

**EXTRACTION AND CHARACTERIZATION OF POLYSACCHARIDES FROM
CULTIVATED MYCELIUM OF *Inonotus obliquus***

Master thesis in Technology
University of Turku
Department of Biochemistry
Master's Degree Programme in Food Development
July 2020

Kirushaanki Shadakopan

The originality of this thesis has been checked in accordance with the University of Turku quality assurance system using the Turnitin OriginalityCheck service.

UNIVERSITY OF TURKU
Department of Biochemistry

KIRUSHAANKI SHADAKOPAN: Extraction and Characterization of Polysaccharides
from Cultivated Mycelium of *Inonotus obliquus*

Master's thesis in Technology, 77 p., 8 appendix pages

Food Development

June 2020

The medicinal value and the presence of diverse bioactive components have drawn the attention of researchers to medicinal and edible mushrooms in the recent years. *Inonotus obliquus* is a white rot fungus and an obligate parasite of the *Betula* species. It forms a sterile conk on the infected stem, called Chaga, which has been long consumed as a folk remedy in Finland and East-European countries. The health benefits of Chaga consumption have been connected to the presence of polysaccharides in the extracts, which have shown immunomodulating, anti-tumor and antioxidant activities.

The slow growth of Chaga in nature has led to the research of alternative methods for the polysaccharide production such as submerged cultivation of the mycelium, which is fast, easy to control and cost effective. In this study, polysaccharides of *Inonotus obliquus* mycelium, cultivated with the supplementation of sea buckthorn press cake, were extracted and purified. The extraction process included aqueous and alkaline extractions, followed by ethanol precipitation and different purification passages. The obtained polysaccharides were characterized in their total sugar, protein and phenolic contents, monosaccharide composition, and molecular weight. FT-IR spectroscopy was used for structural analysis.

The obtained results indicated the presence of glucose as the major component, along with substantial amounts of mannose and galactose. Other monomers such as xylose and arabinose were also found. Small amounts of galacturonic acid present in the monomer composition was connected to the pectin contained in the supplemented sea buckthorn. The molecular weights of the main polymer populations were determined to be 6.80×10^5 Da, 1.17×10^3 Da, 6.51×10^4 Da, and 1.51×10^4 Da for hot water and alkaline extracts of (0+0.5) and (1+2) fractions respectively. Furthermore, the FT-IR spectra confirmed the presence of proteins, uronic acids, and α - and β -glucans.

Keywords: Chaga, *Inonotus obliquus*, polysaccharides, mycelium

Contents

Abbreviations	7
1 INTRODUCTION	8
2 REVIEW OF THE LITERATURE.....	10
2.1. Chaga.....	10
2.2. Bioactive components of Chaga.....	11
2.2.1. Triterpenoids and sterols.....	11
2.2.2. Betulin and Betulinic acid.....	13
2.2.3. Phenolic compounds	15
2.2.4. Melanins.....	16
2.2.5. Polysaccharides.....	17
2.3. Biological activities of Chaga polysaccharides.....	20
2.3.1. Antioxidant activity	20
2.3.2. Anti-tumor activity	21
2.3.3. Immunostimulating activity.....	22
2.3.4. Anti-hyperglycaemic activity	22
2.4. Submerged cultivation.....	22
2.5. Mycelium polysaccharides and bioactivities.....	25
2.6. Extraction of polysaccharides	26
2.6.1. Aqueous extraction	26
2.6.2. Alkaline extraction.....	27
2.7. Purification of polysaccharides	27
2.7.1. Ethanol Precipitation.....	27
2.7.2. Sevag method.....	27
2.7.3. Dialysis	28
2.7.3. Freeze–thawing.....	28
2.8. Characterization of polysaccharides.....	29
2.8.1. FT-IR spectroscopy.....	29
2.8.2. Gas chromatography	30
2.8.3. High performance size exclusion chromatography	31

3 AIM OF THE STUDY.....	32
4 MATERIALS AND METHODS.....	33
4.1. Mycelium cultivation	33
4.2. Extraction of polysaccharides	33
4.2.1. Ethanol extraction	33
4.2.2. Aqueous extraction	35
4.2.3. Alkaline extraction.....	35
4.3. Purification	35
4.3.1. Ethanol precipitation.....	35
4.3.2. Sevag method.....	35
4.3.3. Dialysis	36
4.3.4. Freeze-thawing.....	36
4.4. Characterization of polysaccharides.....	36
4.4.1. Total sugar content.....	36
4.4.2. Total phenolic content	37
4.4.3. Total protein content.....	37
4.4.4. FT-IR spectroscopy.....	37
4.4.5. Analysis of monomer composition of polysaccharides	38
4.4.6. Molecular weight analysis	39
4.5. Statistical analysis	39
5 RESULTS AND DISCUSSION	40
5.1. Extraction and purification of polysaccharides	40
5.2. Characterization of polysaccharides.....	41
5.2.1. Total sugar content.....	42
5.2.2. Total protein content.....	43
5.2.3. Total phenolic content	43
5.2.4. FT-IR spectroscopy.....	44
5.2.5. Monomer composition.....	47
5.2.6. Molecular weight analysis	52
6 CONCLUSIONS.....	55
References	57

Abbreviations

ABTS	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
ANOVA	Analysis of variance
BSA	Bovine serum albumin
BAEE	4-hydroxy-3,5-dimethoxybenzoic acid 2-hydroxy- 1-hydroxymethyl ethyl ester
CA	caffeic acid
DB	3,4-dihydroxybenzaldehyde
DBL	3,4-dihydroxybenzalacetone
DPPH	2,2-diphenyl-1-picrylhydrazyl
DTA	2,5-dihydroxyterephthalic acid
FT-IR	Fourier-transform infrared
GC-FID	Gas chromatography with flame ionization detector
HPSEC	High performance size exclusion chromatography
HW	Hot water
LLS	Laser light scattering
MWCO	Molecular weight cut off
PCA	protocatechuic acid
RC	Regenerated cellulose
RID	Refractive index detector
SA	syringic acid
SD	Standard deviation
TFA	Trifluoroacetic acid
Tri-Sil	Sililation reagent (HDMS/TMCS/Pyridine)

1 INTRODUCTION

A wide range of edible and medicinal mushroom species are present in nature and they have been consumed in different parts of the world for a long time. Many species of the edible mushrooms have shown health promoting properties and diverse medicinal mushrooms are also consumed as food (Guillamón *et al.*, 2010).

Over the years, medicinal mushrooms such as *Ganoderma lucidum* (Reishi), *Lentinus edodes* (Shiitake), *Schizophyllum commune* (Split gill), and *Inonotus obliquus* (Chaga) have been used in different parts of the world, including East Asia and Northern Europe (Giavasis, 2014; Wasser, 2002).

In recent years, these edible and medicinal mushrooms have gained much attention due to the presence of diverse bioactive components and their medicinal value. Studies have associated the positive health effects of mushroom consumption to the presence of phenolics, minerals, proteins and carbohydrates. Previous research has shown immunomodulating, antimicrobial, and antitumor activities in relation to these bioactive components (Seo, 2011; Giavasis, 2014).

In particular, polysaccharides obtained from mushrooms has shown wide diversity of biological activities and have gained much attention in the medicinal and biochemical research areas in the recent past years (Shi, 2016). Studies have shown most of the mushrooms belonging to the family Basidiomycetes to contain high content of bioactive polysaccharides (Mizuno, 1999; Wasser, 2002).

The slow growth of these fungal bodies in nature has resulted in researchers finding alternatives to grow mycelium in order to obtain the bioactive components. Submerged cultivation is one of the most used technique. It has also been proved to be a fast, easy to control and cost-effective method (Xu *et al.*, 2014b). Supplementation of fibrous materials and lipids in the culture media have been shown effective in promoting growth of mycelium of *I. obliquus* (Yang *et al.*, 2000; Park *et al.*, 2002). In the laboratory of Food Chemistry and Food Development, sea buckthorn (*Hippophaë rhamnoides* L.) berry press cakes have been

used as supplement in submerged cultivation of *I. obliquus*, and mycelium has been harvested and dried.

Current thesis work focuses on the extraction and purification of polysaccharides from *Inonotus obliquus* (*I. obliquus*) mycelium cultivated with the supplementation of sea buckthorn press cake in the medium. The obtained polysaccharides were characterized in their total sugar, protein and phenolic contents, monosaccharide composition, and molecular weight. FT-IR spectroscopy was used for structural analysis. The following review includes examination of recent research on the polysaccharides of *I. obliquus*, their bioactivities and characterization in detail.

2 REVIEW OF THE LITERATURE

2.1. Chaga

Inonotus obliquus (Fr.) Pilát is a white rot fungus belonging to the family *Hymenochaetaceae* of Basidiomycetes. It is an obligate parasite fungus of the *Betula* (birch) species. The fungus causes the decay of lignin, cellulose and hemicellulose, and forms a charcoal black sterile conk (sclerotia) (**Figure 1**), commonly known as 'Chaga', on infected stems (Cui *et al.*, 2005). *I. obliquus*, while mainly parasiting birches, has also been reported to grow on trees such as elm, alder, and ash (Zhong *et al.*, 2009; Song *et al.*, 2013). Sterile conks of Chaga are found within the northern hemisphere, with a distribution covering 40° N–68° N latitude. Most commonly known in the regions of Baltic countries, North America, Northern Europe, Siberia, Northern China, Korea, and Japan (Lee *et al.*, 2007).

The heartwood of the trees decays as the Chaga grows. In the occurrence of host death, the Chaga loses its nutrient supply and the growth stops. *I. obliquus* usually forms one to two sterile conks in the branches and the stem of the host trees (Lee *et al.*, 2008). Two structures, the outer pseudo-sclerotial plate and the inner granular core usually characterize the sterile conks formed by infection of *I. obliquus*. The outer structure of Chaga commonly shows hard, irregular charcoal black appearance, while the internal structure is usually light yellow in color with paler granules of mycelium. The Chaga conks can be 3-40 cm long and show slow growth in nature (Balandaykin & Zmitrovich, 2015). A previous study on Chaga has revealed the growth period of a sterile conk with diameter of 10 cm to be approximately 10-15 years (Ham *et al.*, 2009). In addition, Shashkina and coworkers stated the chemical composition of Chaga as follows; water 13.2%, proteins 2.4%, lipids 2.4%, ash 12-15% (50% K₂O), and carbohydrates 71.9% (Shashkina *et al.*, 2006).

Chaga has long been used as a folk medicine in China, Russia, Finland and North and East-European countries. The usage of Chaga as a folk medicine dates back to the 16th century (Song *et al.*, 2013), while the use of Chaga in Finland has been reported to be as early as in 17th century as a substitute for coffee (Halmetoja, 2014). The study of Siberian folk medicine conducted by Saar found that diseases related to heart, liver, and stomach, psychophysical fatigue, and viper bite are traditionally relieved with Chaga decoctions (Saar, 1991). The

health benefits of Chaga consumption have been connected to the presence of bioactive components, which have shown strong immunomodulating, anti-tumor, antioxidant activities and anti-hyperglycaemic activity (Wang *et al.*, 2015; Wold *et al.*, 2018).



Figure 1. *Inonotus obliquus* sterile conk (Wong, 2019).

2.2. Bioactive components of Chaga

Several studies have shown the presence of diverse bioactive compounds in the sterile conk of *I. obliquus*. These bioactive compounds include polyphenolic components, flavonoids, vitamins, melanins, polysaccharides, and triterpenes (Nakajima *et al.*, 2009; Vamanu *et al.*, 2018; Chen *et al.*, 2020). These bioactive components have shown several biological activities, including antioxidant, anti-tumor, antiviral, antimicrobial, hypoglycaemic, and immunomodulating effect (Mizuno *et al.*, 1999; Cui *et al.*, 2005; Kim *et al.*, 2006). Extracts obtained from Chaga have been used against diseases such as diabetes, tuberculosis, and various types of cancer (Koyama *et al.*, 2008).

2.2.1. Triterpenoids and sterols

2.2.1.1. Triterpenes

Oxygenated triterpenes have been reported as one of the main active components in Chaga. Several terpenes have been identified in Chaga by the researchers over the years. These included lanosterol, inotodiol, trametenolic acid, inonotsuoxides A and B, inonotsulides A,

B, and C, inonotsutriols A, B, and C, and spiroinonotsuoxodiol (Handa *et al.*, 2012; Sagayama *et al.*, 2019). First triterpenes identified in Chaga were lanosterol (**Figure 2**) and its derivative inotodiol (**Figure 3**). Following that, 40 other triterpene compounds have been detected in the *I. obliquus* sclerotia (Nikitina *et al.*, 2016).

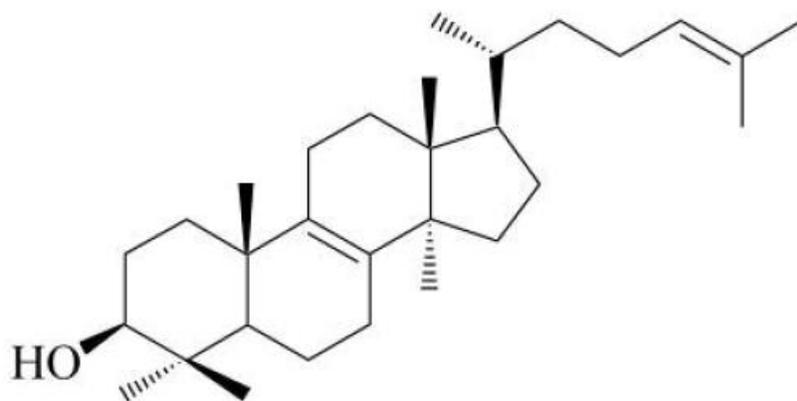


Figure 2. Lanosterol (Nomura *et al.*, 2008).

Triterpene extracts have shown size reduction in sarcoma tumors and antimetastatic effect on sarcoma cells (Nikitina *et al.*, 2016). Further, Zhao and coworkers have reported triterpenoids obtained from Chaga to exhibit strong cytotoxicity against A549 tumour cell lines (Zhao *et al.*, 2015).

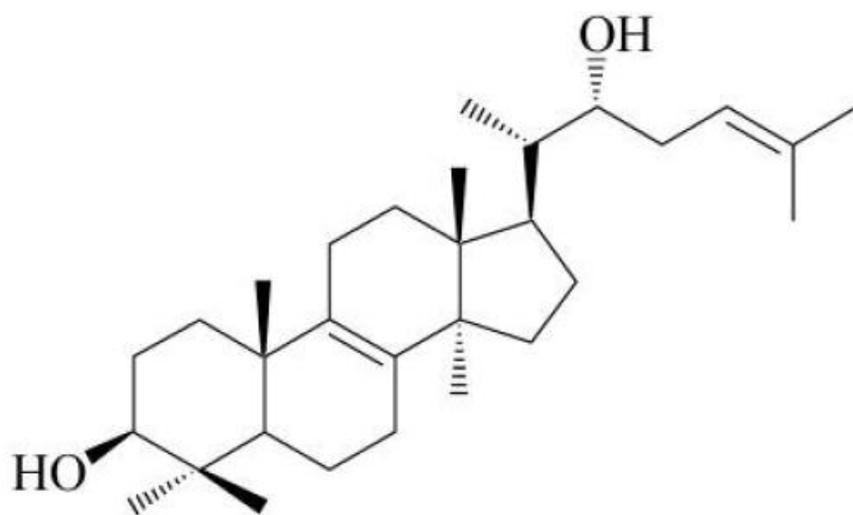


Figure 3. Inotodiol (Nomura *et al.*, 2008)

Further, anti-proliferative activity on B16F1 melanoma cells and 60% growth inhibition of lung cancer cells NC1-H460 were observed in ethanol extracts of Chaga. In addition to the antitumor activities, inotodiol exhibited hypoglycaemic properties, anti-inflammatory and antimutagenic effect (Nikitina *et al.*, 2016).

2.2.1.2. Sterols

Sterols are also present in low amounts in Chaga. Along with ergosterol (**Figure 4**), other steroid compounds namely sitosterol, stigmasterol, sitostanol, and cholesterol have also been found in trace amounts. Chaga chloroform extract containing steroid compounds has shown reduction in proliferation of P388 leukemia cells (Nikitina *et al.*, 2016). Further, a research on bioactive components of Chaga revealed sterols to present strong antioxidant activity (Zhong *et al.*, 2009). Ergosterol has shown antitumor activity against prostate carcinoma. (Nikitina *et al.*, 2016). Further, antioxidative (Shao *et al.*, 2010) and anti-inflammatory (Kuo *et al.*, 2011) have been observed in ergosterol.

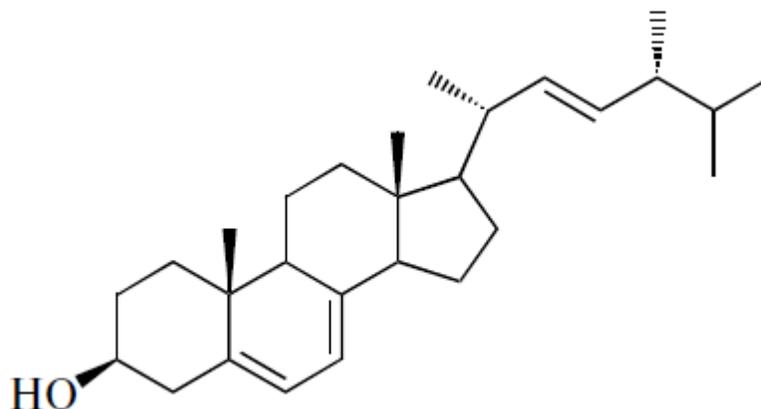


Figure 4. Ergosterol (Nikitina *et al.*, 2016)

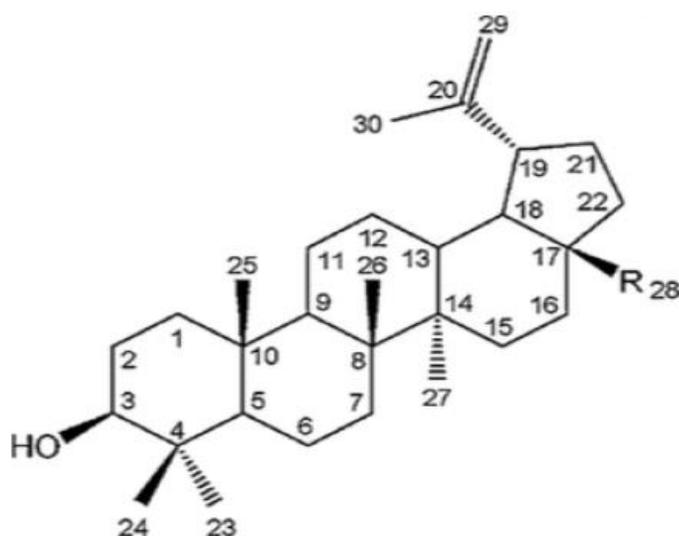
2.2.2. Betulin and Betulinic acid

Studies have revealed betulin and betulinic acid (**Figure 5**) as active compounds with high content in Chaga. A study has reported the presence of betulin in the outer bark of birch trees to be 20-30% of dry outer bark weight (Šiman *et al.*, 2016). The high content of betulin in the birch trees is considered as the main reason for the presence of betulin in Chaga (Abyshev

et al., 2007). Previous research conducted in our lab has shown an amount of betulin of 34.7 mg/ 100 g of Chaga.

