporator (Rotavapor R3000, Buchi, Switzerland). The resulting residue was then dissolved and filtered in accordance with the procedure defined in section 4.2.4.1. Extraction was carried out in triplicate.

4.2.4.2.2 Reflux Extraction (RE)

Chaga powder (1 g) was mixed with 25 mL of aqueous ethanol (70%) in a round-bottom flask. The extraction mixture was then refluxed in a water bath at 70°C for 3 h. The resulting residue was dissolved and filtered in accordance with the procedure defined in section 2.3.1. Extraction was carried out in triplicate.

4.2.4.2.3 Soxhlet Extraction (SE)

Chaga (1 g) was continuously extracted with 500 mL of aqueous ethanol (70%) for 48 h at 70 °C in a Soxhlet apparatus. The resulting residue was then dissolved and filtered in accordance with the procedure defined in section 2.3.1. Extraction was carried out in triplicate.

4.2.5 Determination of total phenolic content (TPC)

The total phenolic content (TPC) of the extracts was determined by the Folin–Ciocalteu method described by (Jaramillo-Flores et al., 2003). Briefly, 20 μL of supernatant was mixed with 90 μL of a 10-fold diluted Folin–Ciocalteu reagent in a 96-well microplate. After standing for 5 min at room temperature, 90 μL of 6% sodium carbonate (Na2CO3) solution was added and the mixture was incubated at room temperature for 90 min. The absorbance was measured at 750nm in a spectrophotometric microplate reader (Bio-Tek

ELx808, Vermont, USA). The absorbance of the extract was compared with a gallic acid standard curve for estimating the concentration of TPC in the sample. The TPC was expressed as milligrams of gallic acid equivalent per gram of dry weight Chaga (mg GAE/g DW).

4.2.6 Determination of Antioxidant Activity (DPPH)

The antioxidant activity of the extract was measured with the 1,1-diphenyl-2-picrylhydrazyl DPPH method according to the procedure reported by (Nakajima et al., 2007). A solution of DPPH was freshly prepared by dissolving 11 mg DPPH in 100 mL methanol (about 0.28 mM). The extract (150 µL) with varying concentrations (60-220 mg/mL) and DPPH solution (150 µL) was mixed in a 96-well microplate and incubated for 30 min at room temperature. The decrease in absorbance was measured at 517 nm using a spectrophotometric microplate reader (Bio-Tek ELx808, Vermont, USA). The percentage inhibition of radicals was calculated using the following formula:

$$\%inhibition = \frac{(A_{control-} A_{sample})}{A_{control}} \times 100$$

where $A_{control}$ is the absorbance of DPPH solution without extract; and A_{sample} is the absorbance of the sample with DPPH solution. The half-maximal inhibitory concentration (IC_{50}) was reported as the amount of antioxidant required to decrease the initial DPPH concentration by 50%. At minimum, all tests were performed in triplicate, and graphs were plotted using the average of three determinations.

4.2.7 Determination of Total Neutral Carbohydrate Contents

The carbohydrate content of the polysaccharide extracts was determined with a slightly modified phenol-sulphuric acid method (Masuko et al., 2005). Briefly, 1 mL of

sample solution, 0.05 mL of 80% phenol, and 5mL of concentrated sulphuric acid were mixed and shaken. After the mixture was kept at room temperature for 10 min, the absorbance was measured at 490 nm. The total carbohydrate content was calculated with D-glucose as standard.

4.2.7.1 Determination of Uronic Acid Content

The uronic acid contents of the polysaccharide extracts were measured according to the method of Blumenkrantz (Allen & Brock, 2000) using D-galacturonic acid as a standard. Briefly, 0.2 mL of sample solution and 1.2 mL of sulphuric acid\ tetraborate solution were mixed and shaken. The mixture was kept at 100 °C for 5 min, 20 mL of m-hydroxydiphenyl reagent was added, the absorbance was measured within 5 minutes at 520 nm.

4.2.7.2 Determination of Protein Content

The total protein content of the polysaccharide extracts was measured by the method of Bradford (Emami Bistgani et al., 2017) with bovine serum albumin as a standard. Briefly, 10 µL of sample solution and 200 µL of Bradford reagent were mixed. After the mixture allowed to stand at room temperature for 5 minutes, the absorbance was read at 595 nm.

4.2.8 Cell Culture

The RAW264.7 cell line derived from murine macrophages was purchased from the American Type Culture Collection (Rockville, MD, USA). The cells were maintained at 37 °C in a humidified atmosphere of 5% (v/v) CO2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with glutamine (1 mM), 10% fetal bovine serum (FBS FBS; ATCC; Manassas, VA, USA), penicillin (50 U/mL), and streptomycin (50 µg/m).

Medium was changed every two days. In all experiments, cells were grown to 70-80% confluence and subjected to no more than 20 cell passages.

4.2.8.1 Measurement of Cell Viability

Cell viability was assessed by the MTT 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay. The assay is based on the ability of mitochondria in viable cells to reduce the yellow tetrazolium salt MTT to purple formazan crystals. The method was performed according to the manufacturer's procedure (Mosmann, 1983) with some modifications. The cells were cultured in 96-well plates at a density of 1×10^4 cells/well for 24 h then the cells were treated with the samples at different concentrations (50, 100, and 150 µg/mL (Chaga extract) or 25, 50, and 100 µM (vanillic acid, caffeic acid, and syringic acid) or 5, 10, and 20 µM (protocatechuic acid and protocatechuic aldehyde) for 24 h in a humidified 5% CO_2 atmosphere at 37 °C. After the incubation period, the media was removed and 100 µL of fresh medium and 10 µL of MTT solution were added to each well, and the plate was incubated for 2h at 37 °C. Finally, the cell culture medium was discarded, and the formazan blue formed in the cell were resuspended in 200µL solubilization solution. The quantity of formazan (an indicator of cell viability) is measured by recording changes in absorbance at 540 nm using a spectrophotometric microplate reader (Bio-Tek ELx808, Vermont, USA). Of note, extracts and standards were dissolved in 0.05% DMSO. Cells treated with 0.05% DMSO were used as control and cells were treated with 2µM gallic acid was used as positive control. All experiments were performed in triplicate. % Cell viability was calculated using:

$$\%\ Cell\ viability\ = \frac{Absorbance\ of\ the\ extract}{Absorbance\ of\ the\ media} \times 100$$

4.2.8.2 Measurement of NO production

Inhibitory effects of Chaga extracts and the pure phenolic acid standards on the production of NO in RAW 264.7 cells were evaluated using a method modified from the previously reported (Sun et al., 2003). RAW 264.7 cells in 10% FBS-DMEM (without phenol red) were seeded at (1×10^5 cells/well) in 12 well plates. Cells were incubated for 24h at 37 °C. Cells were then treated with varying concentrations of samples (50, 100, and 150 μg/mL (Chaga extract) or 25, 50, and 100 μM (vanillic acid, caffeic acid, and syringic acid) or 5, 10, and 20 μM (protocatechuic acid and protocatechuic aldehyde) for 2h. The cells were then treated with LPS (1 μg/mL; Sigma–Aldrich,) for 24 h at 37 °C. After 24 h, 100 μL of cell culture medium was mixed with 100 μL of Griess reagent, incubated at room temperature for 15 min and the absorbance was measured at 540 nm in an ELISA microplate reader (Bio-Tek ELx808, Vermont, USA). The values were compared with a sodium nitrite standard curve (5-100μM).

4.2.8.3 Cytokine Measurement

To assess the anti-inflammatory effect of Chaga extracts and the pure phenolic acid standards on the expression of TNF-α, IL-6, and IL-1β were quantified using ELISA kits (e-Bioscience, Inc., Cincinnati, OH, USA). The assays were performed according to instructions provided by the manufacturer.

4.2.9 Statistical Analysis

Statistical analyses were performed with SPSS v25 (SPSS, Chicago, IL, USA). All results were expressed as the mean ± the standard error of triplicate analysis. Statistical significance was determined using one-way analysis of variance for independent means, followed by Tuckey's test. Differences were considered significant at $P < 0.05$.