The anticancer properties of betulin and betulinic acid have been studied and the results reported antitumor activity along with antioxidant effects that lowered the oxidative stress at cellular level (Kim, 2005). Further, a study on anti-inflammatory effect of betulinic acid reported the reduction in the expression of vascular endothelium growth factor by reducing Sp protein against breast carcinoma MDA-MB-231 and MDA-MB-468 cell lines (Weber *et al.*, 2014).

In addition, anticancer activity has been observed in betulinic acid isolated from the methanolic extract of *Dillenia indica* (elephant apple) L. fruits. The obtained extract was reported to exhibit anti-leukemic activity against human leukemic cell lines namely U937, HL60 and K562 (Moghaddam *et al.*, 2012). Further, antimalarial (Bringmann *et al.*, 1997), anti-bacterial (Woldemichael *et al.*, 2003), and anti-HIV activities (Fujioka *et al.*, 1994) were observed in betulinic acid.



R=CH₂OH (Betulin)

R=COOH (Betulinic acid)

Figure 5. Betulin and betulinic acid (Rizhikovs *et al.*, 2015)

2.2.3. Phenolic compounds

Phenolic compounds have been proved to exhibit antioxidant activity in diverse plant sources over the years. Phenolic compounds present in Chaga have been studied by Nakajima and colleagues and the extracts were reported to contain protocatechuic acid (PCA), caffeic acid (CA), 2,5-dihydroxyterephthalic acid (DTA), 3,4-dihydroxybenzalacetone (DBL), 3,4-dihydroxybenzaldehyde (DB), syringic acid (SA), and 4-hydroxy-3,5-dimethoxybenzoic acid 2-hydroxy-1-hydroxymethyl ethyl ester (BAEE) (**Figure 6**) (Nakajima *et al.*, 2009). Further, a study on the phenolic compound concentration of edible mushrooms reported the concentration of phenolic compounds on Chaga to be 50 $\mu\text{g/g}$ PCA, 51 $\mu\text{g/g}$ homogentisic acid, 263 $\mu\text{g/g}$ *p*-hydroxybenzoic acid, 22 $\mu\text{g/g}$ ferulic acid, 38 $\mu\text{g/g}$ naringin, 52 $\mu\text{g/g}$ quercetiin and 53 $\mu\text{g/g}$ kaempferol (Kim *et al.*, 2008).

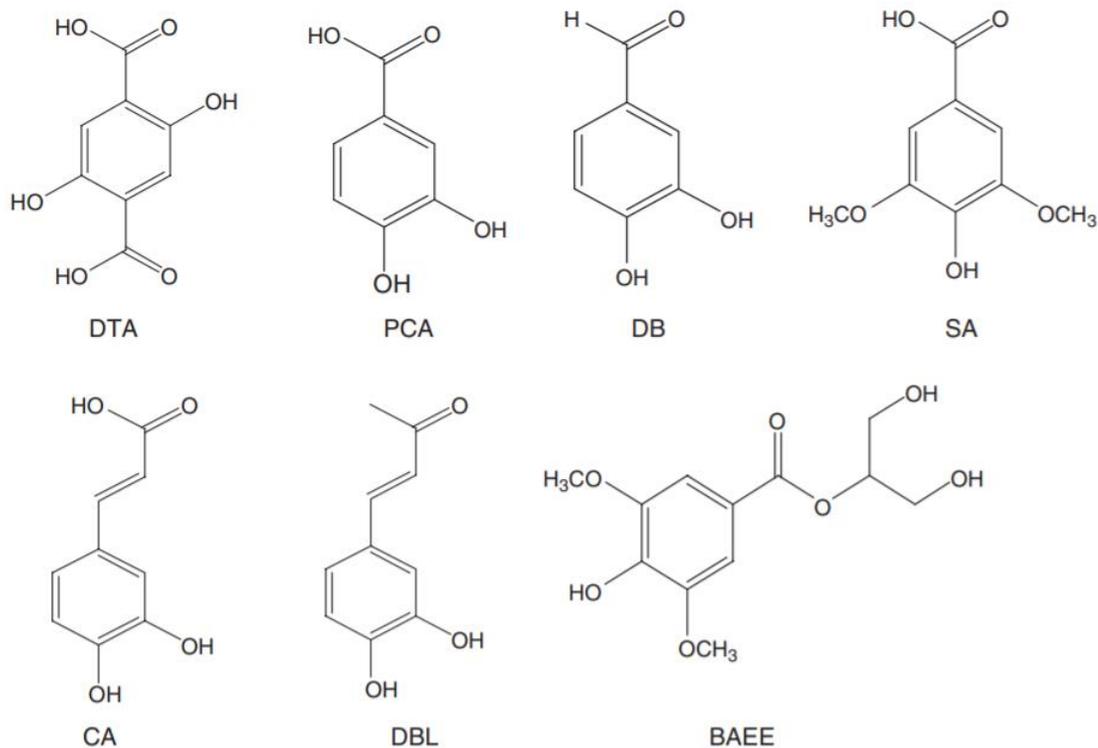


Figure 6. Structure of small phenolic compounds extracted from chaga (Nakajima, 2009)

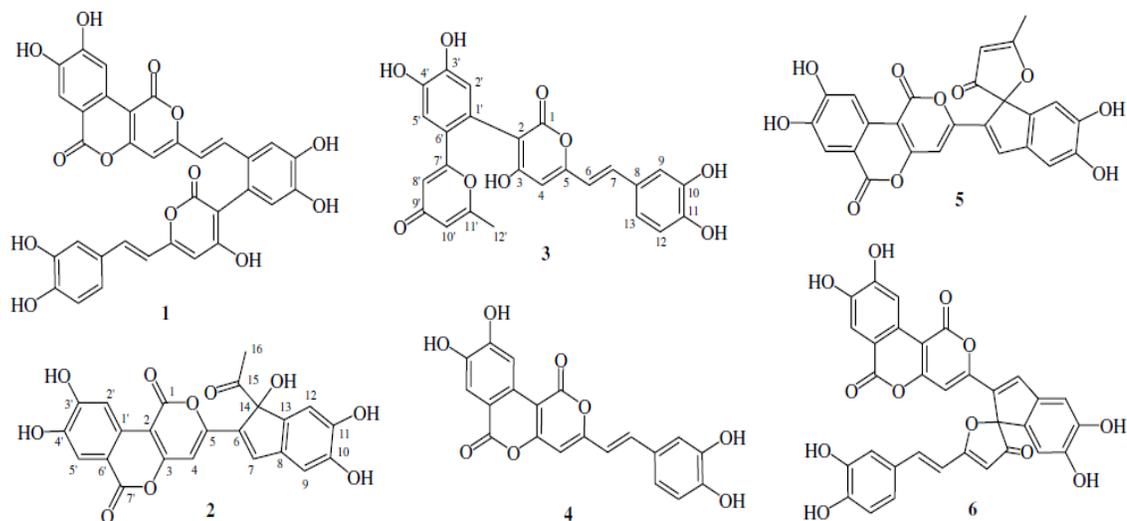


Figure 7. Structures of phenolic compounds 1. Inonoblin A, 2. Inonoblin B, 3. Inonoblin C, 4. Phelligridin D, 5. Phelligridin E, and 6. Phelligridin G (Lee *et al.*, 2007)

Lee and coworkers studied the antioxidant activities of polyphenols extracted from *Inonotus obliquus* sclerotia *in vitro*. The study revealed inonoblins A, B, and C and phelligridins D, E, and G (**Figure 7**) as the major antioxidant polyphenols present in Chaga. The scavenging activities of the extracts were tested against ABTS radical cation and DPPH radical and their antioxidant activity resulted to be higher than the control antioxidant compound, BHA (Lee *et al.*, 2007).

2.2.4. Melanins

Melanins (**Figure 8**) are polymeric pigments present in animals, plants and microorganisms. Differences were observed between the physiochemical properties of melanins obtained from naturally grown Chaga and the melanin synthesized by fungus *I. obliquus* (Zhong *et al.*, 2009). Further, studies on the melanin pigments reported differences between the structures of pigments. Melanin obtained from Chaga were named as allomelanin while the melanin isolated from cultivated *I. obliquus* was named as eumelanin (Zhong *et al.*, 2009).

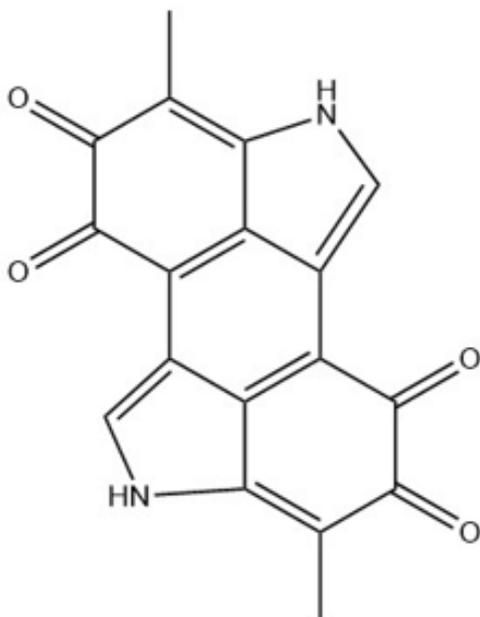


Figure 8. An example of a melanin structure (Gyori *et al.*, 2019)

An *in vitro* study on the antioxidant activity of melanins extracted from *Cinnamomum burmannii* and *Osmanthus fragrans* was conducted by Huang and colleagues. The antioxidant activity was determined by ammonium molybdate method and the results reported higher antioxidant activity to be present in the extracts than BHT (Huang *et al.*, 2010).

2.2.5. Polysaccharides

Polysaccharides (**Figure 9**) are long chain carbohydrates formed by the connection of monosaccharides through glycosidic bonds. The diversity in structures, properties (such as solubility in water) and functions of polysaccharides are mostly based on the composition of monomeric units, type and position of glycosidic linkage, monomers configuration and degree of branching (Shi, 2016).

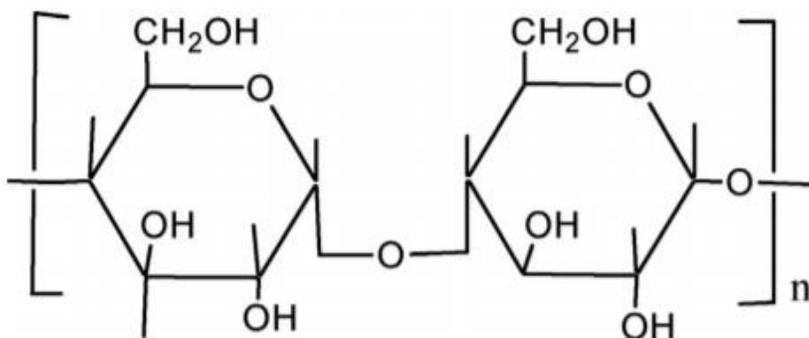


Figure 9. An example of a polysaccharide structure – Amylose (Hassan *et al.*, 2018)

Polysaccharides are found naturally in various sources including plants, fungi, algae, and bacteria. Polysaccharides are present as either energy storage polymer such as starch or as cell wall structural component such as cellulose (**Figure 10**) (Giavasis, 2014). The isolation and purification of the polysaccharides mainly depends on these structural variations present in the polysaccharides (Shi, 2016).

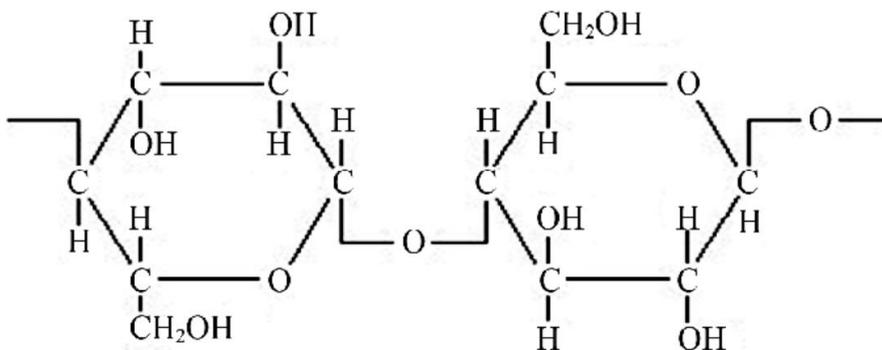


Figure 10. Structure of cellulose (Richards *et al.*, 2012)

Polysaccharides synthesized by fungi are either present on the cell surface, where they constitute the core of the fungal cell wall, or are released to the extracellular medium. Among the several types of polysaccharides present in fungi, glucans are the most abundant. A diverse structural variability is observed in glucans based on the number and distribution of glycosidic bonds, anomeric configuration and branching. These diversities also affect the molecular weight and the characteristics of the glucans. The three main structural types of polysaccharides are α -glucans, β -glucans, and mixed linkage α/β -glucans (Synytsya &

Novak, 2013). Among these, β -glucans (**Figure 11**) are considered to be the most common polysaccharide present in fungi (Kroon-Baternburg & Kroon, 1997; Kataoka *et al.*, 2002; Chan *et al.*, 2009).

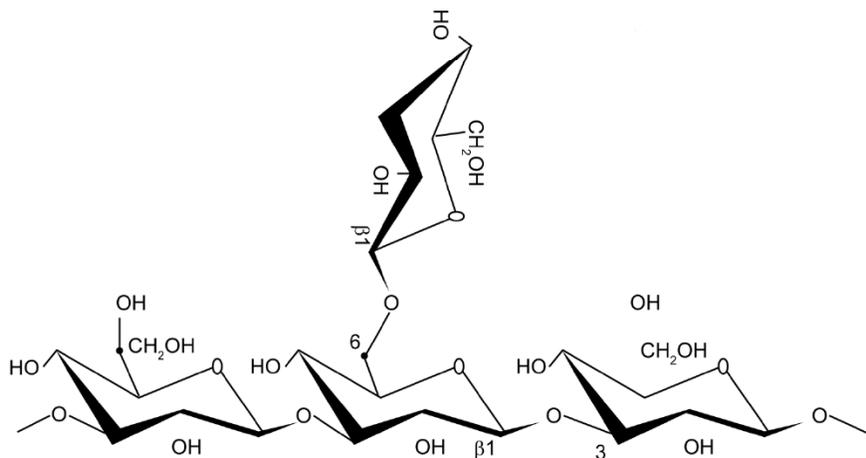


Figure 11. An example of β -glucan structure (Chan *et al.*, 2009)

Fungal β -glucans consists of glucose units linked to each other by β - (1 \rightarrow 3) glycosidic bonds in abundance (Kataoka *et al.*, 2002). These polymers vary among each other according to the branching and the chain length. The branching of glucan polymers is mainly categorized based on two groups namely β -(1 \rightarrow 4) and β -(1 \rightarrow 6) glycosidic chains. Studies have shown different conformations of β -glucans, namely triple helix, single helix or random coils in aqueous solution, and the immune-regulating activity of β -glucans is affected by these conformational complexities. Further, studies have suggested the immunomodulatory activities to be higher when the β -glucans possess high degree of structural complexity (Chan *et al.*, 2009).

Polysaccharides have been identified as one of the major components present in Chaga (Zhong *et al.*, 2009). The monomers present in Chaga are reported to be glucose, mannose, galactose, rhamnose, xylose, and arabinose (Huang *et al.*, 2012; Du *et al.*, 2013; Wold *et al.*, 2018). Further, studies on structure of Chaga polysaccharides revealed the most common glucose structures present in the extracts as (1 \rightarrow 3) Glc and (1 \rightarrow 6) Glc (Wold *et al.*, 2018).

In addition, the presence of -OH groups, C-O-C linkages, and carboxylic acid groups have been observed in FT-IR spectroscopy of Chaga polysaccharide extracts (Xu *et al.*, 2014a; Wold *et al.*, 2018).

The biological activities of polysaccharides including β -glucans present in the medicinal and edible mushrooms have been studied in the past years. The polysaccharides in medicinal mushrooms have been considered as the key component responsible for their physiological functions (Wasser, 2002). Studies on polysaccharides of *Lentinus edodes* (Chihara, 1990) (Shiitake) and *Ganoderma lucidum* (Reishi) reported immunomodulating activities of the β -glucans present in the extracts (Jiang *et al.*, 2016). Similarly, several studies have revealed the presence and activities of polysaccharides in *I. obliquus sclerotia* (Mizuno *et al.*, 1999; Kim *et al.*, 2006). Biological activities of Chaga polysaccharides are discussed further in the following section.

2.3. Biological activities of Chaga polysaccharides

Ma and colleagues stated polysaccharides as one of the main bioactive components present in Chaga (Ma *et al.*, 2013). Based on several findings, it has been reported that fungal polysaccharides usually exhibited low toxicity and diverse pharmacological activities. Several studies have proven the biological activities of polysaccharides, especially in relation to the function of the immune system of organisms (Kim, 2005; Won *et al.*, 2011). Further, recent studies on Chaga polysaccharides have shown anti-tumor, antioxidant, and immunomodulating activities and several clinical assessments have shown potential usage of Chaga extracts as adjuvants during the treatment of cancer, diabetes, and AIDs (Song *et al.*, 2008; Chen *et al.*, 2011).

2.3.1. Antioxidant activity

Antioxidant activity is considered as one of the most important biological activities portrayed by fungal polysaccharides. Antioxidant activity present in the fungal polysaccharides have been studied in recent years in relation to the nutraceutical and pharmaceutical effects of polysaccharides (Song *et al.*, 2008; Xu *et al.*, 2011).

A study conducted by Huang and colleagues reported the increase in superoxide anion scavenging activity with the increase in concentrations of polysaccharide aqueous extracts obtained from the *I. obliquus*. Further the results concluded the presence of antioxidant activity in *I. obliquus* polysaccharides and the activity were observed in a concentration-dependent manner (Huang *et al.*, 2012). Similar results were observed by Hu and co-workers, where concentration depended results were observed in scavenging activities for DPPH and the hydroxyl radicals in Chaga polysaccharides (Hu *et al.*, 2016).

Xu and colleagues studied the antioxidant activity of Chaga along with *I. obliquus* mycelium. The results showed dose-dependent hydroxyl radical scavenging activity. Further, deproteinization increased the activity of polysaccharides extracted from the sclerotia. However, the scavenging activity of sclerotial polysaccharides was found to be lower than vitamin C, a well-known natural antioxidant (Xu *et al.*, 2011).

2.3.2. Anti-tumor activity

The polysaccharides present in fungi have long been known to act as tumor growth inhibitors. Basidiomycete fungi such as *Ganoderma lucidum*, *Lentinus edodes*, and *Schizophyllum commune* contains β -glucans that has shown antitumor and immunomodulatory effects (Smith *et al.*, 2003). Previous research on fungal polysaccharides has indicated the inhibition of oncogenesis and tumor metastasis by the polysaccharides extracted from mushrooms. In addition, the extracted polysaccharides have shown beneficial activity in clinics when combined together with chemotherapy (Kalac, 2016).

Chaga has been widely used for many centuries in Russia and Baltic countries to treat cancer (Zhong *et al.*, 2009). Wang and colleagues conducted *in vitro* cytotoxicity tests on hot water extracts of Chaga purified with DEAE-cellulose anion exchange and Sephadex G-25 gel permeation chromatography. The obtained results indicated that the tumour cell apoptosis was induced by the extracts. Additionally, the activation of nuclear transcription factor NF- κ B in A549 cancer cells was inhibited by the obtained Chaga extracts (Wang *et al.*, 2015).

In another study, the antitumor activity of *I. obliquus* polysaccharide was studied on mice. The results indicated direct inhibition against proliferation of tumor cells. Activity as high as

the positive control 5-Fluorouracil was observed even at lower dosage of polysaccharides from *I. obliquus* (Chen *et al.*, 2010b).

2.3.3. Immunostimulating activity

Several studies on immunostimulant activity of mushroom polysaccharides have suggested the polysaccharides to stimulate T cells, natural killer cell, B cells, and macrophages, playing a role on the enhancement of the immune system (Won *et al.*, 2011). A study on the immunological activities of Chaga polysaccharides attributed the potential to activate macrophages to mainly lignin-carbohydrate complexes. The obtained results showed lignin-carbohydrate complexes to enhance the phagocytic activity of RAW 264.7 macrophages (Niu *et al.*, 2016).

Xu and colleagues studied the stimulation of cytokine production in relation to the *I. obliquus* sclerotia polysaccharides. The obtained results showed the enhancement of proliferation in human peripheral blood mononuclear cells and the stimulation of cytokine production in the presence of extracted sclerotia polysaccharides. This suggested the immunomodulatory activity of Chaga polysaccharides (Xu *et al.*, 2014a).

2.3.4. Anti-hyperglycaemic activity

Polysaccharides extracted from Chaga have also shown anti-hyperglycaemic activities by reducing the glucose levels in the blood. A study on the water-soluble polysaccharides of Chaga reported their ability to inhibit alpha glucosidase *in vitro*, which indicates potential for inducing the inhibition of glucose absorption *in vivo* (Chen *et al.*, 2010b). Another study on aqueous extracts of *I. obliquus* and *Cordyceps militaris* reported the possible use of extracts in treating diabetes, in accordance to the observed effect against different diabetes, in particular types 1 and 2 without causing any severe side effects (Noh *et al.*, 2003).

2.4. Submerged cultivation

Submerged cultivation is an alternative technique for the production of mycelium of medicinal fungi. The microorganisms are grown in liquid medium, which is aerated and stirred. Fungi such as *I. obliquus* (Kim *et al.*, 2006), *Aspergillus fumigatus* (Saqib *et al.*,

2010), and *Ganoderma tsugae* (Peng *et al.*, 2005) has mostly been cultivated with submerged cultivation.

In recent years, submerged cultivation technique has been most widely used in producing mycelium and metabolites of medicinal mushrooms. Submerged cultivation has shown efficient production and has also been used as an alternative in the industrial production of polysaccharides. In particular, submerged cultivation has shown potential as a technique for the production of fungal polysaccharides. Submerged cultivation is fast, cost effective, and easy to control method to produce polysaccharides with lower contamination rates. Further, the submerged cultivation has showed higher production of polysaccharides than the polysaccharides obtained from Chaga (Chen *et al.*, 2020) and the fruiting body of *Lyophyllum decastes* (Pokhrel & Ohga, 2007). Further, the higher production of polysaccharides through submerged mycelium culture, compared to the production through fruit bodies has paved a way for the commercial production of many medicinal mushroom polysaccharides (Litchfield, 1967).

A study on the polysaccharides obtained from submerged cultivation of several edible mushrooms under different culture media showed that polysaccharides could be extracted from both mycelial biomass and growth medium. However, the structural, and physiochemical properties between the polysaccharides obtained from mushrooms, mycelium, and biomass free culture broth differed from each other (Kim *et al.*, 2001).

Chaga has shown slow growth in nature, and great difficulty has been observed in artificially cultivating the sclerotia (Sun *et al.*, 2011). The slow growth of *I. obliquus* has made unfeasible to obtain large quantities of polysaccharides from such sources as natural sterile conks of Chaga for the industrial application (Chen *et al.*, 2007). Submerged cultivation has been used as the effective alternative to produce *I. obliquus* mycelium from which high quantities of polysaccharides are isolated.

Several studies have reported in the last years the submerged cultivation of *I. obliquus* mycelium aimed to extraction of intracellular polysaccharides and exopolysaccharides. Exopolysaccharides and intracellular polysaccharides are the two categories of fungal

polysaccharides where the former is bound to the cell surface as a capsule or a slime, and the latter is extracted from the mycelium (Mahapatra & Banerjee, 2013).

A study (Xu & Zhu, 2011) was conducted on exopolysaccharides and intracellular polysaccharides produced by *I. obliquus* mycelium under submerged cultivation. The results indicated higher production of polysaccharide from the submerged cultivation. Further, a study compared polysaccharides extracted from mycelia of *I. obliquus* harvested from submerged cultivation with those from Chaga, revealing higher content of polysaccharides in cultivated mycelia and stronger antioxidant activity of mycelium polysaccharides than Chaga polysaccharides (Xu *et al.*, 2014a).

Previous studies on the submerged cultivation of *I. obliquus* mycelium has shown enhancement in mycelial production with the supplementation of lignocellulosic biomass in the cultivation medium, and this was due to the ability of *I. obliquus* to decompose lignocellulose (Xu & Zhu, 2011; Xu *et al.*, 2014b). Previous studies have shown the use of fibre supplements such as wheat straw, rice straw, sugarcane bagasse (Xu *et al.*, 2014b), and corn stover (Xu & Zhu, 2011) to increase the mycelium yield of submerged cultivation of *I. obliquus*.

Further, several studies have been conducted to analyse the effects of fatty acids on mycelial growth and polysaccharide production in submerged cultivation. A study on the mycelium of *Ganoderma lucidum* revealed increase in the mycelia and polysaccharide production in the presence of plant oils in the culture medium (Yang *et al.*, 2000). In addition, effects of plant oils and fatty acids in the production of exo-biopolymer and the mycelial growth of *Cordyceps militaris* was studied by Park and colleagues. The results reported substantial increase in the mycelial growth of *Cordyceps militaris* with the supplementation of plant oils in the culture medium (Park *et al.*, 2002).

Northern berries are widely used in the food consumption, and they are mostly processed into juices, jams or purees. However, the remaining press residues which contains high quantities of fibres and bioactive compounds are not frequently used in the food applications.

Sea buckthorn (*Hippophae rhamnoides* L.) is a berry belonging to the family *Elaeagnaceae*, and previous studies have shown the presence of fatty acids and fibres in the press residues

of sea buckthorn berries after juice pressing (Yang & Kallio, 2001; Linderborg *et al.*, 2011). In the current study, the press cake of sea buckthorn was utilized in replacement of lignocellulosic fibre used on previously (Xu *et al.*, 2014b) to enhance the submerged cultivation yield of *I. obliquus* mycelium.

2.5. Mycelium polysaccharides and bioactivities

Mycelium has been used as an effective alternative to fruiting body for the extraction of polysaccharides. It is important to verify the nutritional and medicinal values of cultivated mycelia in comparison to the mushroom fruiting bodies. From previous studies, mannans have been reported as the major components of bioactive hetero polysaccharides. Several studies have reported the antitumor and immunomodulating activity of mannans *in vivo* and *in vitro* (Kiho *et al.*, 1992; Mizuno, 1999). It has been reported that α -(1 \rightarrow 3) mannans, glucomannan, and galactoglucomannan are the highly present components in mycelia obtained from submerged cultivation of *Dictyophora indusiata*, *Agaricus blazei*, and *Lentinula edodes* respectively while the hetero polysaccharide obtained from the mycelia of *Phellinus linteus* primarily contained mannose as the monomer unit (Wasser, 2002).

Peng and coworkers have shown that the cultured mycelium of *Ganoderma tsugae* is a better source of bioactive components than the wild fruiting body, especially regarding polysaccharides (Peng *et al.*, 2005). Their study further showed that polysaccharide extracts of *G. tsugae* mycelium possess anti-tumor activities *in vivo*. Additionally, a research was carried out on the immunomodulatory effect of exopolysaccharides produced by *Laetiporus sulphureus* mycelium on human leukemia U937 cells. The exopolysaccharides showed crucial effect on apoptosis mediators Bax and Bad, indicating the immunomodulatory effect (Seo *et al.*, 2011).

A study (Xu *et al.*, 2011) on polysaccharide extracts of sterile conk and cultured mycelium of *I. obliquus* has shown significant enhancement in the antioxidant activity for the polysaccharides obtained from *I. obliquus* mycelium. The results showed prominent hydroxyl radical scavenging activity and inhibition of lipid peroxidation for both the extracellular and intracellular polysaccharide extracts obtained from the cultured mycelium of *I. obliquus*.

Furthermore, a study was conducted by Xu and coworkers to investigate the immunomodulatory activity of polysaccharides obtained from *I. obliquus* mycelium. The polysaccharides extracted from the mycelium were observed to enhance the cell proliferation and stimulate the secretion of cytokines by human peripheral blood mononuclear cells. The study also suggested that the mycelium polysaccharides of *I. obliquus* could replace the polysaccharides of Chaga in the development of immunomodulator nutraceuticals (Xu *et al.*, 2014a).

Further, a research on anti-cancer effect with *in vitro* and *in vivo* assays was conducted by Kim and co-workers on α -fucoglucomannan extracted from cultivated mycelia of *I. obliquus*. They reported *in vivo* inhibition of the tumor growth in BDF1 mice. Moreover, the results indicated the activity *in vivo* was the result of immunostimulation rather than cytotoxic activity. Intracellular polysaccharides obtained from cultivated mycelia of *I. obliquus* would then have potential in clinical use for cancer prevention and treatment (Kim *et al.*, 2006).

2.6. Extraction of polysaccharides

Fungal polysaccharides could be extracted from fruiting bodies, mycelial biomass or as exopolysaccharides released directly in the cultivation liquid. In addition, mushroom spores and sclerotium are also considered as good sources of polysaccharides (Ruthes *et al.*, 2015). Cultivated mycelium and sterile conk of *I. obliquus* are the major sources of polysaccharides considered in this research. The extraction of polysaccharides is mainly carried out with two media, aqueous and aqueous diluted alkali.

2.6.1. Aqueous extraction

Aqueous extraction is the most commonly used method for the extraction of polysaccharides from the fruit bodies, as most of the fungal polysaccharides show high solubility in hot water. Further, the stability of polysaccharides in hot water minimizes degradation during the process of extraction. Nonpolar compounds such as lipids, phenols, and terpenes present in the fruit bodies are usually extracted beforehand using organic solvents such as ethanol, acetone, or mixtures of CHCl_3 : MeOH, enhancing the purity of the polysaccharides subsequently extracted (Giavasis, 2014; Shi, 2016).

2.6.2. Alkaline extraction

Certain acidic polysaccharides and high molecular weight polysaccharides shows insolubility in hot water while they are easily dissolved by dilute alkali solutions. For this reason, solutions of 2%-15% (w/v) NaOH, KOH or Na₂CO₃ are often used at 80°C for the extraction of the polysaccharides. The concentrations of the alkaline solutions and the extraction temperatures differ based on the samples and solutions. (Chen *et al.*, 2015; Ruthes *et al.*, 2015; Shi, 2016). In general, water soluble polysaccharides are first extracted using hot water and then the remaining polysaccharides are extracted using dilute alkali solution (Shi, 2016).

2.7. Purification of polysaccharides

In general, glucans form complexes with other polysaccharides and molecules resulting in the interference of its chemical analysis and bioactivity. Therefore, following the extraction of polysaccharides, several purification steps should follow to eliminate other substances such as proteins, phenolic compounds, monosaccharides, and other molecules in order to obtain pure polysaccharide fractions. In this study, purification methods such as ethanol precipitation, sevag method, dialysis, freeze thawing and freeze-drying were applied.

2.7.1. Ethanol Precipitation

The most common step for the purification of polysaccharides is the precipitation with cold ethanol. This step is based on the reduction of the dielectric constant of the solvent by the addition of ethanol to the polysaccharide extracts, as the dielectric constant of ethanol is lower than that of water. Thus, the addition of ethanol results in the aggregation and precipitation of polysaccharides in the extract (Liu *et al.*, 2016).

2.7.2. Sevag method

Sevag method is a process used to remove the proteins from the crude extract by denaturation. Chloroform: n-butanol mixture is generally used to denature the protein in the polysaccharide extract. After the addition of solvent mixture, the sample is violently shaken. Once the solution is allowed to stand, proteins form a jelly layer between the upper aqueous and lower organic phase. The lower phase is discarded, and the protein layer is removed by

centrifugation. For the efficient removal of proteins, it is usually repeated three times (Shi, 2016).

2.7.3. Dialysis

The crude extracts typically contain a wide range of impurities such as inorganic salts and low molecular weight nonpolar compounds. Several purification methods are used to remove these substances, and dialysis is one of the most common method used in the purification of polysaccharides (Shi, 2016).

Dialysis is the technique where impurities and unwanted compounds in solution are removed by selective and passive diffusion through a membrane with pores of defined dimensions. Small particles diffuse into the dialysate, which is normally 200 to 500 times larger in volume than the sample, while the particles that are larger than the membrane pores remain in the sample solution. Moreover, changing the dialysate within regular time intervals allows more substances to diffuse into the dialysate and this allows to minimize the concentration of smaller particles within the sample solution (Shi, 2016).

In addition, the semi permeable membranes used as for dialysis are generally designed for protein purification. Hence, there has been difficulty in distinguishing the correct molecular weight cut off (MWCO) membrane for polysaccharide purification. Further, the polysaccharides also differ from the elution of proteins, due to the different conformations attained by the polysaccharides in solutions along with their molecular weight, and this could influence the purification process by dialysis (Ruthes *et al.*, 2015). The MWCO of the membrane should be chosen carefully for the polysaccharides and the dialysis process should not prolong more than 36 hours as it might result in the dialysis bag getting moldy. The process of dialysis is mostly done in closed systems and has been proved as an effective method of purification with little or no loss of sample yield (Shi, 2016).

2.7.3. Freeze–thawing

Freeze-thawing is a simple method where the crude extract dissolved in a concentrated aqueous solution is frozen and then slowly thawed at 4°C. The process separates water-soluble and -insoluble glucans. The solubility of the polymers is based on the differences

between the degrees of branching of polysaccharides. Molecules that have a linear structure or fewer branches tend to precipitate, while those with a higher degree of branching remain in the fraction soluble in cold water (Ruthes *et al.*, 2015).

2.8. Characterization of polysaccharides

The high diversity in composition, linkages, configuration, and molecular weight of polysaccharides results in the requirement of diverse techniques to analyze the polysaccharides (Hu *et al.*, 2016; Wang *et al.*, 2015). Further, it has also been observed from previous studies, that the bioactivities of polysaccharides are affected by the composition, linkages, configuration, and molecular weight (Zhang *et al.*, 2011; Bae *et al.*, 2013). Polysaccharides are characterized through several techniques, the most commonly used being gas chromatography, liquid chromatography, mass spectrometry, infrared spectroscopy, and nuclear magnetic resonance (NMR) (Mulloy *et al.*, 2009).

2.8.1. FT-IR spectroscopy

Fourier-transform infrared spectroscopy (FT-IR) is a common technique used in the structural analysis of carbohydrates. The spectra obtained from FT-IR provides information such as physical and chemical properties of the compounds present in the sample. Further, specific wavenumbers in the spectra highlights the presence of molecules bound to the polysaccharides or fraction impurities, such as proteins (amide bands) or remainder phenolic compounds (aromatic C=C bands) and lipids in the polysaccharide extract (Ruthes *et al.*, 2015). FT-IR spectra also provides the information on prevailing anomeric configuration and glycosidic linkages in the sample.

Diverse fungal polysaccharides and their glucans have been analyzed with FT-IR spectroscopy (Synytsya *et al.*, 2009). The infrared spectrum of polysaccharides includes two major spectral regions, namely the ‘sugar region’ between 1200-950 cm^{-1} and the ‘anomeric region’ between 950-750 cm^{-1} . Sugar region mainly includes the glycosidic bonds and pyranoid rings with highly overlapped bands of C-O and C-C stretching vibrations, while the anomeric region includes the weaker bands of complex skeletal vibrations assigned to the glycosidic linkages of polysaccharides. In addition, bands in the region of 2500-3700 cm^{-1}

are produced by the stretching of the O-H and N-H bonds, and the bands between 1540-1650 cm^{-1} corresponds to the 'protein region' (Synytsya & Novak, 2014).