4.3 RESULTS

4.3.1 Chemical Composition

The yield and the bioactive content of the crude extracts from the powdered form of Chaga were significantly higher ($p < 0.05$) than those of the bagged form at all extraction temperatures (Table 4- 1). For example, P6 yielded 30.21 ± 0.01% crude polysaccharide that contains 17.56 ± 0.01% carbohydrate, 10.23 ± 1.02% protein, 4.12 ± 0.44% uronic acid, and 17.61 ± 0.05% phenolic content, while B6 gave 17.33 ± 0.03% crude polysaccharide containing 11.56 ± 0.01% carbohydrate, 7.11 ± 0.5% protein, 2.57 ± 0.71% uronic acid, and 8.33 ± 0.05% phenolic content. The results also indicated that there was no significant effect of brewing time on the yield and the chemical content of the crude extracts of both bagged and powdered form. For example, B3 extract resulted in a carbohydrate content of 10.26 ± 0.08%, which was comparable to the carbohydrate content of (11.56 ± 0.01% and 11.81 ± 0.04%) obtained from B6 and B10 extracts, respectively. Similarly, P3, P6, and P10 extracts gave carbohydrate content of 17.02 ± 0.01%, 17.56 ± 0.01%, and 18.31 ± 0.05%, respectively. The same trends were observed for the yield, protein, uronic acid, and total phenolic content of crude polysaccharide extracts of Chaga tea obtained from different brewing times.

Table 4-2 Major chemical content of the crude polysaccharide extracts from the sclerotia of Chaga. The data represent the mean ± SD of triplicate experiments.

Sample	Yield%	Carbohydrate %	Protein%	Uronic acid%	TPC%
P3	30.66±0.05[a]	17.02 ± 0.01[a]	10.53±1.66[a]	4.11±0.47[a]	16.77±0.13[ab]
P6	30.21±0.01[a]	17.56 ± 0.01[a]	10.23±1.02[a]	4.12±0.44[a]	17.61±0.05[a]
P10	31.33±0.04[a]	18.31 ± 0.05[a]	11.02±1.28[a]	4.11±0.26[a]	17.04±0.03[a]
B3	15.53±0.01[c]	10.26 ± 0.08[b]	7.14± 1.83[b]	3.01±0.53[b]	6.49 ±0.04[d]
B6	17.33±0.03[b]	11.56 ± 0.01[b]	7.11 ± 0.5[b]	2.57±0.71[b]	8.33 ±0.05[c]
B10	17.33±0.02[b]	11.81 ± 0.04[b]	7.23± 2.01[b]	2.84±1.22[b]	8.33 ±0.06[c]

4.3.2 Cell Viability

RAW 264.7 cells were treated with various Chaga extracts or pure phenolic standards: (50, 100, and 150 μg/mL Chaga extracts) or (25, 50, and 100 μM vanillic acid (VA) or caffeic acid (CA) or syringic acid SA) or (5, 10, and 20 μM protocatechuic acid PA or protocatechuic aldehyde PCA) to assess their effects on cell viability using the MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay. This assay measures the activity of mitochondrial succinate-tetrazolium reductase of living cells and their ability to cleave the tetrazolium salts to formazan crystals resulting in a color change that can be monitored spectrophotometrically (Mosmann, 1983). The data were expressed as percent cell viability compared to control (0.05% DMSO). The results showed that Chaga extracts and the pure standards did not cause any cytotoxicity at the examined

concentrations in RAW 264.7 cells (Figure 4-1). Therefore, subsequent experiments were performed at these concentrations.

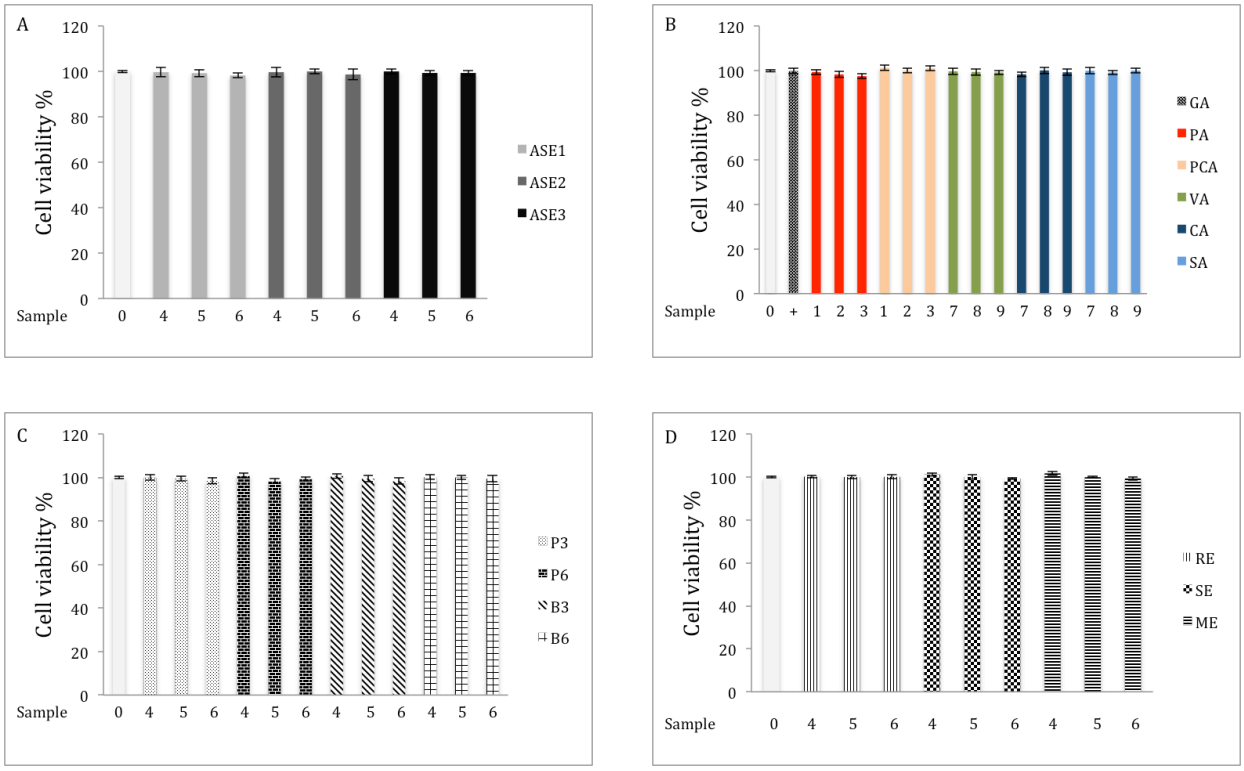


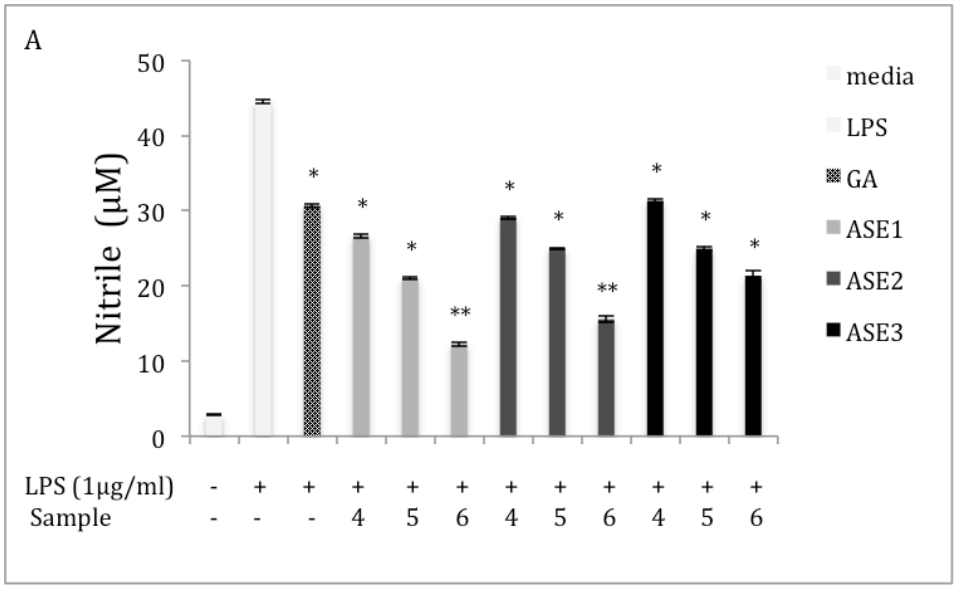
Figure 4-1 Effects of different samples on cell viability of RAW264.7 cells. Extracts were obtained by (A) ASE conditions, (B) pure phenolic acid standards, (C) crude polysaccharide extracts, and (D) different extraction methods. Cells were stimulated with 1 μg/mL of LPS plus varying concentrations of samples (0 = media, GA = 2 μM; 1 = 5 μM; 2 = 10 μM; 3 = 20 μM; 4 = 50 μg/mL; 5 = 100 μg/mL; 6 = 150 μg/mL; 7= 25 μM; 8 = 50 μM; and 9 = 100 μM).

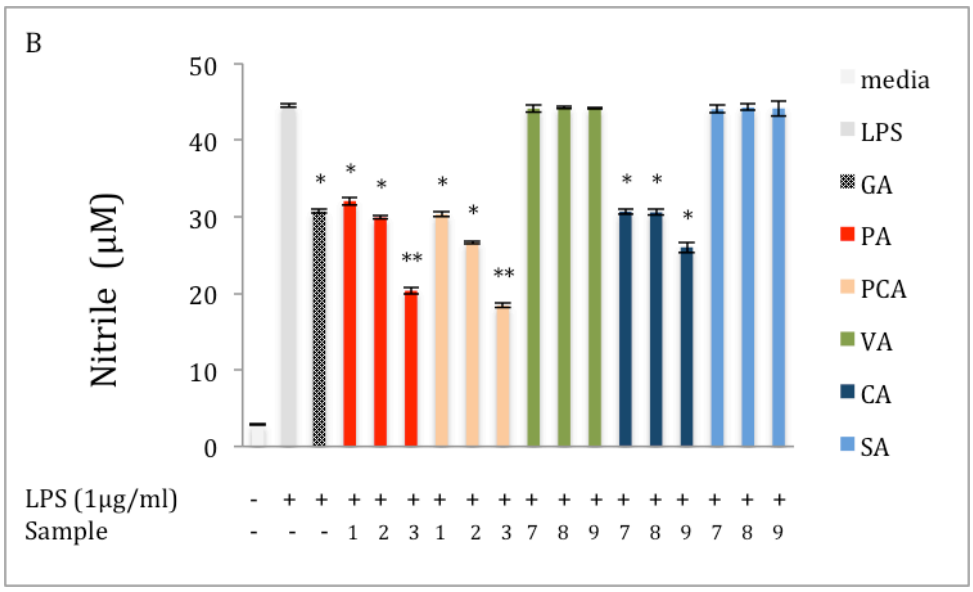
4.3.3 Inhibition of NO Production in LPS-Stimulated RAW 264.7 Macrophages

We examined the inhibitory effects of various Chaga extracts and pure phenolic acid standards on NO production on LPS-induced RAW 264.7 cells. Stimulation of the cells with 1 μg/mL LPS increased the NO levels to 44.53 ± 0.23 μM compared to 4.8 ± 0.12 μM in the negative control group (Figure 4-2). All ASE extracts significantly ($p < 0.05$ and $p < 0.01$) reduced the level of NO production at all tested concentrations (Figure 4-2A). For example, at 150 μg/mL, ASE1, ASE2, and ASE3 reduced the concentration of NO released from RAW 264.7 cells by 66.82%, 61.61%, and 43.34%, respectively, compared with the LPS group. We further investigated the inhibitory effect of the main phenolic acids found in Chaga extracts on the inhibition of NO production. Results showed that treating the LPS induced cells with various concentrations of PA, PCA, and CA significantly ($p < 0.05$ and $p <0.01$) reduced the production of NO, while treatments with SA and VA did not exhibit any effect on the induced cells (Figure 4-2B). For example, at the highest examined concentrations, the levels of NO released from the induced cells decreased by 54.35%, 58.66%, and 41.60%, after treatments with PA, PCA, and CA, respectively. However, the VA and SA treatments did alter the level of NO productions at any concentration.

All crude polysaccharide extracts separated from Chaga tea in the powder form showed significant inhibitory effect ($p < 0.05$ and $p < 0.01$) on NO production Figure 4-2C. However, crude polysaccharide extracts separated from the bag form of Chaga tea showed significant inhibitory effect ($p <0.05$), on NO production only at the highest examined concentration of the extracts compared to the LPS group. For example, at 150 μg/mL, P6 and B6 extracts reduced the nitrile concentration in the supernatants by 67.76% and 37.31%, respectively, compared to the LPS group

To investigate the effect of the conventional extraction methods on the inhibition of NO production, RAW264.7 cells were incubated with extracts obtained by different extraction methods in the presence of 1 μg/mL of LPS. There was no significant effect of the ME extract on the inhibition of NO production. However, both RE and SE extracts showed significant inhibition ($p < 0.05$ and $p < 0.01$) on the production of NO, compared to the LPS group (Figure 4-2D). For example, at 150 μg/mL, RE and SE extracts reduced the nitrile concentration in the supernatants by 51.42% and 52.61%, respectively, compared to the LPS group.