2.8.2. Gas chromatography

The carbohydrate composition of polysaccharides can be analyzed with different methods. Some of these methods are thin layer chromatography (TLC), high performance liquid chromatography (HPLC), and gas chromatography (Shi, 2016). In this study, gas chromatography with flame ionization detector (GC-FID) was used to analyze the monosaccharide composition.

Homo and heteropolysaccharides are made of anhydrosugar residues covalently bound by glycosidic linkages. Hence, accurate analysis of the monosaccharide composition requires depolymerization of polysaccharides. The most commonly used depolymerization methods are acid hydrolysis, acid methanolysis, and enzymatic hydrolysis. Acid hydrolysis is mostly used to depolymerize cell wall polysaccharides, while acid methanolysis efficiently depolymerizes hemicelluloses, and enzymatic hydrolysis of polysaccharides are mostly used on delignified wood samples (Willför *et al.*, 2009). The current study included the acid hydrolysis of extracted polysaccharides prior to the GC analysis.

Further, carbohydrates should be derivatized in order to perform gas chromatography. Commonly, silylation, fluoroacylation, or acetylation derivatizations are carried prior to analysis. Among these methods, silylation is considered to be the simplest method based on its rapid reaction mechanism, and it is applicable to all carbohydrate classes (Ruthes *et al.*, 2015).

Xu and coworkers analysed the monosaccharide composition of polysaccharides extracted from Chaga and *I. obliquus* mycelium using GC-FID. They reported the presence of monomers glucose, galactose, xylose, rhamnose, arabinose, and mannose in the extracts. Glucose was reported as the major monomer in intracellular polysaccharide extracts (Xu *et al.*, 2014).

2.8.3. High performance size exclusion chromatography

High performance size exclusion chromatography (HPSEC) allows the determination of the molecular weight of the polysaccharides (Ruthes *et al.*, 2015). The principle behind HPSEC is the separation of molecules by size through a filtration gel. This gel contains spherical beads with pores of specific size distribution. These pores separate the molecules by including or excluding them depending on their sizes. Larger molecules are not entering the pores, hence elute earlier, while smaller molecules enter into pores of multiple size, hence elute later. Every SEC column is characterized by an elution limit, the upper limit of molecular weight beyond which the molecules elute together at the same retention volume, and a penetration limit, the limit below a certain pore size where the molecules penetrates completely. (Giddings, 1967).

The molecular weight of a specific polysaccharide population can be determined by calibrating the analytical system with a set of standard polymers, such as dextrans or pullulans, of defined molecular weight. Doing so, it is possible to draw a relationship between molecular weight and retention time (or elution volume) of the polymer. This relationship is linear if $\log(M)$ is used for calibration. Further, HPSEC is coupled with refractive index detectors (RID). RID measures the changes in refractive index of the mobile phase, which are due to the elution of molecules. Moreover, the refractive index of a liquid depends on the temperature and pressure. Hence, stable temperature must be maintained within the analytical system (Cheong *et al.*, 2015).

HPSEC has been used to determine the molecular weight of Chaga polysaccharides in previous studies. (Xu *et al.*, 2014a; Wold *et al.*, 2018). The results obtained from HPSEC reported the molecular weight of Chaga polysaccharides to range between 60-96 kDa (Xu *et al.*, 2014a; Wold *et al.*, 2018).

3 AIM OF THE STUDY

The aim of the present study was to extract and purify polysaccharides from *Inonotus obliquus* mycelium, which was cultivated with the supplementation of sea buckthorn press cake. The polysaccharide extracts were characterized, and their physico-chemical and chemical properties were analyzed. The characterization process included quantification of the total sugar, protein and phenolic contents, structural analysis by FT-IR spectroscopy, and monosaccharide composition and molecular weight analyses. The comparison of the results provided an insight on the production of polysaccharides from the mycelium of *I. obliquus* cultivated in liquid medium.

4 MATERIALS AND METHODS

4.1. Mycelium cultivation

I. obliquus mycelium was cultivated in triplicate in control and sea buckthorn containing media. The control liquid cultivation medium contained (g/200 mL) maltose 3, glucose 3, peptone 0.4, beef extract 0.26, MgSO₄ 0.3, KH₂PO₄ 0.5, and vitamin B1 0.002. The press cake medium contained the same components of control medium, in addition to different concentrations of sea buckthorn press cake, namely 0.5, 1, 2, and 6 g/200 mL (dry weight/volume of culture medium). Two different cultivation times, 200 h and 250 h, were used to grow the mycelium. The culture was incubated in a rotatory shaker with a rotation speed of 140 rpm at 27°C. Each cultivation was performed in triplicate.

A previous study on the monomer composition of the cultivated mycelia indicated no significant difference in the monomer composition between mycelial samples cultivated in control medium and control medium supplemented with press cake at dosage of 0.5 g/200 mL, nor between mycelia cultivated with sea buckthorn press cakes added at two different dosage, 1 and 2 g/200 mL. Hence, the mycelia cultivated without sea buckthorn and with 0.5 g/200 mL sea buckthorn dosage were pooled together for polysaccharide analysis. The mycelia obtained with 1 and 2 g/200 mL press cake dosages were pooled together, in order to gain a sufficient amount of samples for extraction and further purification of polysaccharides. The two mycelia pools were dried at 70°C and milled with a mortar prior to polysaccharide extraction.

4.2. Extraction of polysaccharides

Figure 12 presents a detailed scheme consisting of series of extractions and purification steps of polysaccharides from mycelia of *I. obliquus*.

4.2.1. Ethanol extraction

Milled *I. obliquus* mycelia were extracted three times using 95% ethanol with a solid: liquid ratio of 1:5, at room temperature for 6 h in order to remove free sugars and phenols and to deactivate endogenous enzymes. The obtained ethanolic mixture was filtered and the supernatant was removed. The mycelial residue was subjected to aqueous extraction.

Inonotus obliquus cultivated in supplemented medium (0, 0.5, 1 and 2g/200ml) with cultivation times 200 h and 250 h

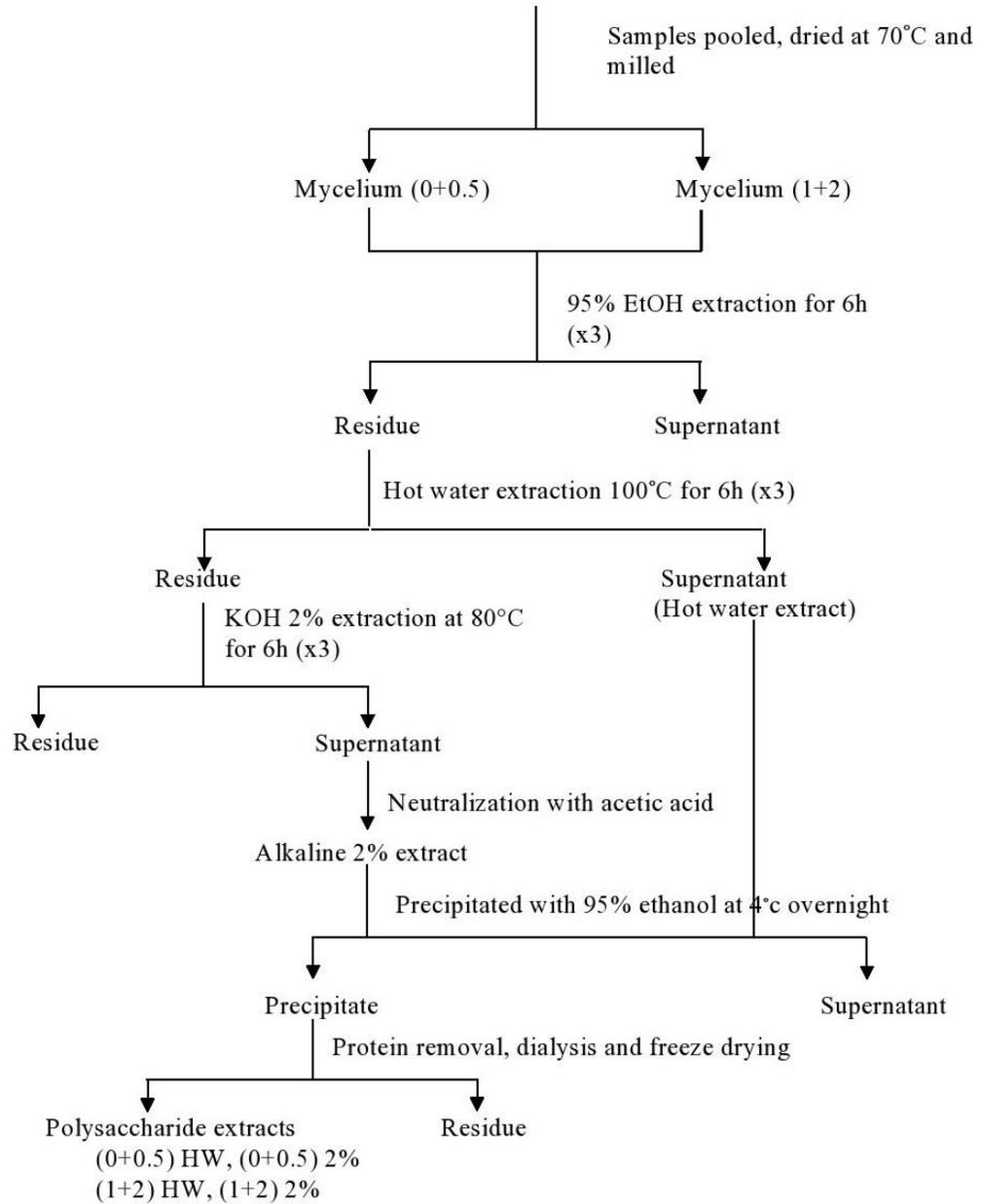


Figure 12. Scheme of extraction and purification of polysaccharides from *I. obliquus* mycelium

4.2.2. Aqueous extraction

Following the ethanol extraction, the solid residue was extracted with deionized water at a solid: liquid ratio of 1:5 at 100°C for 6 h with reflux. Extraction was performed three times. Then, the extracts were combined, filtered and centrifuged to remove water-insoluble materials. The supernatant constituted the hot water extract, and the solid extraction residue was further subjected to alkaline extraction.

4.2.3. Alkaline extraction

The combined residues obtained from aqueous extraction were subjected to alkaline extraction, using KOH (2% w/v) at 80°C three times. The same separation procedure reported for the hot water extract was followed, and the alkaline extract were obtained from the supernatant. Following the extraction, the alkaline extracts were neutralized with acetic acid.

4.3. Purification

4.3.1. Ethanol precipitation

95% ethanol was slowly added to both hot water and alkaline extracts containing polysaccharides of *I. obliquus*, and the mixtures were set at 4°C overnight for precipitation. The precipitates were collected after centrifugation at 6289×g 4°C for 20 mins. The precipitates were further purified by Sevag method, dialysis, and freeze-thawing.

4.3.2. Sevag method

Sevag method (Shi, 2016) was carried out to remove proteins from the polysaccharide extracts. A chloroform:n-butanol mixture 4:1 (v/v) was added to the aqueous polysaccharide solution in the ratio of 3:1 (v/v) in a separatory funnel. This mixture was shaken, and the lower phase was discarded. Further, the remaining upper phase and the layer of emulsion formed between the upper and lower phases were centrifuged and the upper solvent phase was collected while the jelly layer of denatures proteins was discarded. This was proceeded three times for both hot water and alkaline polysaccharide extracts.

4.3.3. Dialysis

Following the Sevag method, the solution was dialyzed (Song & Tang, 2016) in order to remove the impurities such as salts and molecules of low molecular weights, less than 12,000 Da, in this case. First, the dialysis membrane (Spectra/Por®, molecular weight cut off 12,000 Da) was cut into tubes of suitable size (about 20 cm) and soaked in deionized water for 15 min. Then, the soaked membrane tubes were filled with hot water and alkaline extracts and kept under magnetic stirring for 24 hours.

4.3.4. Freeze-thawing

The polysaccharide solutions obtained from dialysis were subjected to freezing and thawing cycles at 4°C three times to separate soluble and insoluble polysaccharides (Ruthes *et al.*, 2014). Then, the frozen soluble polysaccharide extracts were freeze-dried using VirTis bulk tray drier (SP scientific, NY, USA). The purified polysaccharide extracts were coded (0+0.5) HW (hot water extract from mycelia cultivated with control medium and supplemented with sea buckthorn press cake at dosage of 0.5 g/200 mL), (0+0.5) 2% (alkaline extract from mycelia cultivated with control medium and supplemented with sea buckthorn press cake at dosage of 0.5 g/200 mL), (1+2) HW (hot water extract from mycelia cultivated with supplemented sea buckthorn press cake at dosage of 1 g/200 mL and 2 g/200 mL), and (1+2) 2% (alkaline extract from mycelia cultivated with supplemented sea buckthorn press cake at dosage of 1 g/200 mL and 2 g/200 mL).

4.4. Characterization of polysaccharides

4.4.1. Total sugar content

The total sugar content of the polysaccharide fractions was determined by the phenol-sulfuric acid method (Masuko *et al.*, 2005) with modifications. Glucose was used as the standard solution for the calculations. 400 µL of each of the extracts (0+0.5) HW, (0+0.5) 2%, (1+2) HW, and (1+2) 2% were pipetted into test tubes, followed by the addition of 1200 µL concentrated H₂SO₄ and vortexing for 30s. Following this, 240 µL of 5% phenol solution was added to the mixture and vortexed for 10s. Then the samples were moved to incubation in a water bath for 5 mins at 90°C. Following this, the samples were moved into an ice bath for 2 mins and then left in room temperature for 5 mins to let the temperature stabilize. Then,

250 μL of the samples were pipetted onto microplate wells and the absorbance was measured at 490 nm using a Hidex microplate reader.

4.4.2. Total phenolic content

Modified Folin-Ciocalteu microplate assay (Magalhães *et al.*, 2010) was used to determine the content of phenolic compounds in the purified polysaccharide fractions (0+0.5) HW, (0+0.5) 2%, (1+2) HW, and (1+2) 2%. According to the protocol, Folin-Ciocalteu reagent was diluted with deionized water at a ratio of 1:5 (v/v). Then, 50 μL of sample fractions were added to the microplate, followed by the addition of 50 μL diluted Folin-Ciocalteu reagent. Following this, 100 μL of 0.35M NaOH were added to the solutions and the absorbance was measured at 760 nm using a Hidex microplate reader. Total phenolic content was determined based on a standard curve of gallic acid.

4.4.3. Total protein content

A modified Lowry method (Markwell *et al.*, 1978) was used to analyze the total protein content of the purified hot water and alkaline extracts. Stock solution A was prepared by adding 2% Na_2CO_3 , 0.4% NaOH, 0.16% sodium tartrate and 1% sodium dodecyl sulfate (SDS), and 4% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ was used as reagent B. Further, Folin-Ciocalteu phenol reagent was diluted 1:1 with deionized water. Reagent C was obtained by mixing reagent A and B in the ratio of 100:1.

A sample volume of 1 mL containing 10 to 100 μg of protein was added to 3 mL of reagent C, followed by the addition of 0.3 mL diluted Folin-Ciocalteu reagent. The mixture was vortexed, and the absorbance at 660 nm was measured using a Hidex microplate reader. Total protein content of the fractions was determined based on a standard curve of Bovine Serum Albumin (BSA).

4.4.4. FT-IR spectroscopy

Infrared (IR) spectra of the purified polysaccharide extracts were determined using a FT-IR spectrometer (Bruker Vertex 70). The instrument was equipped with a single bounce (angle of incidence 45°) diamond attenuated total reflection accessory (VideoMVP, Harrick).

Spectra were recorded directly on the freeze-dried polysaccharide extracts, scanning the frequency range 5000-450 cm^{-1} with a resolution of 2 cm^{-1} (Beltrame *et al.*, 2019).

4.4.5. Analysis of monomer composition of polysaccharides

GC-FID was used to determine the monomer composition of the purified polysaccharide extracts (Huang *et al.*, 2012). First, the polysaccharide fractions were hydrolyzed by adding 2 M Trifluoroacetic acid (TFA) to the samples to reach a concentration of 1 mg/mL. These mixtures were let to react at 100°C for six hours. Then, the samples were filtered using 0.45 μm regenerated cellulose (RC) filters. Following this, 0.5 mL of the filtered samples were added with 0.1 mL internal standard (1 mg/mL myo-inositol) solution, and the mixtures were dried in autosampler vials at 70°C with N_2 flow. These dried samples were kept overnight in the vacuum desiccator before the silylation process.

Hydrolyzed samples were subjected to silylation by adding 500 μL of Tri-Sil (Thermo Scientific, Bellefonte, PA, USA), and then vortexed for 7 mins. Then the samples were incubated at 70°C for one hour and then the clear upper phase were pipetted into autosampler vials with glass inserts.

The silylated samples were injected to GC- FID (Shimadzu GC-2010 Plus) with a SPB-1 column (30 m \times 0.25 mm \times 0.25 μm , Supelco, USA) for the analysis. GC analysis was performed under the following conditions: split injection 1:15; injector temperature 210°C; FID temperature 290°C. The temperature in the oven was programmed as follows: initial temperature of 150°C was maintained for 2 min, then increased to 210°C with a rate of 4°C/min and then increased to 275°C, at a rate of 40°C/min and maintained for 5 min. Helium was used as a carrier gas. The monomer compositions were identified by matching the GC retention time with standard compounds and the relative amount of each monomer was calculated based on proportion of compound peak area to the internal standard peak area of inositol and the correction factors determined using standard compounds and internal standards. Xylose, galactose, rhamnose, glucose, mannose, arabinose, fucose, and galacturonic acid were used as standards.

4.4.6. Molecular weight analysis

A Waters 2690 HPLC instrument equipped with a TSK-GMPW column (30 cm×7.5 mm i.d.) was used in the HPSEC analysis to determine the molecular weights of the purified mycelial polysaccharide extracts (Ruthes *et al.*, 2013) with modifications. The analytical system was coupled with a Waters 2487 UV detector and a Shimadzu 20A refractive index detector. The samples were dissolved in the mobile phase (0.1 M NaNO₃ 0.02% NaN₃) to a concentration of 1 mg/mL and 50 µL of the solutions were injected in the HPSEC-RID system. The system was calibrated with a pullulan standard kit (Pullulan Kit, Polymer Standards Service, Germany) with molecular weights ranging from 342 Da to 7.08×10⁵ Da. The flow rate was 0.5 mL/min, and the sample and column temperatures were 40°C.