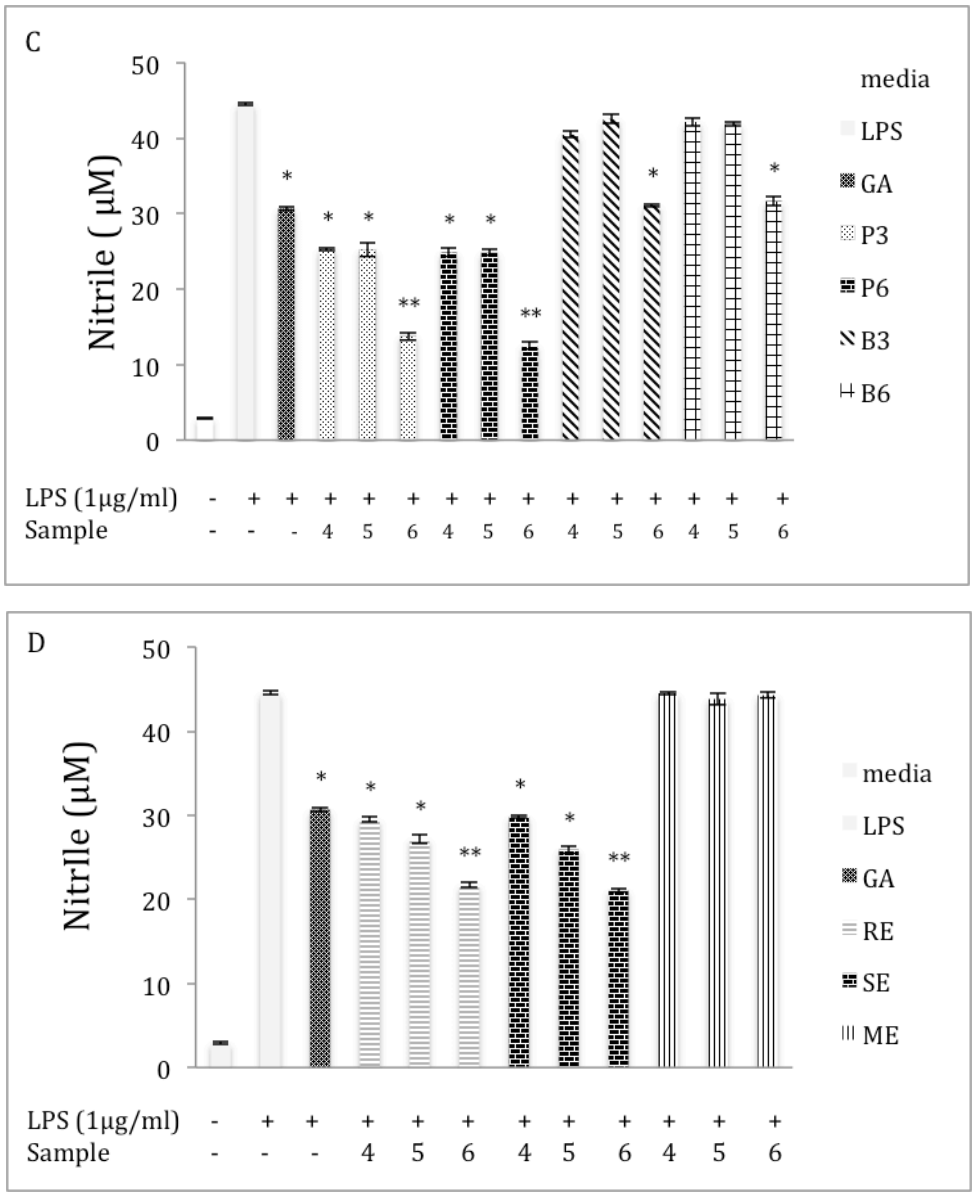


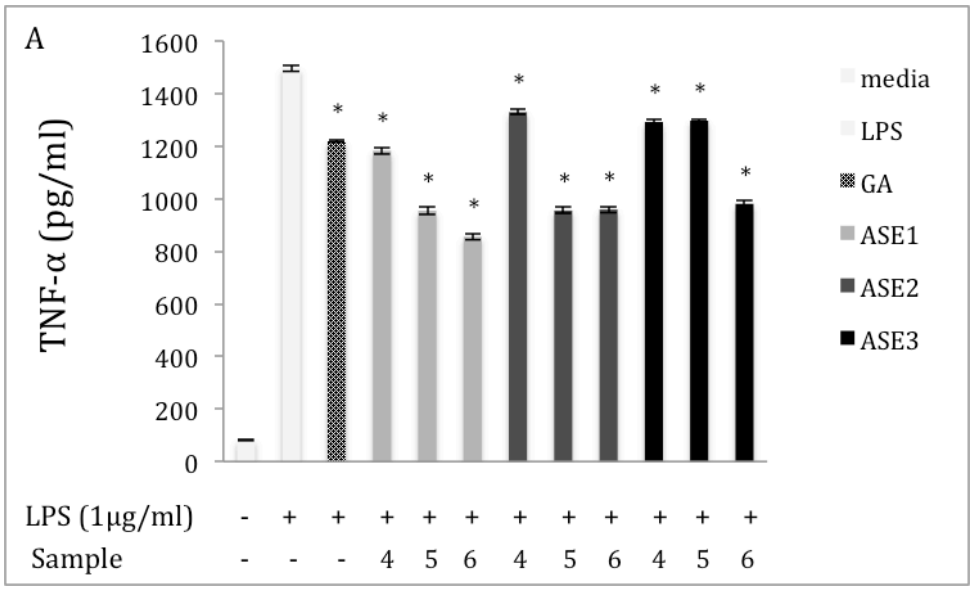
Figure 4-2 Effect of various samples on production of nitric oxide (NO) in macrophage RAW 264.7 cells (A) extracts obtained by ASE (B) pure phenolic standards (C) crude polysaccharide extracts and (D) different extraction methods. Cells were cultured in the absence or presence of LPS (1 μg /mL) with various concentrations of different samples for 24h (0 = media; GA=2 μM; 1= 5 μM; 2=10 μM; 3= 20 μM; 4 = 50 μg/mL; 5=100 μg/mL; and 6= 150 μg/mL; 7= 25 μM; 8 = 50 μM; and 9 = 100 μM). NO production was measured by the Griess reagent and was represented as mean ± standard error (SE) in the bars. Significant different values at $P < 0.05$ and $p < 0.01$.

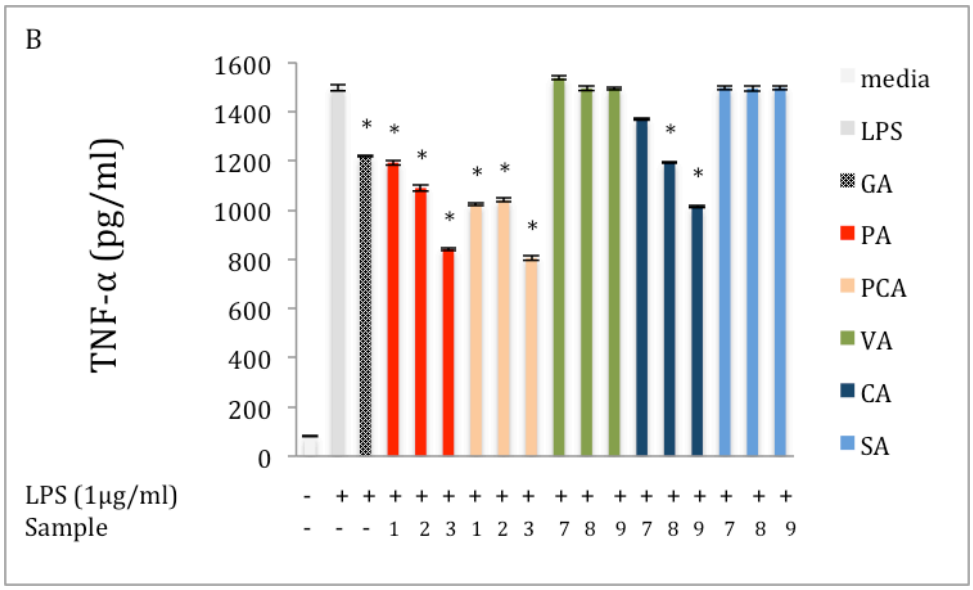
4.3.4 Inhibition of TNF-α Production in LPS-Stimulated RAW 264.7 Macrophages

To evaluate the anti-inflammatory effect of Chaga, we investigated the inhibitory effects of various Chaga extracts and pure phenolic standards on the expression of pro-inflammatory cytokines TNF-α, 1L-6, and IL-1β, in LPS-stimulated RAW 264.7 cells. We found an increase in the expression of TNF-α in the LPS stimulation group compared to the control group (Figure 4-3). Twenty-four hours of incubation with ASE extracts significantly inhibited the level of TNF-α ($P < 0.05$) in the LPS-induced cells, compared with the LPS group. For example, at 150 μg /mL, ASE1, ASE2, and ASE3 extracts reduced the level of TNF-α, released from RAW 264.7 cells by 42.90%, 35.94%, and 31.15%, respectively, compared with the LPS group (Figure 4-3A). The effect of the pure phenolic standards on the expression of TNF-α is presented in Figure 4-3B. All phenolic acids except VA and SA significantly ($p <0.05$) decreased the secretion of TNF-α at all examined concentrations, compared to the LPS treatment. For example, at the highest concentrations, PA, PCA, and CA reduced the expression of TNF-α by 44.3%, 46.7%, and 32.5%, respectively, compared to the LPS treatment. The results from the crude polysaccharide extracts showed that extracts separated from the powder form of Chaga tea had significant inhibitory effects ($P < 0.05$) on the level of TNF-α, compared with the LPS group. For example, at a concentration of 150 μg/mL, P3 and P6 extracts reduced the level of TNF-α in the supernatants by 37.2% and 37.5%, respectively (Figure 4- 3C).

The level of NO was inhibited by Chaga extracts depended on the extraction method. We further examined the effect of the extraction method on the ability of the extracts to attenuate the level of TNF-α. At a concentration of 150 μg/mL, extracts made by RE and SE had significant effects ($p < 0.05$) on the level of TNF-α, compared to LPS treatment. For example, TNF-α expression was inhibited by 22.3%, and 22.1%, after treatment

with 150 µg/mL of RE, and SE, respectively. The results also showed that ME extract had no inhibitory effect on the level of TNF-α at any concentration (Figure 4- 3D).





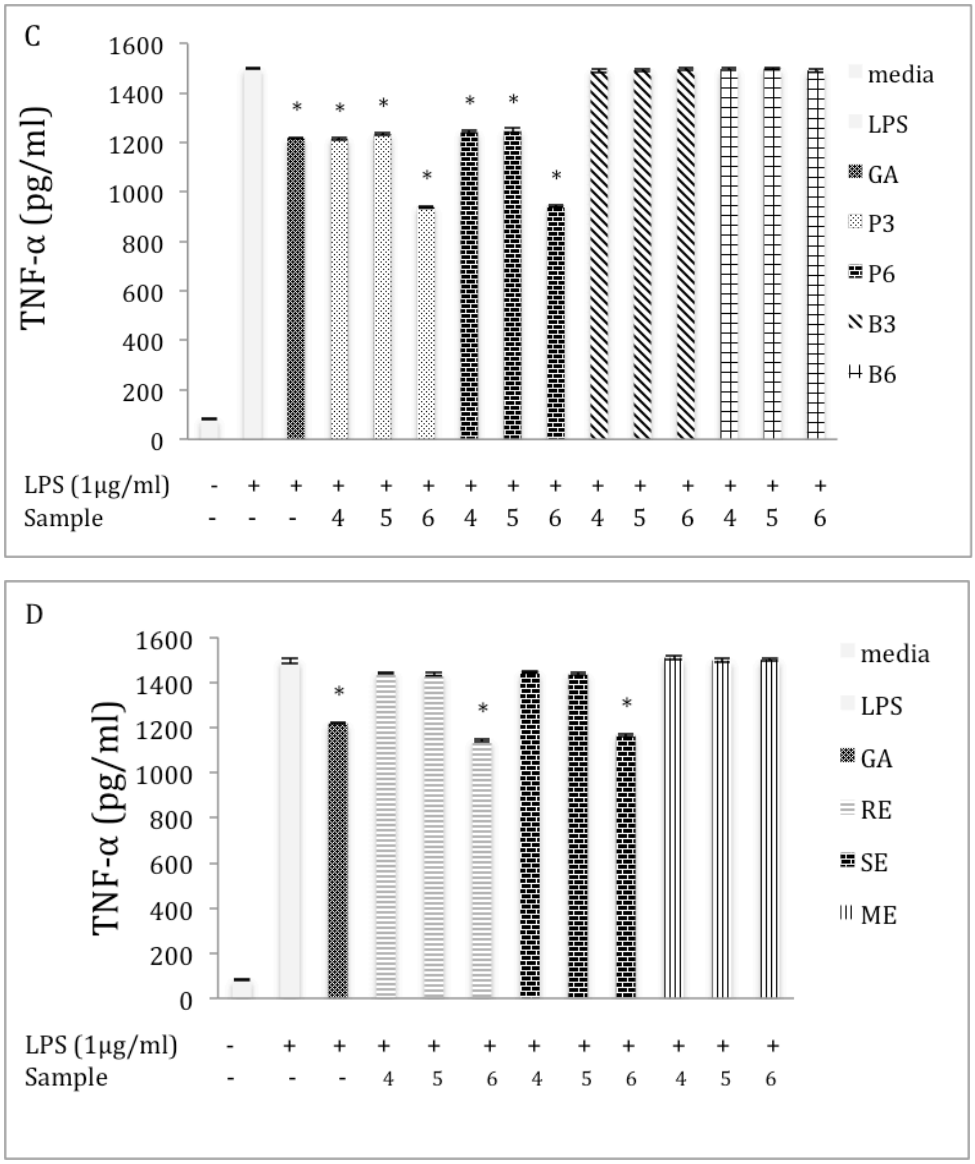
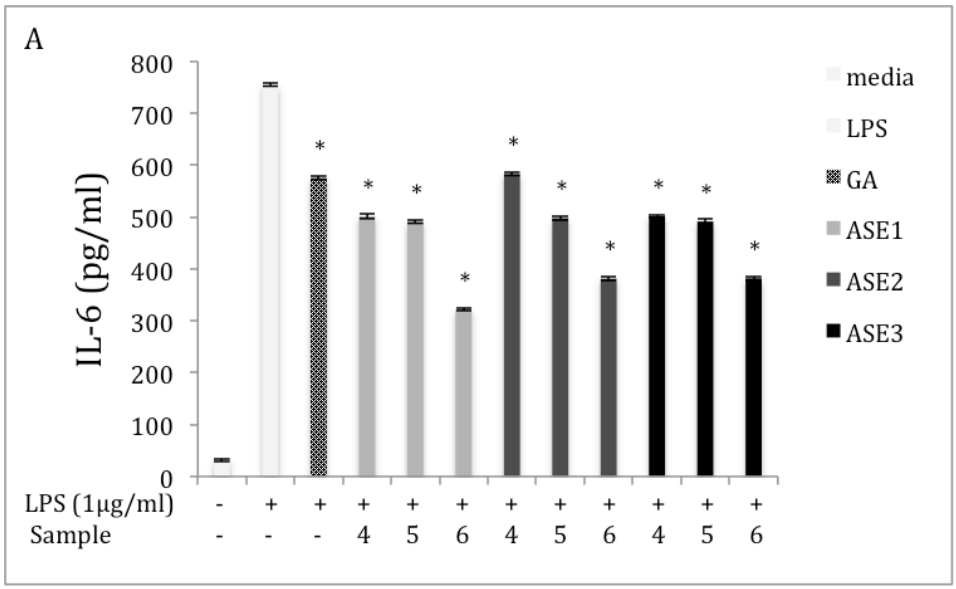


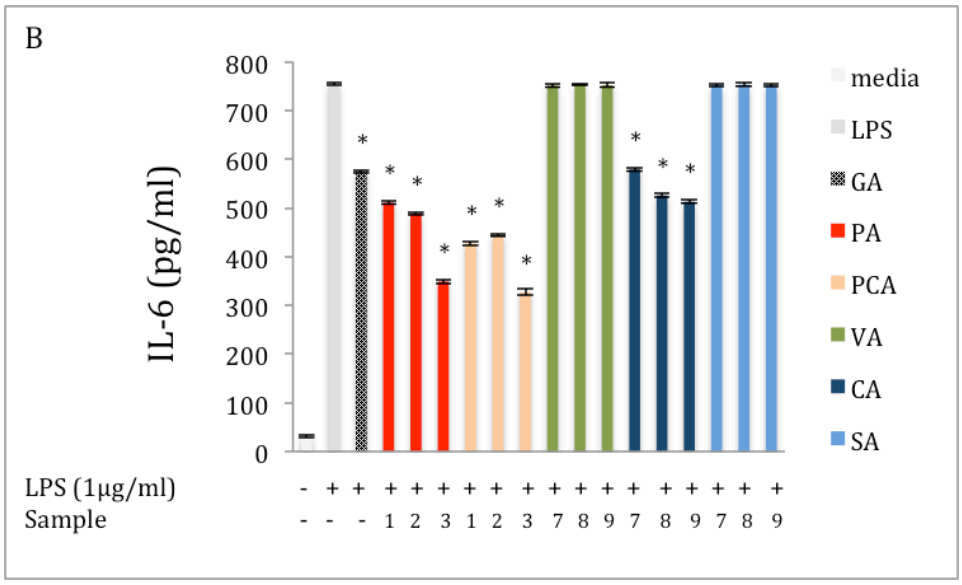
Figure 4-3 Effect of various samples on tumor necrosis factor-α (TNF-α) expression in macrophage RAW 264.7 cells (A) extracts obtained by ASE (B) pure phenolic standards (C) crude polysaccharide extracts and (D) different extraction methods. Cells were cultured in the absence or presence of LPS (1 μg /mL) with various concentrations of different samples for 24h (0 = media; GA=2 μM; 1= 5 μM; 2=10 μM; 3= 20 μM; 4 = 50 μg/mL; 5=100 μg/mL; and 6= 150 μg/mL; 7= 25 μM; 8 = 50 μM; and 9 = 100 μM). TNF-α production was determined through an ELISA. The data represent the mean ± SE of triplicate experiments. Significant different values at $P < 0.05$

4.3.5 Inhibition of IL-6 Production in LPS-Stimulated RAW 264.7 Macrophages

The expression of IL-6 cytokine increased to (755 ± 0.42 pg/mL) in macrophage cells after stimulation with LPS. However, when various Chaga extracts obtained by ASE were added at 50, 100, and 150 μg/mL, these increases were significantly ($p < 0.05$) reduced. For example, at 150 μg/mL, ASE1, ASE2, and ASE3 extracts reduced the level of IL-6 released from RAW 264.7 cells by 57.3%, 49.4%, and 50.4%, respectively, compared with the LPS group (Figure 4-4A). The inhibition activity of phenolic acid standards on the expression of IL-6 in LPS-induced 264.7 RAW cells is displayed in (Figure 4-4B). We observed the same trend of TNF-α expression inhibition for the level of IL-6; all assayed phenolic acids except VA and SA significantly ($p < 0.05$) suppressedthe level of IL-6 at all examined concentration, compared to the LPS treatment. For example, at the highest concentrations, PA, PCA, and CA reduced the expression of IL-6 by 53.7%, 56.6%, and 39.5%, respectively, compared to the LPS treatment. The effect of the polysaccharide extracts on the expression of IL-6 was similar to their effect on TNF-α; the expression of IL-6 was significantly reduced ($p < 0.05$) after treating the induced cells with P3 and P6 extracts at different concentrations. For example, the IL-6 level was reduced by 56.8% and 57.1% after the LPS-induced cells were treated with 150 μg/mL of P3 and concentration (Figure 4-4C). The results in (Figure 4-4D) showed that extracts obtained by SE and RE have significant inhibitory effect ($P < 0.05$) on the expression of IL-6 cytokine, compared with the LPS group. The results in (Figure 4-4D) showed that extracts obtained by SE and RE have significant inhibitory effect ($P < 0.05$) on the expression of IL-6 cytokine, compared with the LPS group. For example, at a concentration of 150 μg/mL, RE and SE inhibited the cytokine

level by 26.6% and 26.9%, respectively, compared with the LPS group. No inhibitory effect of ME extract on the level of IL-6 was observed at any concentration.