4.5. Statistical analysis

Except molecular weight analysis and FT-IR spectroscopy, all the experiments were performed in triplicate and the results were expressed as mean ± standard deviation (SD). The softwares IBM SPSS Statistics 25 and RStudio were used for the statistical analysis.

Normality of the data was tested by Kolmogorov-Smirnov and Shapiro-Wilk and the equality of variance was tested with Levene's test. The significant differences in variances were assessed with the non-parametric Kruskal-Wallis test and one-way analysis of variance (ANOVA). Multiple comparison between all the four extracted polysaccharides of *I. obliquus* was performed by Dunn test for Kruskal-Wallis (with Bonferroni correction) and by Tukey test in ANOVA.

5 RESULTS AND DISCUSSION

5.1. Extraction and purification of polysaccharides

Polysaccharide fractions (0+0.5) HW, (0+0.5) 2%, (1+2) HW, and (1+2) 2% (**Figure 13**) were obtained after aqueous and alkaline extractions from *I. obliquus* mycelia which were cultivated with different dosages of sea buckthorn press cake. The obtained hot water extracts were yellowish brown in color while the alkaline extracts were dark brown in color. The extraction yields (w/w %) (**Table 1**) of the four fractions were calculated based on the weight obtained after purification process and freeze drying and were reported as 2.15%, 4.05%, 1.07%, and 0.95%, respectively.

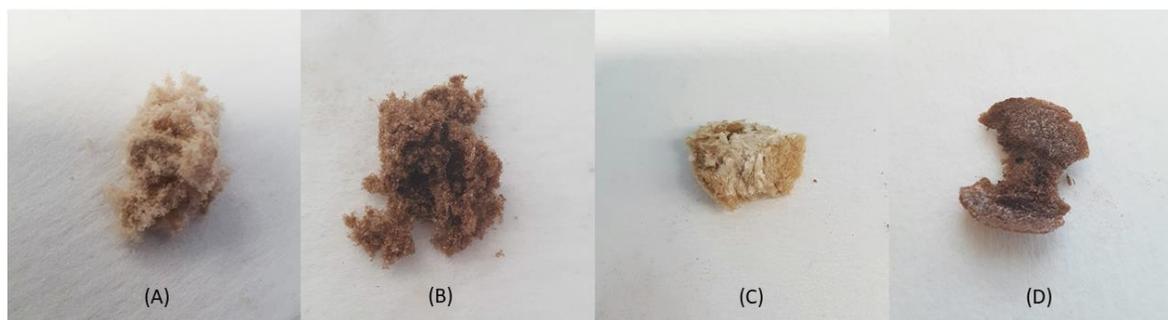


Figure 13. *I. obliquus* mycelium polysaccharide extracts (0+0.5) HW (A), (0+0.5) 2% (B), (1+2) HW (C), and (1+2) 2% (D).

The obtained yields of hot water extract were in agreement with the yields (w/w %) reported by Chen and coworkers (2.12%) and Du and colleagues (2.2%) for Chaga hot water extract (Chen *et al.*, 2010a; Du *et al.*, 2013). In addition, Hu and colleagues reported the yield for aqueous Chaga polysaccharide extract as 9.83% (Hu *et al.*, 2016) indicating the current study to hold a lower yield value in comparison.

Table 1. Aqueous and alkaline extraction yields of *I. obliquus* mycelium

Sample	Initial mycelium amount (g)	Weight of polysaccharide extract after purification and freeze drying (g)	Yield (%_{w/w})
(0+0.5) HW	18.02	0.39	2.15
(0+0.5) 2%	18.02	0.74	4.05
(1+2) HW	35.04	0.37	1.07
(1+2) 2%	35.04	0.33	0.95

5.2. Characterization of polysaccharides

Table 2. Total sugar, phenolic and protein contents of in the purified polysaccharide extracts from mycelia of *I. obliquus*, expressed as percentage of dry weight of mycelia. Values are expressed as mean \pm standard deviation (n=3).

Fraction	Total sugar content (%_{w/w})	Total phenolic content (%_{w/w})	Total protein content (%_{w/w})
Mycelium (0+0.5) HW	80.02 \pm 2.19 ^a	ND [*]	23.43 \pm 1.70 ^a
Mycelium (0+0.5) 2%	76.04 \pm 1.38 ^a	ND [*]	33.38 \pm 4.56 ^b
Mycelium (1+2) HW	74.29 \pm 2.56 ^a	ND [*]	22.78 \pm 0.77 ^a
Mycelium (1+2) 2%	54.98 \pm 2.17 ^a	1.19 \pm 0.94	34.42 \pm 0.93 ^b

^{*}Not detected. Means with different letters in the same column mark significant difference ($p \leq 0.05$)

5.2.1. Total sugar content

The total sugar, phenolic and protein content of the mycelium polysaccharide extracts are shown in **Table 2**. The sugar contents of mycelial polysaccharide extracts were significantly higher than the sugar contents (55-80%) of Chaga polysaccharide extracts, which were $16.00 \pm 0.12\%$ and $19.42 \pm 2.70 \%$ (w/w), for hot water and alkali extract respectively (results of a previous experiment). These results were in agreement with previous comparisons between mycelial and sterile conk polysaccharides (Xu *et al.*, 2011; Xu *et al.*, 2014a). The higher total sugar content of polysaccharides obtained from the mycelium of *I. obliquus* in comparison to the Chaga extracts suggests that submerged cultivation of *I. obliquus* could be a good alternative to produce polysaccharides.

The sugar contents of both hot water extracts were higher than those of the alkaline extracts of their respective groups. This indicates that most of the polymers present in the mycelium are extracted with hot water while only the remaining polymers are extracted through alkaline extraction. In addition, both hot water and alkaline extract fractions of the (0+0.5) pool showed higher sugar content than the corresponding polysaccharide extracts from the (1+2) pool. The results suggest culture medium with lower sea buckthorn content to be more efficient in producing mycelium polysaccharides.

Furthermore, the sugar contents (w/w %) of hot water extracts were higher than those reported for the hot water extracts of *I. obliquus* mycelium cultivated with a control medium (51.67%) and after the supplementation of different lignocellulosic materials, such as rice straw (total sugar content 48.29%), wheat straw (68.14%), or sugarcane bagasse (47.43%) (Xu *et al.*, 2014b). Another study on aqueous polysaccharide extract obtained from submerged cultivation of *I. obliquus* mycelium reports a sugar content (72.4%) in agreement with the results of the current study (Xu *et al.*, 2011). Moreover, the alkaline extracts obtained from the cultivated mycelia in the current study had a sugar content higher than the reported content of an alkaline extract of Chaga (41.9%) (Wold *et al.*, 2018).

5.2.2. Total protein content

The content of remaining proteins in the extracts after the removal of free proteins by Sevag method were analyzed by Lowry method. A study by Kim and colleagues has shown that the biological activity of polysaccharide extracts is not affected by the proteins bound to the polysaccharides in the extract (Kim *et al.*, 2005).

As shown in **Table 2**, all polysaccharide fractions showed a total protein content lower than the total sugar content of the respective extracts. This is in agreement with previous studies, where the total protein content was lower than the total sugar content of their respective extracts (Xu *et al.*, 2011; Xu *et al.*, 2014b).

Results showed that the polysaccharide-protein complexes extracted with alkali contained more proteins than the ones extracted with hot water. Further, the protein contents (w/w %) of hot water extracts were higher than the ones reported by Xu and coworkers in two different studies (8.65% and 12.9%) obtained from *I. obliquus* mycelium cultivated in submerged culture without a supplementation (Xu *et al.*, 2011; Xu *et al.*, 2014a). In addition, our results showed higher protein content than the one reported by Xu and coworkers for polysaccharides obtained from *I. obliquus* mycelium cultivated with supplementation of wheat straw (15.14%), rice straw (15.27%), and sugarcane bagasse (13.05%) (Xu *et al.*, 2014b).

5.2.3. Total phenolic content

As shown in **Table 2**, no phenolic compounds were detected in the purified polysaccharide fractions (0+0.5) HW, (0+0.5) 2%, and (1+2) HW, while (1+2) 2% showed a phenolic content of $1.19 \pm 0.94\%$ w/w. Previous studies have stated the high total phenolic content of sea buckthorn, reporting the phenolic content to be 55.38 mg GAE/g (Ercisli *et al.*, 2007), 45.78 mg GAE/g (Korekar *et al.*, 2011), and 22.83 g ferulic acid equivalents /kg dry weight (Li *et al.*, 2009). The presence of phenolics in sea buckthorn could explain the presence of phenolics in (1+2) 2% extract in the present study, as no phenolic content was detected in the polysaccharides obtained with lower sea buckthorn supplementation. Further, the phenolic contents of Chaga polysaccharide fractions extracted with hot water and alkali (unpublished results of a separate study) were 8.09% and 17.42% w/w, respectively. These results

indicated the presence of higher phenolic content in Chaga polysaccharides in comparison to the *I. obliquus* mycelium. In addition, phenolic content of the intracellular polysaccharides extracted in the current study was in agreement with findings of previous research on extracted exopolysaccharides. Chen and coworkers reported the phenolic content of exopolysaccharides extracted from submerged cultivation of *I. obliquus* mycelium supplemented with corn flour and corn straw to be 0.08% of the dried extract (w/w) (Chen *et al.*, 2010c).

Further, a research was conducted by Wold and coworkers on Chaga polysaccharides, and they reported the alkaline extract with dark brown color to contain polyphenolic structure; further, they speculated the reason to be the presence of melanin in the extract (Wold *et al.*, 2018). The current study revealed presence of phenolic compounds in alkaline extract of (1+2), which was dark brown in color, and this could be due to the presence of melanins in the extract, as mentioned by Wold and coworkers (Wold *et al.*, 2018).

5.2.4. FT-IR spectroscopy

FT-IR spectroscopy was used for the structural analysis of the polysaccharides extracted from the mycelium of *I. obliquus*. The obtained spectra are shown in **Figure 14**. Different absorption bands were assigned according to the literature (Synytsya *et al.*, 2009; Hu *et al.*, 2016). The highly overlapped intense IR bands of the fractions observed in the region of 930–1240 cm^{-1} indicated the presences of polysaccharides as the major component with higher intensity than other regions. In particular, the intense band at 1150 cm^{-1} was assigned to the C-O-C stretching glycosidic bonds. The IR bands indicating the presence of polysaccharides in (1+2) HW were less pronounced than those of (0+0.5) HW.

The weaker bands at 894 cm^{-1} indicated the presence of β -glucans while the bands found at 856 cm^{-1} indicated the presence of α -glucans (Liu *et al.*, 2007). Among the two anomeric signals, the band assigned to α -glucans showed much higher intensity in all the obtained extracts. In particular, α -glucan signal assigned at 856 cm^{-1} showed relatively same intensity for (0+0.5) 2%, (1+2) HW, and (1+2) 2% extracts, while the α -glucan signal of (0+0.5) HW spectrum showed higher intensity than the other three extracts. In addition, the β -glucan signal assigned at 894 cm^{-1} showed relatively same intensity between spectrum of the two

alkaline extracts, while the β -glucan signal of (0+0.5) HW spectrum showed higher intensity and (1+2) HW showed lower intensity in comparison to the other fractions.

The signals near 1373 and 1080 cm^{-1} could be assigned to β -glucans. Furthermore, other bands ascribable to α -glucans were also found near 1367, 930 and 853 cm^{-1} (Synytsya *et al.*, 2009). Higher intensity was observed for β -glucan signal assigned at 1373 cm^{-1} than the intensities of all the signals assigned to α -glucans in all four extracted polysaccharide fractions. The strong IR bands at 1650 cm^{-1} were attributed to the presence of amide I, while the band at 1545 cm^{-1} was assigned to amide II. The presence of amide I and amide II bands were ascribed to the vibrations of proteins. The amide I and amide II bands of hot water extracts were much less pronounced than the corresponding bands of their alkaline extracts. The results were in agreement with the measured total protein content (**Table 2**), where the alkaline extracts contained more proteins than the hot water extracts in both (0+0.5) and (1+2) fractions. In addition, the IR bands at 1745 cm^{-1} for (1+2) HW and (1+2) 2% were attributed to the presence of carboxylic group belonging to uronic acids. These bands were absent in the (0+0.5) HW and (0+0.5) 2% extracts.

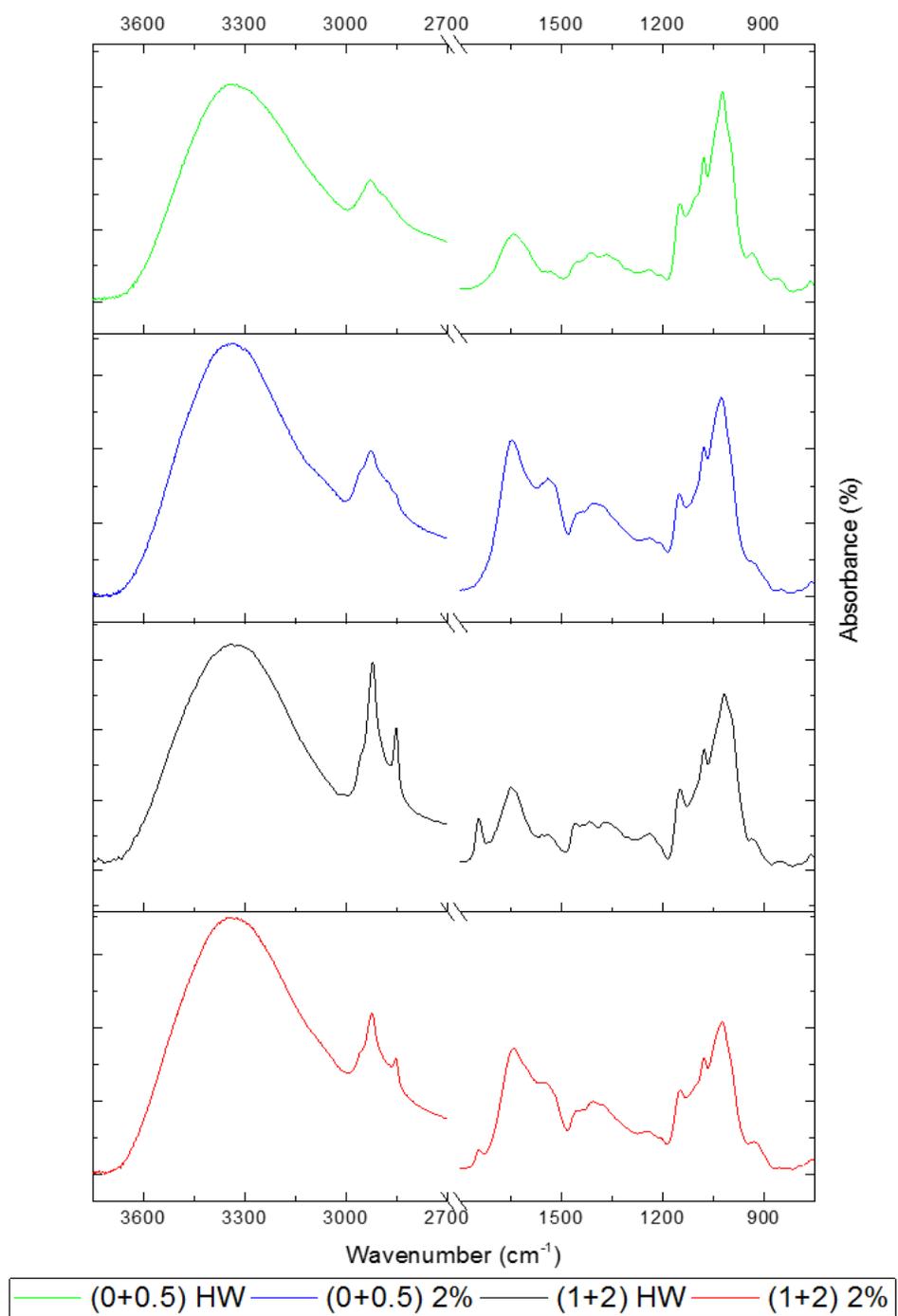


Figure 14. FT-IR spectra of polysaccharide extracts obtained from *I. obliquus*.

5.2.5. Monomer composition

The monomer composition of the polysaccharides extracted from *I. obliquus* was outlined and quantified using GC-FID. Representative chromatograms obtained for hot water and alkaline extracts of fractions (0+0.5) and (1+2) are shown in **Figure 15**. Monomer composition of all the extracted polysaccharide fractions is summarized in **Table 3**. All the polysaccharide extracts were mainly composed of the monomer xylose, galactose, rhamnose, glucose, mannose, arabinose, fucose, and galacturonic acid, in different relative molar percentages.

Table 3. Monomer composition (mol % of total) of polysaccharides extracted from *I. obliquus*. Each value is expressed as mean \pm standard deviation (n=3).

Fraction	Xyl	Gal	Rha	Glc	Man	Ara	Fuc	GalA
Mycelium (0+0.5) HW	1.4 \pm 0.02 ^{ab}	12.90 \pm 0.35 ^{ab}	0.17 \pm 0.00 ^a	63.47 \pm 0.13 ^{ab}	20.38 \pm 0.22 ^{ab}	0.46 \pm 0.04 ^a	1.17 \pm 0.07 ^{ab}	0.44 \pm 0.02 ^{ab}
Mycelium (0+0.5) 2%	1.7 \pm 0.03 ^{ab}	15.80 \pm 0.22 ^a	0.22 \pm 0.01 ^{ab}	56.26 \pm 0.35 ^{ab}	23.92 \pm 0.17 ^{ab}	0.78 \pm 0.03 ^{ab}	1.30 \pm 0.03 ^{ab}	0.20 \pm 0.02 ^a
Mycelium (1+2) HW	0.8 \pm 0.02 ^a	11.66 \pm 0.14 ^{ab}	0.35 \pm 0.08 ^{ab}	65.01 \pm 0.12 ^a	17.85 \pm 0.13 ^a	1.23 \pm 0.01 ^{ab}	1.03 \pm 0.02 ^a	1.99 \pm 0.08 ^b
Mycelium (1+2) 2%	6.7 \pm 0.06 ^b	10.90 \pm 0.24 ^b	0.53 \pm 0.02 ^b	49.96 \pm 0.26 ^b	27.00 \pm 0.38 ^b	1.98 \pm 0.04 ^b	1.57 \pm 0.03 ^b	1.19 \pm 0.06 ^{ab}

Different letters in the same column mark significant difference ($p \leq 0.05$).