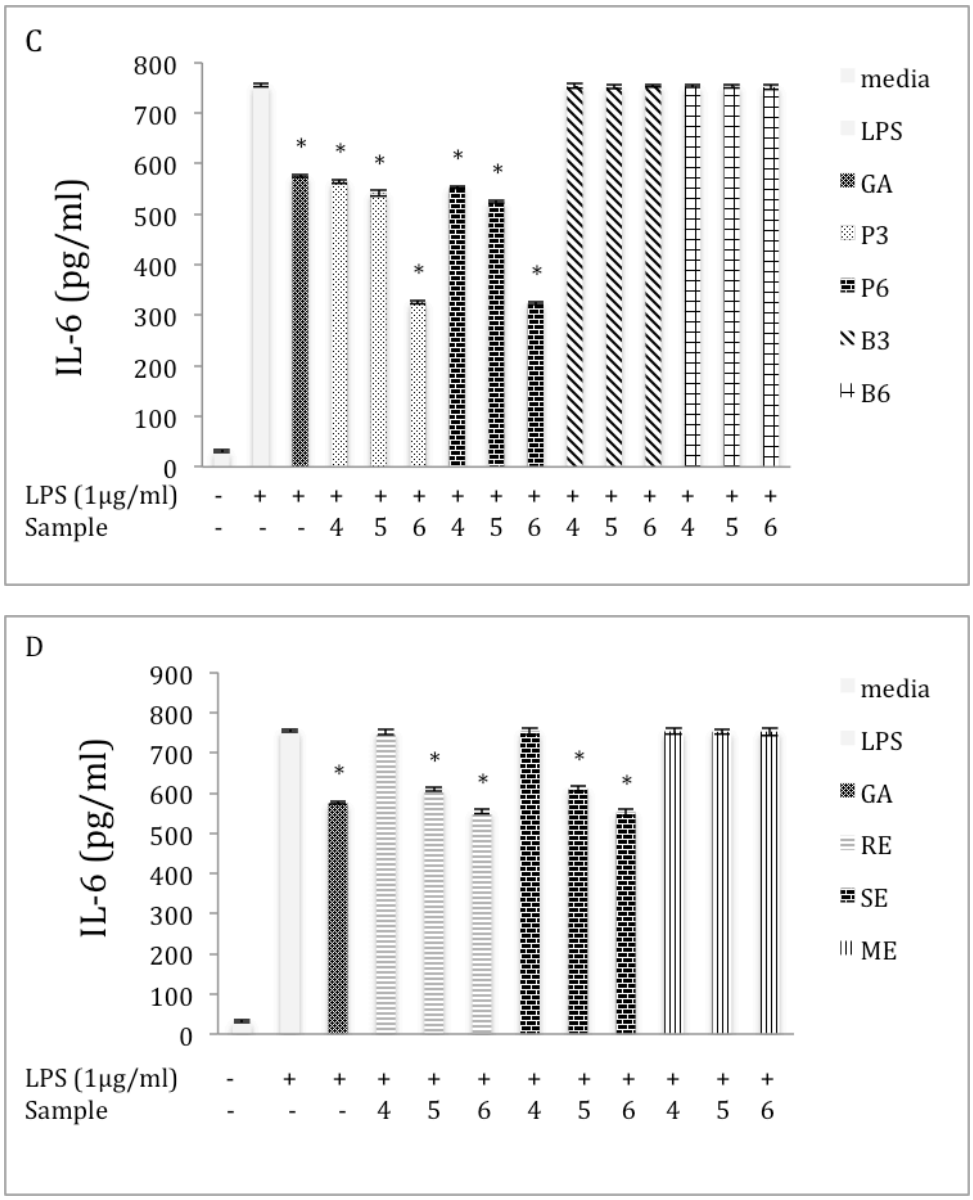
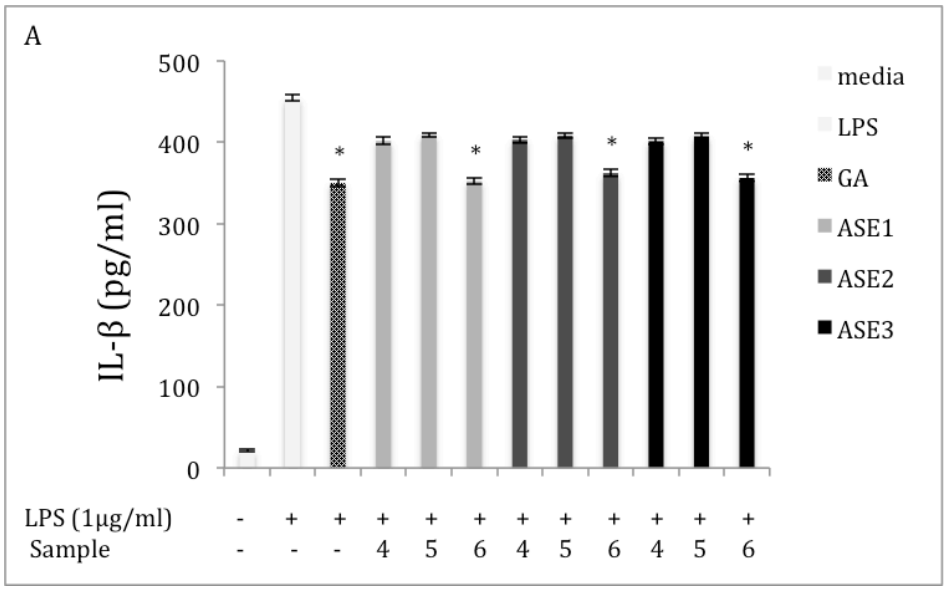


Figure 4-4 Effect of various samples on IL-6 expression in macrophage RAW 264.7 cells (A) extracts obtained by ASE (B) pure phenolic acid standards (C) crude polysaccharide extracts and (D) different extraction methods. Cells were cultured in the absence or presence of LPS (1 µg /mL) with various concentrations of different samples for 24h (0 = media; GA=2 µM; 1= 5 µM; 2=10 µM; 3= 20 µM; 4 = 50 µg/mL; 5=100 µg/mL; and 6= 150 µg/mL; 7= 25 µM; 8 = 50 µM; and 9 = 100 µM). IL-6 production was determined through an ELISA. The data represent the mean ± SD of triplicate experiments. Significant different values at $P < 0.05$.