Glucose was the major monomer of all four polysaccharide fractions of *I. obliquus*. A decrease in relative percentage was observed in the alkaline extracts compared to the hot water extracts in both the pools (0+0.5) and (1+2), suggesting that most of the glucans were extracted initially by hot water and the remaining was extracted during alkaline extraction process. Overall, (1+2) HW had the highest content of glucose while (1+2) 2% had the lowest content of glucose. Mannose was present as the second major monomer in all the four

fractions and, in contrast to glucose, its relative content was higher in the alkali fractions of the relative mycelium pools.

The reported monomer compositions were in agreement with the previous studies on polysaccharides of *I. obliquus* mycelia, where glucose and mannose were found as the major monosaccharides (Kim *et al.*, 2005; Huang *et al.*, 2012; Xu *et al.*, 2014a; Xu *et al.*, 2014b). A previous study on *I. obliquus* mycelia cultivated with the supplementation of lignocellulosic biomass reported the glucose content of extracted polysaccharides to be 71%, 72.1%, and 62.8% for mycelia grown in culture supplemented with wheat straw, rice straw, and sugarcane bagasse, respectively (Xu *et al.*, 2014b). The glucose content of all the polysaccharide fractions in the current study was slightly lower than the glucose content of the above-mentioned mycelia cultures supplemented with wheat straw and rice straw. However, the hot water extracts of current study were higher than the glucose content of polysaccharides obtained from mycelium cultivated with the supplementation of sugarcane bagasse. Another study on polysaccharides extracted from *I. obliquus* mycelium cultivated without supplements in the medium reported the glucose content to be 37.8% (Xu *et al.*, 2014a), indicating higher glucose content in all the extracted polysaccharides of current study with the sea buckthorn supplementation in the culture medium than the glucose content of polysaccharides extracted from *I. obliquus* mycelium cultivated without supplements in the medium. The difference in the glucose content between the studies could be speculated to be due to the different cultivation time, supplementations used in the culture, and extraction and purification processes.

The mannose content of the all the polysaccharide extracts obtained from the supplementation of sea buckthorn press cake in the mycelia culture (18-27%) was higher than the reported content of polysaccharides obtained after supplementation of wheat straw (11%), rice straw (13.2%), and sugarcane bagasse (18%) in the mycelia culture medium (Xu *et al.*, 2014b). The content of rhamnose was the lowest among the monosaccharides in all the fractions. This was in agreement with previous studies (Xu *et al.*, 2014b). Further, a noticeable increase was observed in the content of xylose of the (1+2) 2% fraction, compared to the other fractions. Arabinose showed an increase in relative content from hot water to alkali fractions and from 0+0.5 to 1+2 mycelia pool. It could be speculated that the presence

of sea buckthorn in the culture medium affected the monomer composition of the polysaccharides, with monomers present in sea buckthorn being absorbed by the mycelia. Xu and coworkers as well reported that monomer composition of the *I. obliquus* mycelia was affected by the presence of the lignocellulose supplements in the culture medium (Xu *et al.*, 2014b). Overall, the relative content all the monomers except glucose increased from aqueous extract to alkaline extract. Similar results were reported by Wold and colleagues for the aqueous and alkaline polysaccharide extracts obtained from Chaga (Wold *et al.*, 2018).

In addition, galacturonic acid was found in smaller amounts in the extracts containing lower sea buckthorn supplementation in the medium (0+0.5), while it was relatively higher in the polysaccharides obtained from (1+2) mycelial pool. This suggests that galacturonic acid present in the polysaccharides extracted from the (1+2) pool is due to the pectin released in the medium by the press cake.

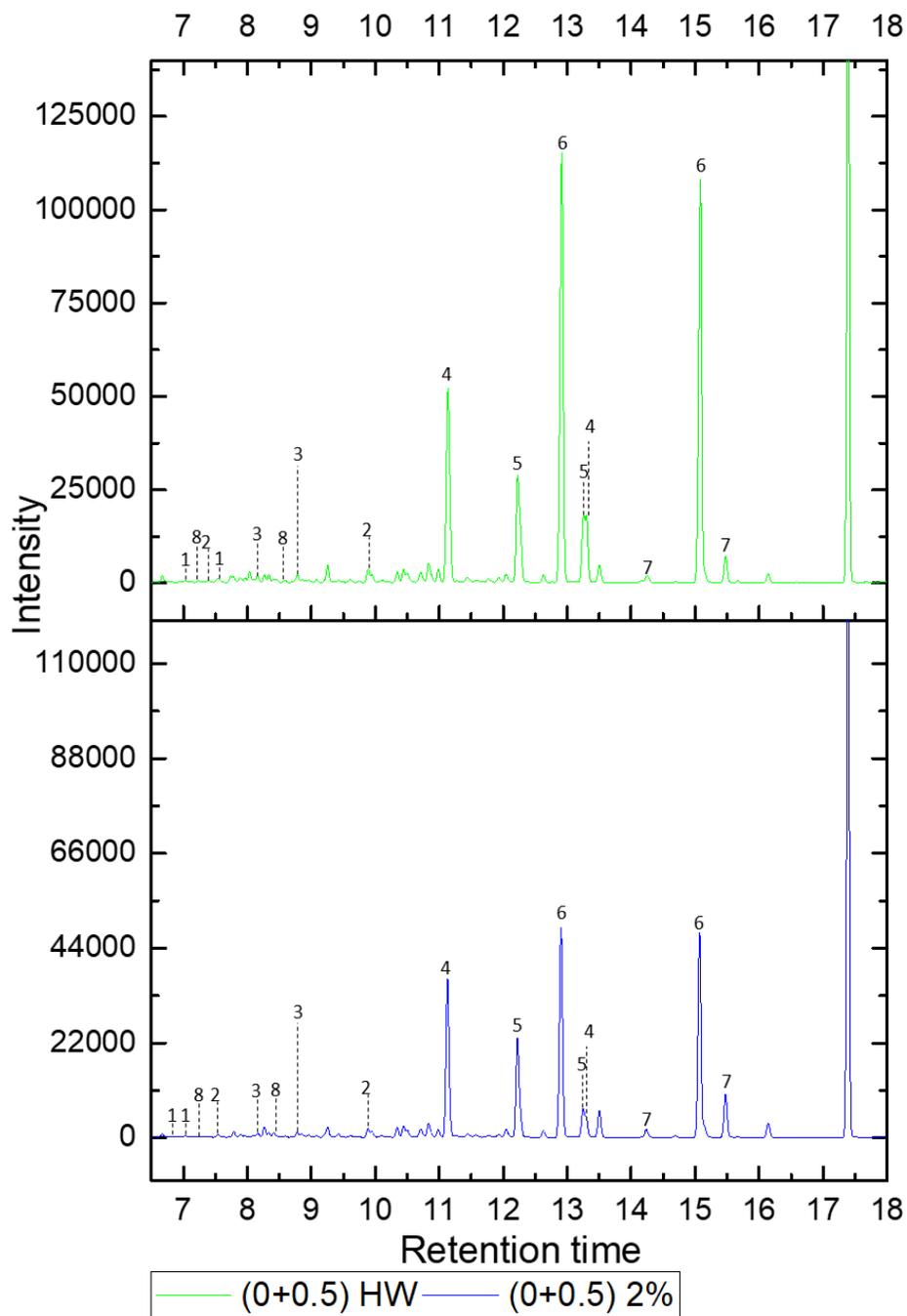


Figure 15. GC-FID chromatograms of polysaccharide extracts obtained from *I. obliquus*. Peaks were identified as (1) Arabinose, (2) Xylose, (3) Fucose, (4) Mannose, (5) Galactose, (6) Glucose, (7) Galacturonic acid, and (8) Rhamnose

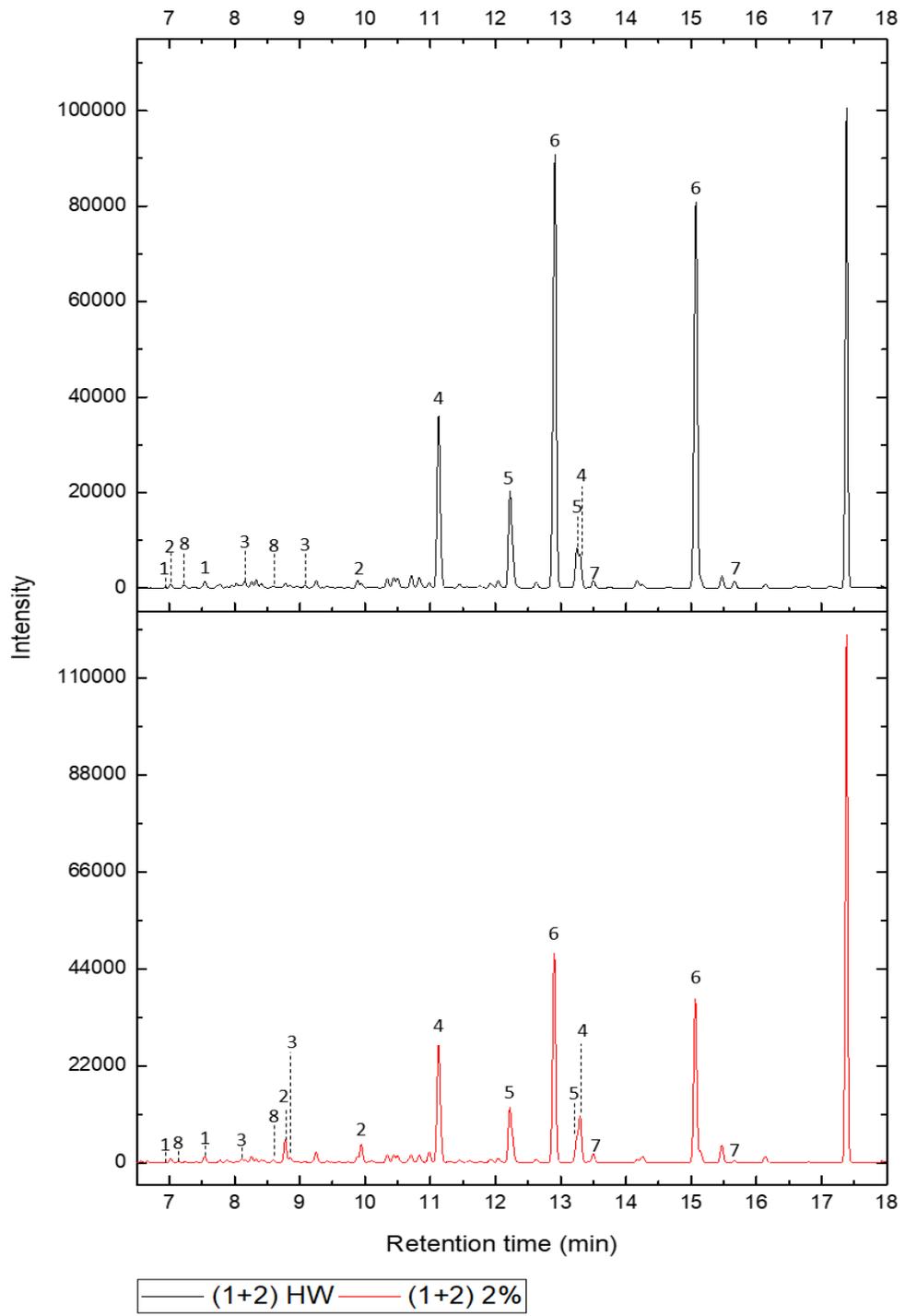


Figure 15. (Continued)

5.2.6. Molecular weight analysis

Molecular weights of the extracted polysaccharides were determined by HPSEC coupled with RID detector. The obtained chromatograms are shown in **Figure 16**. Both (0+0.5) fractions showed two major peaks, while three polymer populations were observed in the (1+2) fractions. Molecular weights of the extracted fractions were determined according to the calibration curve obtained from pullulan standard kit. Values are reported in **Table 4**.

The larger polymer populations were observed to be 6.80×10^5 , 2.80×10^4 , 6.51×10^4 , and 1.54×10^4 Da for fractions (0+0.5) HW, (0+0.5) 2%, (1+2) HW, and (1+2) 2% respectively. Previous research has reported the molecular weight of hot water polysaccharides obtained from *I. obliquus* mycelium to be 1×10^6 Da and 1.2×10^5 Da, respectively (Kim *et al.*, 2006; Xu *et al.*, 2014a). On the other hand, three different studies on hot water extract of Chaga have reported the molecular weight of the polysaccharides to be 1.5×10^5 Da, 4.9×10^4 Da, and 3.25×10^4 Da, respectively (Huang *et al.*, 2012; Chen *et al.*, 2015; Yang *et al.*, 2016). The current study on hot water extracts of *I. obliquus* mycelia revealed (0+0.5) HW to contain larger molecular weight in main population while, (1+2) HW showed a smaller molecular weight in the main population than the above-mentioned literature on hot water extracts of *I. obliquus* mycelium.

Kim and coworkers studied the polysaccharide extracts obtained from submerged culture of *I. obliquus* mycelium in absence of supplement and reported the glucose content to be decreasing with the increase in the molecular weight of *I. obliquus* mycelial polysaccharides in the extract. The glucose content dropped from 98.12% to 60.08% when the molecular weight of the polysaccharides increased from <10 kDa to >50 kDa (Kim *et al.*, 2005).

The molecular weight of alkaline polysaccharide extracts obtained from Chaga were reported as 6.1×10^4 Da and 2×10^6 Da, in two different studies (Niu *et al.*, 2016; Wold *et al.*, 2018). The current study reported lower molecular weights for the main populations of alkaline extracts than that was reported for Chaga alkaline extracts.

Kim and coworkers studied the immunostimulating activity of *I. obliquus* mycelial polysaccharides and reported the extracts containing the higher molecular weight ($\geq 5 \times 10^4$ Da) to show higher immune stimulating activity than the other extracts (Kim *et al.*, 2005). In

addition, Xu and coworkers reported the polysaccharide extracts of *I. obliquus* mycelia with higher molecular weight in the range of 5×10^5 to 2×10^6 Da to have a higher effect in the stimulation of cytokine production (Xu *et al.*, 2014a). Based on the previous studies, it could be speculated that the aqueous extracts of (0+0.5) and (1+2) pools which contains higher molecular weight in the main population could possess higher immune stimulating activity than the alkaline extracts.

Further, the obtained results indicated a reduction in the molecular weight of the main populations of respective hot water and alkaline extracts with the increase in the sea buckthorn amount used as the supplement in the culture medium. Hence, it could be speculated that the use of sea buckthorn in the medium has a negative influence on the biological activity of extracted polysaccharides.

Table 4. Molecular weight of polysaccharides extracted from *I. obliquus*

Fraction	Mw (Da)	Area %
Mycelium (0+0.5) HW	6.80×10^5	75.00
	1.60×10^4	25.00
Mycelium (0+0.5) 2%	4.10×10^5	26.00
	2.80×10^4	74.00
Mycelium (1+2) HW	2.00×10^5	18.55
	6.51×10^4	79.48
	6.70×10^3	1.97
Mycelium (1+2) 2%	1.40×10^6	8.62
	2.02×10^5	21.40
	1.54×10^4	69.98

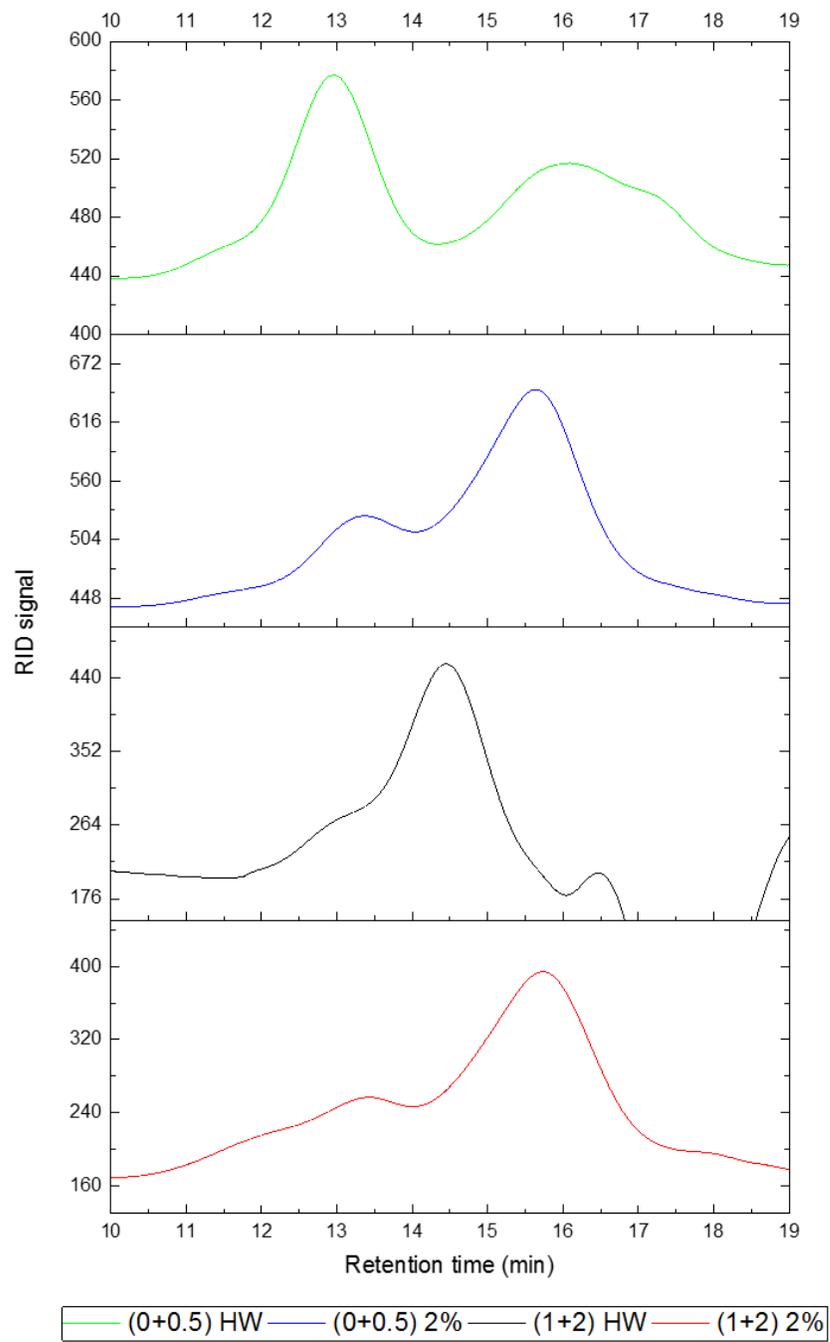


Figure 16. HPSEC chromatograms of polysaccharide extracts obtained from *I. obliquus*.