4.3.6 Inhibition of IL-β Production in LPS-Stimulated RAW 264.7 Macrophages

Our data indicated an increase in the expression of IL-β in the LPS-stimulation group compared to the control group (Figure 4-5). At 150 μg/mL, all Chaga extract obtained by ASE displayed significant inhibitory effect ($p<0.05$) on the level of IL-1β as compared to the LPS group (Figure 4-5A). For example, ASE1 reduced the level of IL-1β by 22.6% compared to the LPS group. Similarly, the level of IL-1β decreased significantly ($p <0.05$) after treating the LPS-induced cells with the highest concentrations of PA and PCA, respectively (Figure 4-5B). For example, the concentration of IL-1β in the supernatants was decreased by 22.6% and 21.5%, after incubated the LPS-induced cells with highest concentration of PA, PCA, respectively. The effect of the polysaccharide extracts on the expression of IL-β is presented in (Figure 4-5C). Polysaccharide extracts from the Chaga obtained by the tea bag form had no ability to attenuate the level of inflammatory cytokine. However, at 150 μg/mL, P6 significantly ($p <0.05$) reduce the level of IL-1β by 21.5% , compared to the LPS treatment. (Figure 4-5D) presents the effect of the extraction method on the inhibition activity of the extracts against IL-1β in the LPS-induced macrophages. None of the extracts obtained by the conventional methods affected the expression of the IL-1β at any concentration, compared to the LPS group.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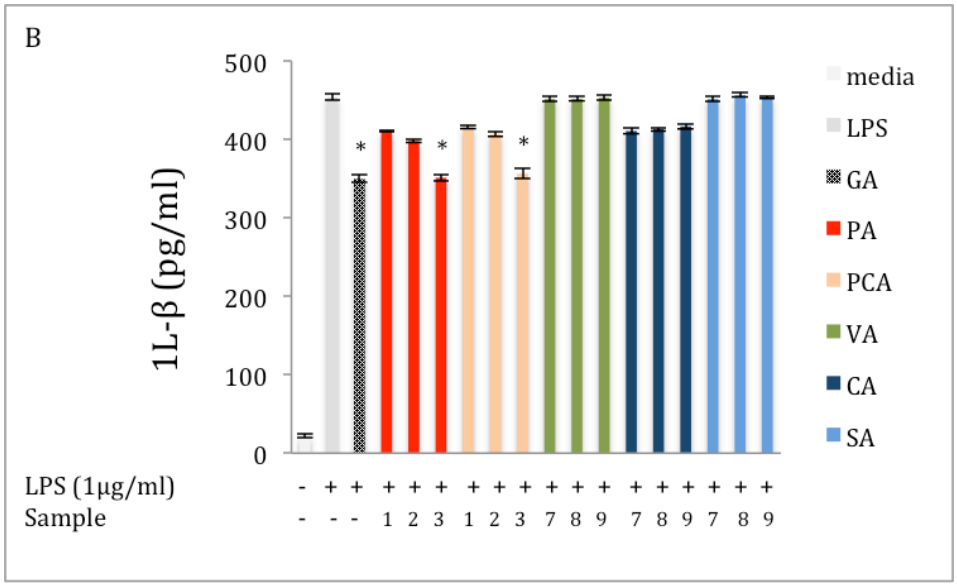
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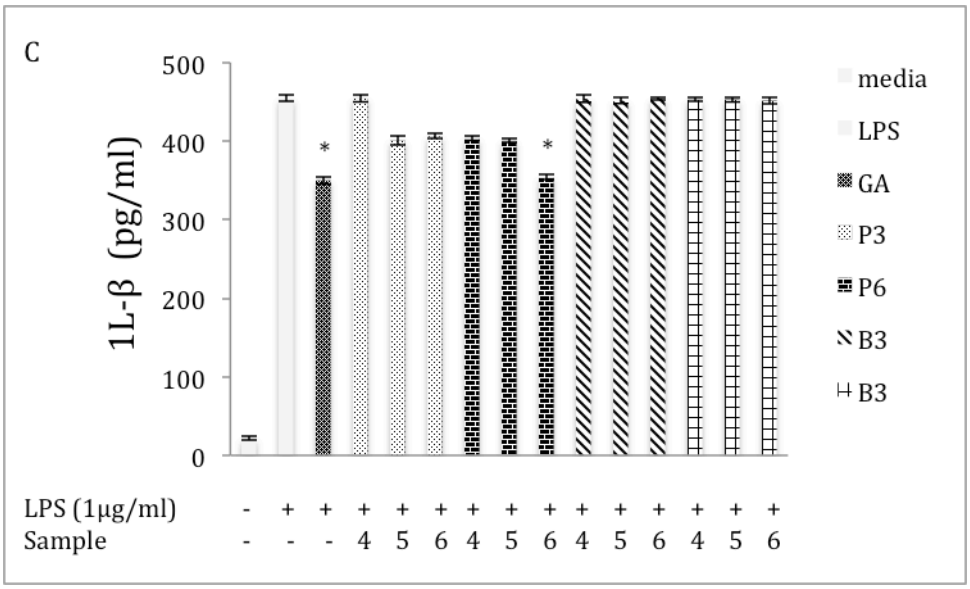

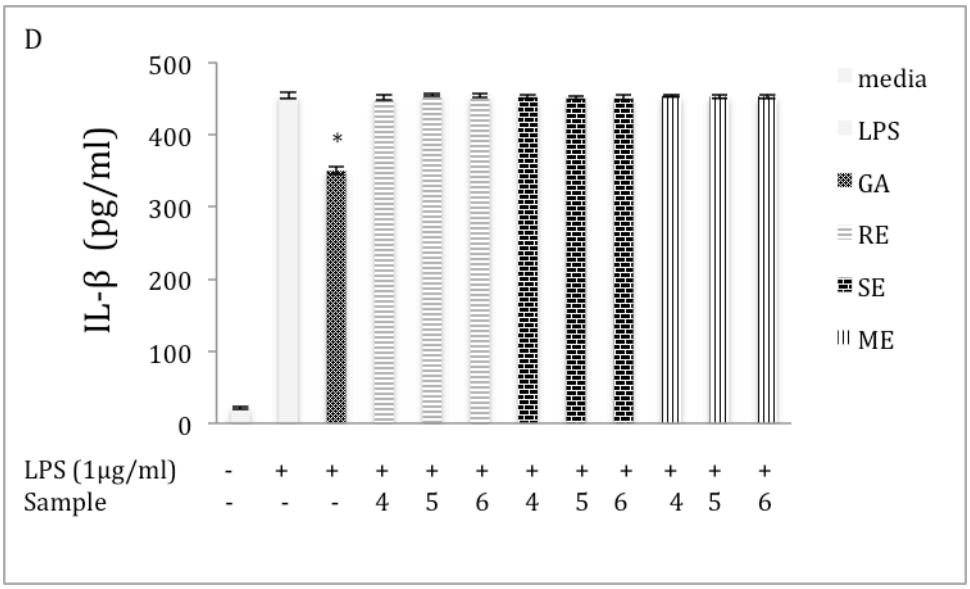

Figure 4-5 Effect of various samples on IL-β expression in macrophage RAW 264.7 cells (A) extracts obtained by ASE (B) pure phenolic acid standards (C) crude polysaccharide extracts and (D) different extraction methods. . Cells were cultured in the absence or presence of LPS (1 μg /mL) with various concentrations of different samples for 24h (0 = media; GA=2 μM; 1= 5 μM; 2=10 μM; 3= 20 μM; 4 = 50 μg/mL; 5=100 μg/mL; and 6= 150 μg/mL; 7= 25 μM; 8 = 50 μM; and 9 = 100 μM). IL-β production was determined through an ELISA. The data represent the mean ± SD of triplicate experiments. Significant different values at $P < 0.05$.

4.4 DISCUSSION

Inflammation is a physiological immune response of body tissues against physical, chemical, and biological stimuli such as tissue injury, chemical toxins, or pathogens (Jeong & Jeong, 2010). Lipopolysaccharide is an endotoxin, an integral outer membrane component of gram-negative bacteria, and the most potent trigger for microbial initiators of inflammatory response (Dobrovolskaia & Vogel, 2002). Macrophages are essential for the initiation and resolution of pathogen- or tissue damage-induced inflammation. Macrophages are cells that play a vital role in the immune system and are associated with inflammatory diseases. Macrophages activated by LPS treatment produce wide variety of inflammatory markers mostly for the primary protection of the host including NO TNF-α, IL-6, and IL-1β. However, excess and uncontrolled production of these inflammatory product lead to excessive inflammatory response and oxidative stress (Dai et al., 2019; Han et al., 2002; Park et al., 2007). Anti-inflammatory agents produce anti-inflammatory effect through regulating cytokines and these inflammatory mediators. Therefore, monitoring the expression of these mediators is vital for understanding the inflammatory process and provides a measure to evaluate the effects of anti- inflammatory agents (Taofiq et al., 2016).

Nitric oxide is a multi-functional mediator that plays an important role in cellular signaling and a variety of physiological functions in many cells and tissues, including the brain, the vasculature, and the immune system (Paige & Jaffrey, 2007). Evidence indicates that overproduction of NO due iNOS is a significant contributor to the inflammatory processes and may provide an indicator of the degree of inflammation (Alderton et al., 2001; Barkett & Gilmore, 1999). Therefore, the inhibition of NO

overproduction may be an essential measure for assessing the anti-inflammatory effects of drugs (Kim et al., 2006).