6 CONCLUSIONS

Production of Chaga and extraction of polysaccharides from naturally grown sclerotia is hampered by the slow growth of Chaga in the nature. Submerged cultivation presents an alternative method for the production of polysaccharides from cultured *I. obliquus* mycelium. In the present study, polysaccharides were extracted from the mycelium of *I. obliquus*, cultivated with supplementation of sea buckthorn press cake, using hot water and alkaline solution. After purification, the physio-chemical and chemical properties of the extracted fractions were determined by chemical methods, GC-FID, HPSEC and FT-IR spectroscopy.

Our results showed that the total sugar contents of the polysaccharide extracts from *I. obliquus* mycelia were higher than that of Chaga polysaccharide extracts. This suggests the cultivated mycelium of *I. obliquus* could be a good alternative for the production of polysaccharides. In addition, presence of proteins in the extracts was revealed through FT-IR spectroscopy and Lowry method. Results obtained from Lowry method reported the presence of proteins after the protein removal with Sevag method, and this indicates the protein to be covalently bound to the polysaccharides in the extracts. Further, the total phenolics were observed to be absent in the extracts of (0+0.5) fraction while present in the extracts of (1+2) fraction. It could then be speculated that higher amounts of sea buckthorn in the culture medium are responsible for the presence of phenolics in the (1+2) fractions.

The study on the monomer composition of the extracted polysaccharides indicated the presence of glucose as the major monomer in all the extracted fractions. The result was in agreement with the previous research, which has shown that glucose is the major monomer of both Chaga polysaccharides and *I. obliquus* mycelium polysaccharides. Substantial amounts of galactose and mannose were also found in the extracts along with smaller amounts of monomers such as xylose, arabinose, rhamnose and fucose. Galacturonic acid was found in small quantity in the extracts. Its presence in the polysaccharide extracts was connected to the pectin contained in the supplemented sea buckthorn. In addition, IR bands obtained from the FT-IR spectroscopy confirmed the presence of proteins, uronic acids and α - and β -glucans in the obtained extracts. Further, α -glucans were observed to be more abundant than β -glucans in all the polysaccharide extracts.

Further, the molecular weights of the main polymer populations were determined to be 6.80×10^5 Da, 1.17×10^3 Da, 6.51×10^4 Da and 1.51×10^4 Da for water and alkaline extracts of (0+0.5) and (1+2) fractions respectively. In addition, a reduction in the molecular weight of the main populations was observed in the respective hot water and alkaline extracts with the increase in the amount of sea buckthorn supplemented in the culture medium. The results obtained from monomer composition and molecular weight analysis suggests the macromolecular properties of mycelia polysaccharides to be affected by the presence of sea buckthorn in the medium.

Higher immunostimulating activity and stimulation of cytokine production have been reported by the polysaccharide extracts with higher molecular weight in previous studies. Hence, the current study could suggest the extracts of (1+2) pool with higher molecular weight to have a higher bioactivity. Further, the increase in the sea buckthorn supplementation could influence the bioactivity of the extracts by increasing the molecular weight of the polysaccharide extracts.

The current findings show a favorable production of polysaccharides through *I. obliquus* mycelia with a supplementation of sea buckthorn press cake in the medium. Hence, the current study indicates a potential application of polysaccharide production, that could be used in the mycelium nutraceuticals. Further studies would be required to verify the influence of extraction medium and use of supplement on the biological activities of extracted polysaccharides.

References

- Abyshv, A. Z., Agaev, É M., & Guseinov, A. B. (2007). Studies of the chemical composition of birch bark extracts (Cortex betula) from the Betulaceae family. *Pharmaceutical Chemistry Journal*, 41(8), 419-423. doi:10.1007/s11094-007-0091-5
- Bae, I. Y., Kim, H. W., Yoo, H. J., Kim, E. S., Lee, S., Park, D. Y., & Lee, H. G. (2013). Correlation of branching structure of mushroom β -glucan with its physiological activities. *Food Research International*, 51(1), 195-200. doi:10.1016/j.foodres.2012.12.008
- Balandaykin, M. E., & Zmitrovich, I. V. (2015). Review on Chaga Medicinal Mushroom, *Inonotus obliquus* (Higher Basidiomycetes): Realm of Medicinal Applications and Approaches on Estimating its Resource Potential. *International Journal of Medicinal Mushrooms*, 17(2), 95–104. doi: 10.1615/intjmedmushrooms.v17.i2.10
- Bao, X.-F., Wang, X.-S., Dong, Q., Fang, J.-N., & Li, X.-Y. (2002). Structural features of immunologically active polysaccharides from *Ganoderma lucidum*. *Phytochemistry*, 59(2), 175–181. doi: 10.1016/s0031-9422(01)00450-2
- Beltrame, G., Trygg, J., Rahkila, J., Leino, R., & Yang, B. (2019). Structural investigation of cell wall polysaccharides extracted from wild Finnish mushroom *Craterellus tubaeformis* (Funnel Chanterelle). *Food Chemistry*, 301, 125255. doi: 10.1016/j.foodchem.2019.125255
- Bringmann, G., Saeb, W., Assi, L., François, G., Narayanan, A. S., Peters, K., & Peters, E. (1997). Betulinic Acid: Isolation from *Triphyophyllum peltatum* and *Ancistrocladus heyneanus*, Antimalarial Activity, and Crystal Structure of the Benzyl Ester*. *Planta Medica*, 63(03), 255-257. doi:10.1055/s-2006-957666
- Campbell, W. A. and Davidson, R. W. 1938. A poria as the fruiting stage of the fungus causing the sterile conks on birch. *Mycologia* 30:553-560.
- Chan, G. C., Chan, W. K., & Sze, D. M. (2009). The effects of β -glucan on human immune and cancer cells. *Journal of Hematology & Oncology*, 2(1). doi:10.1186/1756-8722-2-25
- Chen, H., Chen, Y., Liu, S., Liou, B., & Chen, C. (2020). The Influence of Submerged Fermentation of *Inonotus obliquus* with Control Atmosphere Treatment on Enhancing

Bioactive Ingredient Contents. *Applied Biochemistry and Biotechnology*. doi:10.1007/s12010-020-03273-2

Chen, Y., Gu, X., Huang, S., Li, J., Wang, X., & Tang, J. (2010a). Optimization of ultrasonic/microwave assisted extraction (UMAE) of polysaccharides from *Inonotus obliquus* and evaluation of its anti-tumor activities. *International Journal of Biological Macromolecules*, 46(4), 429-435. doi:10.1016/j.ijbiomac.2010.02.003

Chen, Y., Huang, Y., Cui, Z., & Liu, J. (2015). Purification, characterization and biological activity of a novel polysaccharide from *Inonotus obliquus*. *International Journal of Biological Macromolecules*, 79, 587–594. doi: 10.1016/j.ijbiomac.2015.05.016

Chen, H., Lu, X., Qu, Z., Wang, Z., & Zhang, L. (2010b). Glycosidase Inhibitory Activity And Antioxidant Properties Of A Polysaccharide From The Mushroom *Inonotus Obliquus*. *Journal of Food Biochemistry*, 34, 178-191. doi:10.1111/j.1745-4514.2009.00322.x

Chen, H., Yan, M., Zhu, J., & Xu, X. (2010c). Enhancement of exo-polysaccharide production and antioxidant activity in submerged cultures of *Inonotus obliquus* by lignocellulose decomposition. *Journal of Industrial Microbiology & Biotechnology*, 38(2), 291-298. doi:10.1007/s10295-010-0772-z

Chen, C., Zheng, W., Gao, X., Xiang, X., Sun, D., Wei, J., & Chu, C. (2007). Aqueous Extract of *Inonotus obliquus* (Fr.) Pilat (Hymenochaetaceae) Significantly Inhibits the Growth of Sarcoma 180 by Inducing Apoptosis. *American Journal of Pharmacology and Toxicology*, 2(1), 10-17. doi:10.3844/ajptsp.2007.10.17

Cheong, K., Wu, D., Zhao, J., & Li, S. (2015). A rapid and accurate method for the quantitative estimation of natural polysaccharides and their fractions using high performance size exclusion chromatography coupled with multi-angle laser light scattering and refractive index detector. *Journal of Chromatography A*, 1400, 98-106. doi:10.1016/j.chroma.2015.04.054

Chihara, G. (1990). Lentinan and its Related Polysaccharides as Host Defence Potentiators: Their Application to Infectious Diseases and Cancer. *Immunotherapeutic Prospects of Infectious Diseases*, 9-18. doi:10.1007/978-3-642-76120-1_2

- Cui, S. W. (2000). Polysaccharide Gums from Agricultural Products. doi:10.1201/9781482279016
- Cui, Y., Kim, D.-S., & Park, K.-C. (2005). Antioxidant effect of *Inonotus obliquus*. *Journal of Ethnopharmacology*, 96(1-2), 79–85. doi: 10.1016/j.jep.2004.08.037
- Du, X., Mu, H., Zhou, S., Zhang, Y., & Zhu, X. (2013). Chemical analysis and antioxidant activity of polysaccharides extracted from *Inonotus obliquus sclerotia*. *International Journal of Biological Macromolecules*, 62, 691–696. doi: 10.1016/j.ijbiomac.2013.10.016
- Elisashvili, V. (2012). Submerged Cultivation of Medicinal Mushrooms: Bioprocesses and Products (Review). *International Journal of Medicinal Mushrooms*, 14(3), 211-239. doi:10.1615/intjmedmushr.v14.i3.10
- Ercisli, S., Orhan, E., Ozdemir, O., & Sengul, M. (2007). The genotypic effects on the chemical composition and antioxidant activity of sea buckthorn (*Hippophae rhamnoides* L.) berries grown in Turkey. *Scientia Horticulturae*, 115(1), 27-33. doi:10.1016/j.scienta.2007.07.004
- Fujioka, T., Kashiwada, Y., Kilkuskie, R. E., Cosentino, L. M., Ballas, L. M., Jiang, J. B., . . . Lee, K. (1994). Anti-AIDS Agents, 11. Betulinic Acid and Platanic Acid as Anti-HIV Principles from *Syzigium claviflorum*, and the Anti-HIV Activity of Structurally Related Triterpenoids. *Journal of Natural Products*, 57(2), 243-247. doi:10.1021/np50104a008
- Giavasis, I. (2014). Bioactive fungal polysaccharides as potential functional ingredients in food and nutraceuticals. *Current Opinion in Biotechnology*, 26, 162–173. doi: 10.1016/j.copbio.2014.01.010
- Giddings, J. C. (1967). Maximum number of components resolvable by gel filtration and other elution chromatographic methods. *Analytical Chemistry*, 39(8), 1027-1028. doi:10.1021/ac60252a025
- Guillamón, E., García-Lafuente, A., Lozano, M., D'arrigo, M., Rostagno, M. A., Villares, A., & Martínez, J. A. (2010). Edible mushrooms: Role in the prevention of cardiovascular diseases. *Fitoterapia*, 81(7), 715-723. doi:10.1016/j.fitote.2010.06.005

- Gyori, Z., Berceanu, M., Cheveresan, A., Farcas, C., Bratu, T., & Crainiceanu, Z. (2019). Melanin and Behaviour of Melanoma-related Cell Lines in the Presence of Ultraviolet B Type Radiation. *Revista De Chimie*, 70(7), 2398-2400. doi:10.37358/rc.19.7.7348
- Ham, S., Kim, S., Moon, S., Chung, M. J., Cui, C., Han, E., . . . Choe, M. (2009). Antimutagenic effects of subfractions of Chaga mushroom (*Inonotus obliquus*) extract. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, 672(1), 55-59. doi:10.1016/j.mrgentox.2008.10.002
- Handa, N., Yamada, T., & Tanaka, R. (2012). Four new lanostane-type triterpenoids from *Inonotus obliquus*. *Phytochemistry Letters*, 5(3), 480-485. doi:10.1016/j.phytol.2012.04.010
- Hassan, B., Chatha, S. A., Hussain, A. I., Zia, K. M., & Akhtar, N. (2018). Recent advances on polysaccharides, lipids and protein based edible films and coatings: A review. *International Journal of Biological Macromolecules*, 109, 1095-1107. doi:10.1016/j.ijbiomac.2017.11.097
- Hu, Y., Sheng, Y., Yu, M., Li, K., Ren, G., Xu, X., & Qu, J. (2016). Antioxidant activity of *Inonotus obliquus* polysaccharide and its amelioration for chronic pancreatitis in mice. *International Journal of Biological Macromolecules*, 87, 348-356. doi: 10.1016/j.ijbiomac.2016.03.006
- Huang, S.-Q., Ding, S., & Fan, L. (2012). Antioxidant activities of five polysaccharides from *Inonotus obliquus*. *International Journal of Biological Macromolecules*, 50(5), 1183-1187. doi: 10.1016/j.ijbiomac.2012.03.019
- Huang, S., Pan, Y., Gan, D., Ouyang, X., Tang, S., Ekunwe, S. I., & Wang, H. (2010). Antioxidant activities and UV-protective properties of melanin from the berry of *Cinnamomum burmannii* and *Osmanthus fragrans*. *Medicinal Chemistry Research*, 20(4), 475-481. doi:10.1007/s00044-010-9341-2
- Jiang, D., Wang, L., Zhao, T., Zhang, Z., Zhang, R., Jin, J., . . . Wang, F. (2016). Restoration of the tumor-suppressor function to mutant p53 by *Ganoderma lucidum* polysaccharides in colorectal cancer cells. *Oncology Reports*, 37(1), 594-600. doi:10.3892/or.2016.5246

- Kalac, P. (2016). Chapter 4. In *Edible Mushrooms: Chemical Composition and Nutritional Value*. Elsevier Science.
- Kataoka, K., Muta, T., Yamazaki, S., & Takeshige, K. (2002). Activation of Macrophages by Linear (1→3)-β-d-Glucans. *Journal of Biological Chemistry*, 277(39), 36825-36831. doi:10.1074/jbc.m206756200
- Khan, M. S., Zhang, X., You, L., Fu, X., & Abbasi, A. M. (2015). Structure and Bioactivities of Fungal Polysaccharides. *Polysaccharides*, 1851-1866. doi:10.1007/978-3-319-16298-0_28
- Kiho, T., Shiose, Y., Nagai, K., & Ukai, S. (1992). Polysaccharides in Fungi. XXX. Antitumor and Immunomodulating Activities of Two Polysaccharides from the Fruiting Bodies of *Armillariella tabescens*. *Chemical & Pharmaceutical Bulletin*, 40(8), 2110-2114. doi:10.1248/cpb.40.2110
- Kim, Y. (2005). Immunomodulatory Activity of the Water Extract from Medicinal Mushroom *Inonotus obliquus*. *Mycobiology*, 33(3), 158. doi:10.4489/myco.2005.33.3.158
- Kim, Y. O., Han, S. B., Lee, H. W., Ahn, H. J., Yoon, Y. D., Jung, J. K., ... Shin, C. S. (2005). Immuno-stimulating effect of the endo-polysaccharide produced by submerged culture of *Inonotus obliquus*. *Life Sciences*, 77(19), 2438–2456. doi: 10.1016/j.lfs.2005.02.023
- Kim, S., Hwang, H., Park, J., Cho, Y., Song, C., & Yun, J. (2002). Mycelial growth and exopolysaccharide production by submerged culture of various edible mushrooms under different media. *Letters in Applied Microbiology*, 34(1), 56-61. doi:10.1046/j.1472-765x.2002.01041.x
- Kim, Y. O., Park, H. W., Kim, J. H., Lee, J. Y., Moon, S. H., & Shin, C. S. (2006). Anti-cancer effect and structural characterization of endo-polysaccharide from cultivated mycelia of *Inonotus obliquus*. *Life Sciences*, 79(1), 72-80. doi:10.1016/j.lfs.2005.12.047
- Kim, M., Seguin, P., Ahn, J., Kim, J., Chun, S., Kim, E., . . . Chung, I. (2008). Phenolic Compound Concentration and Antioxidant Activities of Edible and Medicinal Mushrooms from Korea. *Journal of Agricultural and Food Chemistry*, 56(16), 7265-7270. doi:10.1021/jf8008553

- Kim, D., Yang, B., Hur, N., Das, S., Yun, J., Choi, Y., & Song, C. (2001). Hypoglycemic Effects of Mycelia Produced from a Submerged Culture of *Phellinus linteus* (Berk. et Curt.) Teng (Aphyllphoromycetidae) in Streptozotocin-Induced Diabetic Rats. *International Journal of Medicinal Mushrooms*, 3(1), 6. doi:10.1615/intjmedmushr.v3.i1.30
- Korekar, G., Stobdan, T., Singh, H., Chaurasia, O., & Singh, S. (2011). Phenolic content and antioxidant capacity of various solvent extracts from seabuckthorn (*Hippophae rhamnoides*L.) fruit pulp, seeds, leaves and stem bark. *Acta Alimentaria*, 40(4), 449-458. doi:10.1556/aalim.40.2011.4.4
- Koyama, T., Gu, Y., & Taka, A. (2008). Fungal medicine, *Fuscoporia obliqua*, as a traditional herbal medicine: Its bioactivities, in vivo testing and medicinal effects. *Asian Biomed*, 2:459-469.
- Kroon-Batenburg, L., & Kroon, J. (1997). The crystal and molecular structures of cellulose I and II. *Glycoconjugate Journal*, 14(5), 677-690. doi:10.1023/a:1018509231331
- Kuo, C., Hsieh, C., & Lin, W. (2011). Proteomic response of LAP-activated RAW 264.7 macrophages to the anti-inflammatory property of fungal ergosterol. *Food Chemistry*, 126(1), 207-212. doi:10.1016/j.foodchem.2010.10.101
- Lee, M.-W., Hur, H., Chang, K.-C., Lee, T.-S., Ka, K.-H., & Jankovsky, L. (2008). Introduction to Distribution and Ecology of Sterile Conks of *Inonotus obliquus*. *Mycobiology*, 36(4), 199. doi: 10.4489/myco.2008.36.4.199
- Lee, I., Kim, Y., Jang, Y., Jung, J., & Yun, B. (2007). New antioxidant polyphenols from the medicinal mushroom *Inonotus obliquus*. *Bioorganic & Medicinal Chemistry Letters*, 17(24), 6678-6681. doi:10.1016/j.bmcl.2007.10.072
- Li, W., Hydamaka, A., Lowry, L., & Beta, T. (2009). Comparison of antioxidant capacity and phenolic compounds of berries, chokecherry and seabuckthorn. *Open Life Sciences*, 4(4), 499-506. doi:10.2478/s11535-009-0041-1
- Linderborg, K. M., Lehtonen, H., Järvinen, R., Viitanen, M., & Kallio, H. (2011). The fibres and polyphenols in sea buckthorn (*Hippophaë rhamnoides*) extraction residues delay postprandial