Inflammation is characterized by the production of a wide variety of free radicals, nitrogen reactive species, and cytokines—such as TNF-α, IL-6 and IL-1β—which act as modulators throughout the inflammation process (Adams & Hamilton, 1984). TNF-α stimulates the production of other cytokines such as IL-6, IL-1β. IL-6 is a multifunctional cytokine with pro- and anti-inflammatory properties that plays a central role in immune and inflammatory responses (Beutler & Cerami, 1989). IL-1β is also a multifunctional cytokine that has been implicated in pain, fever, inflammation and autoimmune conditions (Dinarello, 2002). High levels of these cytokines elicit number of physiological effects including septic shock, inflammation, and cytotoxicity (Garlanda et al., 2007; Sánchez-Miranda et al., 2013). Thus, inhibiting the expression of cytokines in macrophage cells is very important during the anti-inflammatory response.

Chaga has been used for its medicinal properties throughout history in many parts of the world. Numerous scientific reports have investigated the chemical composition and the biological activities of Chaga from various geographical locations. However, no previous study has utilized chaga from the United States of America. In this study, the results showed that different extracts of Chaga sclerotia collected from Maine have significant anti-inflammatory activity on LPS-stimulated RAW 264.7 cells. The inhibitory effect was through a decrease in the production of the NO and a down-regulation of TNF-α, IL-6, and IL-1β in RAW 264.7 macrophages; there was no effect on cell viability at a concentration range of (50–150 μg/mL). The results also showed that phenolic extracts obtained from different extraction methods have different anti-inflammatory properties. All samples made by the accelerated solvent extraction method

and conventional methods significantly reduce the level of NO. The results also showed that ME extract did not affect the NO production at any concentration. The inhibitory effect of Chaga extracts could be attributed to the content of phenolic compounds and DPPH scavenging activity. Phenolic compounds have been largely recognized as natural molecules with potential antioxidant activity. It has been demonstrated that oxidative stress can activate a variety of inflammatory mediators that contribute to the inflammation process; oxidative stress inhibited by compounds with high antioxidant activity such as phenolic compounds. Previous studies have also reported that alcohol extracts of Chaga are rich in polyphenolic compounds that possess strong antioxidant activity and can protect cells against oxidative damage. Such extracts have been reported to attenuate inflammation reactions and decrease the production of inflammatory mediators in 264.7 macrophages.

Previous studies have reported small phenolic ingredients as a main constituent of alcoholic extracts of Chaga that contribute significantly to the antioxidant activity. However, no reports have examined the anti-inflammatory effect of these constituents using RAW 264.7 cells. In our previous report, we increased the extraction of phenolic acids from Chaga by optimizing an ASE extraction method. In this study, we investigated the NO-inhibitory activities of small phenolic ingredients in stimulated RAW 264.7 cells. Our results showed phenolic acids affected different inflammatory mediators; some of the compounds tested did not affect the production of NO or the expression of the inflammatory cytokines, while others had a significant effect. For example, both VA and SA did not alter the production of NO and the expression of TNF-α, IL-6, and IL-1β at any of the examined concentrations. However, PA, PCA, and CA significantly reduced the production of NO and attenuated the expression of TNF-α, IL-6, and IL-1β at all the

examined concentrations. This is in accordance with previous studies, which demonstrated anti-inflammatory properties of PA, PCA, and CA (Juman et al., 2012). However other studies have suggested that phenolic compounds with only one phenol ring—such as the tested compounds—have less of an anti-inflammatory effect through inhibition of cytokine production; it has been hypothesized that other mechanisms might be involved in the anti-inflammatory action of phenolics (Miles et al., 2005). Our results suggest that some of the small phenolic compounds present in Chaga might play a vital role in anti-inflammatory activity.

Previous evidence demonstrates that polysaccharides from many sources have a variety of therapeutic effects including antioxidant and anti-inflammation activities. Polysaccharides in Chaga from Russia, China, and South Korea have been reported to act as immune-modulators and possess anti-inflammation properties from in vivo and in vitro studies. In this study, we examined the chemical structure of crude polysaccharides extracted from Chaga tea, in both powder and bagged form, collected from Maine, USA. We evaluated the anti-inflammation effects of these extracts using LPS-stimulated RAW 264.7 cells. The yield and chemical characteristics of the polysaccharide samples are summarized in Table 1. The results indicate that more components of Chaga could be extracted from the powder form than from bagged form. For example, P6 yielded 30% polysaccharide containing 17.56% carbohydrate, 10.23% protein, 4.12% uronic acid, and 17.61% phenolic content; while B6 yielded 17.33% polysaccharide containing 11.81% carbohydrate, 7.23% protein, 2.84% uronic acid, and 8.33% phenolic content. Our data are in agreement with previous reports suggesting that more bioactive constituents can be extracted from different raw sources in powders forms than other forms. We attribute the higher extraction efficiency to the greater surface area of powdered form, which allows

better penetration of the solvents to target analytes and thus higher extraction efficiency. The results show that the crude polysaccharides of Chaga extracts in both forms have high phenolic content, indicating that these phenolic compounds are bound to macromolecules in Chaga such as polysaccharides and melanin. Chaga contains high amounts of water-soluble macromolecule pigments known as melanin. The dark color of the extracts suggests that a relatively high amount of melanin is present. Other reports also suggested that crude polysaccharide extract from chaga have melanin and melanin-associated phenolic compounds.

Our results demonstrate that crude polysaccharides obtained from both the powder and the bagged form significantly inhibit LPS-induced NO production in RAW 264.7 cells. We observed higher NO inhibitory effect of polysaccharides obtained from Chaga tea in the powdered form, in comparison to those in bagged form at the same concentration. Polysaccharides obtained from the powdered form significantly inhibited the production of TNF-α and IL-6; however, none of the crude polysaccharide extracts obtained in either form altered the expression of IL-1β. However, since the extract is crude, it contains high values of phenolic compounds and melanin, which may contribute to the anti-inflammation effect of the extracts.

4.5 CONCLUSION

With increasing interest in research on the health-promoting effects of Chaga *(Inonotus obliquus*), we have shown here that Chaga collected in Maine, USA can exhibit significant anti-inflammatory properties against LPS-activated 264.7 RAW macrophages. Our results suggest that ASE extract and Chaga tea extract obtained from powder form may be a promising method in the development of new anti-inflammatory supplements. Thus, intake of Chaga as a tea might help to attenuate inflammatory reaction. The data

also showed that the metabolites in Chaga such as phenolic acids inhibited NO production in macrophages.

APPENDIX: OVERALL CONCLUSIONS

To our knowledge, there has been no prior study that optimizes extraction parameters for phenolic compound isolation from Maine-harvested Chaga or assessed the effects of the extraction methodology on anti-inflammation benefits. A simple, precise, and accurate HPLC method for the simultaneous quantification of five phenolic acids extracted from Chaga was developed and validated. Using base and acid hydrolysis conditions, with and without AA and EDTA protection, we determined that the majority of phenolic acids occur bound to the cell wall components of the fungus. Thus, a hydrolysis procedure is required to allow for maximum extraction and quantification of total phenolic acid content. The study also highlighted the protective effect of AA and EDTA on phenolic acids during acid and base hydrolysis.

An Accelerated Solvent Extraction (ASE) based green extraction method was also developed to obtain Chaga-derived phenolic compounds. The response surface methodology (RSM) was utilized for optimizing parameters relative to the extraction of phenolic-rich extracts. Maximum TPC (39.32 mg GAL/g DW) and DPPH scavenging activity (76.59%) yields were produced when an extraction temperature of 170 °C and ethanol: water composition of 66% were used in phenolic compound recovery. Additionally, the total phenolic acids content of the extract was increased at these optimized conditions, supporting our findings of the previously mentioned HPLC work.

Chaga extracts produced under several extraction conditions also resulted in varying degrees of phenolic compound isolation, which subsequently affected the degree of anti-inflammatory benefits. This finding confirms that the bioactive advantage of the fungal extract may be attributed, at least in part, to some of the phenolic acids within the Chaga extracts. Therefore, Maine Chaga demonstrates potential for therapeutic value.

FUTURE WORK:

As an outcome of this work, other future studies became evident and are cited below.

- The extracts from different extraction methods require further characterization to identify the amounts and types of phytochemicals present in each, which most likely resulted in different extraction models for TPC and antioxidants.
- The extracts must be characterized for individual TP to determine if the phenols are acting alone, synergistically, or additively to impact (negatively and positively) both antioxidant and anti-inflammatory effects.
- The crude extracts of Chaga contain carbohydrates and minerals

along with the phenolic compounds, necessitating further purification to remove these impurities for further studies and characterization of individual phenolic compounds.

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