- lipemia. *International Journal of Food Sciences and Nutrition*, 63(4), 483-490. doi:10.3109/09637486.2011.636346
- Litchfield, J. H. (1967). Morel mushroom mycelium as a food-flavoring material. *Biotechnology and Bioengineering*, 9(3), 289–304. doi: 10.1002/bit.260090303
- Liu, C., Sun, R., Qin, M., Zhang, A., Ren, J., Xu, F., . . . Wu, S. (2007). Chemical modification of ultrasound-pretreated sugarcane bagasse with maleic anhydride. *Industrial Crops and Products*, 26(2), 212-219. doi:10.1016/j.indcrop.2007.03.007
- Liu, Y., Xu, Z., Zhang, W., Duan, J., Jiang, J., & Sun, D. (2016). Characterization of Fractional Polysaccharides from *Gleditsia sinensis* and *Gleditsia microphylla* Gums. *Molecules*, 21(12), 1745. doi:10.3390/molecules21121745
- Ma, L., Chen, H., Zhu, W., & Wang, Z. (2013). Effect of different drying methods on physicochemical properties and antioxidant activities of polysaccharides extracted from mushroom *Inonotus obliquus*. *Food Research International*, 50(2), 633-640. doi:10.1016/j.foodres.2011.05.005
- Magalhães, L. M., Santos, F., Segundo, M. A., Reis, S., & Lima, J. L. (2010). Rapid microplate high-throughput methodology for assessment of Folin-Ciocalteu reducing capacity. *Talanta*, 83(2), 441–447. doi: 10.1016/j.talanta.2010.09.042
- Mahapatra, S., & Banerjee, D. (2013). Fungal Exopolysaccharide: Production, Composition and Applications. *Microbiology Insights*, 6. doi:10.4137/mbi.s10957
- Markwell, M. A. K., Haas, S. M., Bieber, L., & Tolbert, N. (1978). A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Analytical Biochemistry*, 87(1), 206–210. doi: 10.1016/0003-2697(78)90586-9
- Masuko, T., Minami, A., Iwasaki, N., Majima, T., Nishimura, S.-I., & Lee, Y. C. (2005). Carbohydrate analysis by a phenol–sulfuric acid method in microplate format. *Analytical Biochemistry*, 339(1), 69–72. doi: 10.1016/j.ab.2004.12.001

- Mizuno, T. (1999). The Extraction and Development of Antitumor-Active Polysaccharides from Medicinal Mushrooms in Japan (Review). *International Journal of Medicinal Mushrooms*, 1(1), 9-29. doi:10.1615/intjmedmushrooms.v1.i1.20
- Mizuno, T., Zhuang, C., Abe, K., Okamoto, H., Kiho, T., Ukai, S., . . . Meijer, L. (1999). Antitumor and Hypoglycemic Activities of Polysaccharides from the Sclerotia and Mycelia of *Inonotus obliquus* (Pers.: Fr.) Pil. (Aphyllorphoromycetidae). *International Journal of Medicinal Mushrooms*, 1(4), 301-316. doi:10.1615/intjmedmushr.v1.i4.20
- Moghaddam, M. G., Ahmad, F. B., & Samzadeh-Kermani, A. (2012). Biological Activity of Betulinic Acid: A Review. *Pharmacology & Pharmacy*, 03(02), 119-123. doi:10.4236/pp.2012.32018
- Mulloy B. High-field NMR as a technique for the determination of polysaccharide structures. *Mol Biotechnol* 1996;6:241-65.
- Nakajima, Y., Nishida, H., Matsugo, S., & Konishi, T. (2009). Cancer Cell Cytotoxicity of Extracts and Small Phenolic Compounds from Chaga [*Inonotus obliquus* (persoon) Pilat]. *Journal of Medicinal Food*, 12(3), 501–507. doi: 10.1089/jmf.2008.1149
- Nielsen, S. S. (2010). *Food analysis laboratory manual*. New York: Springer.
- Nikitina, S. A., Habibrakhmanova, V. R., & Sysoeva, M. A. (2016). Chemical composition and biological activity of triterpenes and steroids of chaga mushroom. *Biochemistry (Moscow) Supplement Series B: Biomedical Chemistry*, 10(1), 63-69. doi:10.1134/s1990750816010108
- Niu, H., Song, D., Mu, H., Zhang, W., Sun, F., & Duan, J. (2016). Investigation of three lignin complexes with antioxidant and immunological capacities from *Inonotus obliquus*. *International Journal of Biological Macromolecules*, 86, 587–593. doi: 10.1016/j.ijbiomac.2016.01.111
- Noh, J.Y, Yoon, B.G, & Yoon, J.J (2003). Composition for prevention and treatment of type 1 and type 2 diabetes, containing water extract of *Cordyceps militaris* and *Inonotus obliquus* as effective ingredient. Korean Patent 2003-14648.

- Nomura, M., Takahashi, T., Uesugi, A., Tanaka, R., & Kobayashi, S. (2008). Inotodiol, a lanostane triterpenoid, from *Inonotus obliquus* inhibits cell proliferation through caspase-3-dependent apoptosis. *Anticancer research*, 28(5A), 2691–2696.
- Park, J., Kim, S., Hwang, H., Cho, Y., & Yun, J. (2002). Stimulatory effect of plant oils and fatty acids on the exo-biopolymer production in *Cordyceps militaris*. *Enzyme and Microbial Technology*, 31(3), 250-255. doi:10.1016/s0141-0229(02)00099-6
- Peng, Y., Zhang, L., Zeng, F., & Kennedy, J. F. (2005). Structure and antitumor activities of the water-soluble polysaccharides from *Ganoderma tsugae* mycelium. *Carbohydrate Polymers*, 59(3), 385-392. doi:10.1016/j.carbpol.2004.10.009
- Pokhrel, C., & Ohga, S. (2007). Submerged culture conditions for mycelial yield and polysaccharides production by *Lyophyllum decastes*. *Food Chemistry*, 105(2), 641-646. doi:10.1016/j.foodchem.2007.04.033
- Richards, H. L., Baker, P. G., & Iwuoha, E. (2012). Metal Nanoparticle Modified Polysulfone Membranes for Use in Wastewater Treatment: A Critical Review. *Journal of Surface Engineered Materials and Advanced Technology*, 02(03), 183-193. doi:10.4236/jsemat.2012.223029
- Ruthes, A. C., Carbonero, E. R., Córdova, M. M., Baggio, C. H., Sasaki, G. L., Gorin, P. A. J., ... Iacomini, M. (2013). Fucomannogalactan and glucan from mushroom *Amanita muscaria*: Structure and inflammatory pain inhibition. *Carbohydrate Polymers*, 98(1), 761–769. doi: 10.1016/j.carbpol.2013.06.061
- Ruthes, A. C., Smiderle, F. R., & Iacomini, M. (2015). d -Glucans from edible mushrooms: A review on the extraction, purification and chemical characterization approaches. *Carbohydrate Polymers*, 117, 753–761. doi: 10.1016/j.carbpol.2014.10.051'
- Saar, M. (1991). Fungi in khanty folk medicine. *Journal of Ethnopharmacology*, 31(2), 175-179. doi:10.1016/0378-8741(91)90003-v
- Sagayama, K., Tanaka, N., Fukumoto, T., & Kashiwada, Y. (2019). Lanostane-type triterpenes from the sclerotium of *Inonotus obliquus* (Chaga mushrooms) as proproliferative

- agents on human follicle dermal papilla cells. *Journal of Natural Medicines*, 73(3), 597-601. doi:10.1007/s11418-019-01280-0
- Saqib, A. A., Hassan, M., Khan, N. F., & Baig, S. (2010). Thermostability of crude endoglucanase from *Aspergillus fumigatus* grown under solid state fermentation (SSF) and submerged fermentation (SmF). *Process Biochemistry*, 45(5), 641-646. doi:10.1016/j.procbio.2009.12.011
- Seo, M. J., Kang, B. W., Park, J. U., Kim, M. J., Lee, H. H., Choi, Y. H., & Jeong, Y. K. (2011). Biochemical characterization of the exopolysaccharide purified from *Laetiporus sulphureus* mycelia. *Journal of microbiology and biotechnology*, 21(12), 1287–1293. doi:10.4014/jmb.1106.06046
- Shao, S., Hernandez, M., Kramer, J. K., Rinker, D. L., & Tsao, R. (2010). Ergosterol Profiles, Fatty Acid Composition, and Antioxidant Activities of Button Mushrooms as Affected by Tissue Part and Developmental Stage. *Journal of Agricultural and Food Chemistry*, 58(22), 11616-11625. doi:10.1021/jf102285b
- Shashkina, M. Y., Shashkin, P. N., & Sergeev, A. V. (2006). Chemical and medicobiological properties of chaga (review). *Pharmaceutical Chemistry Journal*, 40(10), 560-568. doi:10.1007/s11094-006-0194-4
- Shi, L. (2016). Bioactivities, isolation and purification methods of polysaccharides from natural products: A review. *International Journal of Biological Macromolecules*, 92, 37–48. doi: 10.1016/j.ijbiomac.2016.06.100
- Šiman, P., Filipová, A., Tichá, A., Niang, M., Bezrouk, A., & Havelek, R. (2016). Effective Method of Purification of Betulin from Birch Bark: The Importance of Its Purity for Scientific and Medicinal Use. *Plos One*, 11(5). doi:10.1371/journal.pone.0154933
- Smith, J. E., Sullivan, R., & Rowan, N. J. (2003). The Role of Polysaccharides Derived from Medicinal Mushrooms in Cancer Treatment Programs: Current Perspectives (Review). *International Journal of Medicinal Mushrooms*, 5(3), 18. doi:10.1615/interjmedicmush.v5.i3.10

- Solomon, P. W., & Alexander, L. W. (1999). Therapeutic effects of substances occurring in higher Basidiomycetes mushrooms: a modern perspective. *Immunobiology*, 19, 65–96
- Song, Y., Hui, J., Kou, W., Xin, R., Jia, F., Wang, N., Liu, H. (2008). Identification of *Inonotus obliquus* and Analysis of Antioxidation and Antitumor Activities of Polysaccharides. *Current Microbiology*, 57(5), 454-462. doi:10.1007/s00284-008-9233-6
- Song, F., Liu, Y., Kong, X., Chang, W., & Song, G. (2013). Progress on Understanding the Anticancer Mechanisms of Medicinal Mushroom: *Inonotus Obliquus*. *Asian Pacific Journal of Cancer Prevention*, 14(3), 1571-1578. doi:10.7314/apjcp.2013.14.3.1571
- Song, X., & Tang, J. (2016). Extraction Optimization, Preliminary Characterization and Bioactivities in Vitro of *Ligularia hodgsonii* Polysaccharides. *International Journal of Molecular Sciences*, 17(5), 788. doi: 10.3390/ijms17050788
- Sun, Y., Yin, T., Chen, X., Zhang, G., Curtis, R. B., Lu, Z., & Jiang, J. (2011). In Vitro Antitumor Activity and Structure Characterization of Ethanol Extracts from Wild and Cultivated Chaga Medicinal Mushroom, *Inonotus obliquus* (Pers.:Fr.) Pilát (Aphyllophoromycetidae). *International Journal of Medicinal Mushrooms*, 13(2), 121-130. doi:10.1615/intjmedmushr.v13.i2.40
- Synytsya, A., Míčková, K., Synytsya, A., Jablonský, I., Spěváček, J., Erban, V., ... Čopíková, J. (2009). Glucans from fruit bodies of cultivated mushrooms *Pleurotus ostreatus* and *Pleurotus eryngii*: Structure and potential prebiotic activity. *Carbohydrate Polymers*, 76(4), 548–556. doi: 10.1016/j.carbpol.2008.11.021
- Synytsya, A., & Novak, M. (2013) Structural Analysis of Fungal Glucans. *Series Title: Biology and Chemistry of Beta Glucan Volume Title: Beta-Glucan, Structure, Chemistry and Specific Application*, 3–28. doi: 10.2174/9781608052608113020004
- Vamanu, E., Livadariu, O., & Ene, M. (2018). Characterization of some bioactive compounds released by *Inonotus obliquus* in submerged culture. *Scientific Bulletin. Series F. Biotechnologies*, XXII.

- Wang, Q., Mu, H., Zhang, L., Dong, D., Zhang, W., & Duan, J. (2015). Characterization of two water-soluble lignin metabolites with antiproliferative activities from *Inonotus obliquus*. *International Journal of Biological Macromolecules*, 74, 507–514. doi: 10.1016/j.ijbiomac.2014.12.044
- Wasser, S. (2002). Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides. *Applied Microbiology and Biotechnology*, 60(3), 258-274. doi:10.1007/s00253-002-1076-7
- Weber, D., Zhang, M., Zhuang, P., Zhang, Y., Wheat, J., Currie, G., & Al-Eisawi, Z. (2014). The efficacy of betulinic acid in triple-negative breast cancer. *SAGE Open Medicine*, 2, 205031211455197. doi:10.1177/2050312114551974
- Willför, S., Pranovich, A., Tamminen, T., Puls, J., Laine, C., Suurnäkki, A., . . . Holmbom, B. (2009). Carbohydrate analysis of plant materials with uronic acid-containing polysaccharides—A comparison between different hydrolysis and subsequent chromatographic analytical techniques. *Industrial Crops and Products*, 29(2-3), 571-580. doi:10.1016/j.indcrop.2008.11.003
- Wold, C. W., Kjeldsen, C., Corthay, A., Rise, F., Christensen, B. E., Duus, J. Ø., & Inngjerdigen, K. T. (2018). Structural characterization of bioactive heteropolysaccharides from the medicinal fungus *Inonotus obliquus* (Chaga). *Carbohydrate Polymers*, 185, 27–40. doi: 10.1016/j.carbpol.2017.12.041
- Woldemichael, G. M., Singh, M. P., Maiese, W. M., & Timmermann, B. N. (2003). Constituents of Antibacterial Extract of *Caesalpinia paraguariensis* Burk. *Zeitschrift Für Naturforschung C*, 58(1-2), 70-75. doi:10.1515/znc-2003-1-213
- Won, D. P., Lee, J. S., Kwon, D. S., Lee, K. E., Shin, W. C., & Hong, E. K. (2010). Immunostimulating activity by polysaccharides isolated from fruiting body of *Inonotus obliquus*. *Molecules and Cells*, 31(2), 165-173. doi:10.1007/s10059-011-0022-x
- Wong, C. (2019, September 05). What Can Chaga Do for You? Retrieved June 13, 2020, from <https://www.verywellhealth.com/what-can-chaga-do-for-you-89553>

- Xu, X., Li, J., & Hu, Y. (2014a). Polysaccharides from *Inonotus obliquus* sclerotia and cultured mycelia stimulate cytokine production of human peripheral blood mononuclear cells in vitro and their chemical characterization. *International Immunopharmacology*, 21(2), 269–278. doi: 10.1016/j.intimp.2014.05.015
- Xu, X., Hu, Y., & Quan, L. (2014b). Production of bioactive polysaccharides by *Inonotus obliquus* under submerged fermentation supplemented with lignocellulosic biomass and their antioxidant activity. *Bioprocess and Biosystems Engineering*, 37(12), 2483–2492. doi: 10.1007/s00449-014-1226-1
- Xu, X., & Zhu, J. (2011). Enhanced phenolic antioxidants production in submerged cultures of *Inonotus obliquus* in a ground corn stover medium. *Biochemical Engineering Journal*, 58-59, 103-109. doi:10.1016/j.bej.2011.09.003
- Yang, B., & Kallio, H. P. (2001). Fatty Acid Composition of Lipids in Sea Buckthorn (*Hippophaë rhamnoides* L.) Berries of Different Origins. *Journal of Agricultural and Food Chemistry*, 49(4), 1939-1947. doi:10.1021/jf001059s
- Yang, F., Ke, Y., & Kuo, S. (2000). Effect of fatty acids on the mycelial growth and polysaccharide formation by *Ganoderma lucidum* in shake flask cultures. *Enzyme and Microbial Technology*, 27(3-5), 295-301. doi:10.1016/s0141-0229(00)00213-1
- Zhao, F., Mai, Q., Ma, J., Xu, M., Wang, X., Cui, T., . . . Han, G. (2015). Triterpenoids from *Inonotus obliquus* and their antitumor activities. *Fitoterapia*, 101, 34-40. doi:10.1016/j.fitote.2014.12.005
- Zheng, W., Zhang, M., Zhao, Y., Miao, K., Pan, S., Cao, F., & Dai, Y. (2011). Analysis of antioxidant metabolites by solvent extraction from sclerotia of *Inonotus obliquus* (Chaga). *Phytochemical Analysis*, 22(2), 95-102. doi:10.1002/pca.1225
- Zhong, X.-H., Ren, K., Lu, S.-J., Yang, S.-Y., & Sun, D.-Z. (2009). Progress of research on *Inonotus obliquus*. *Chinese Journal of Integrative Medicine*, 15(2), 156–160. doi: 10.1007/s11655-009-0156-2

Appendix

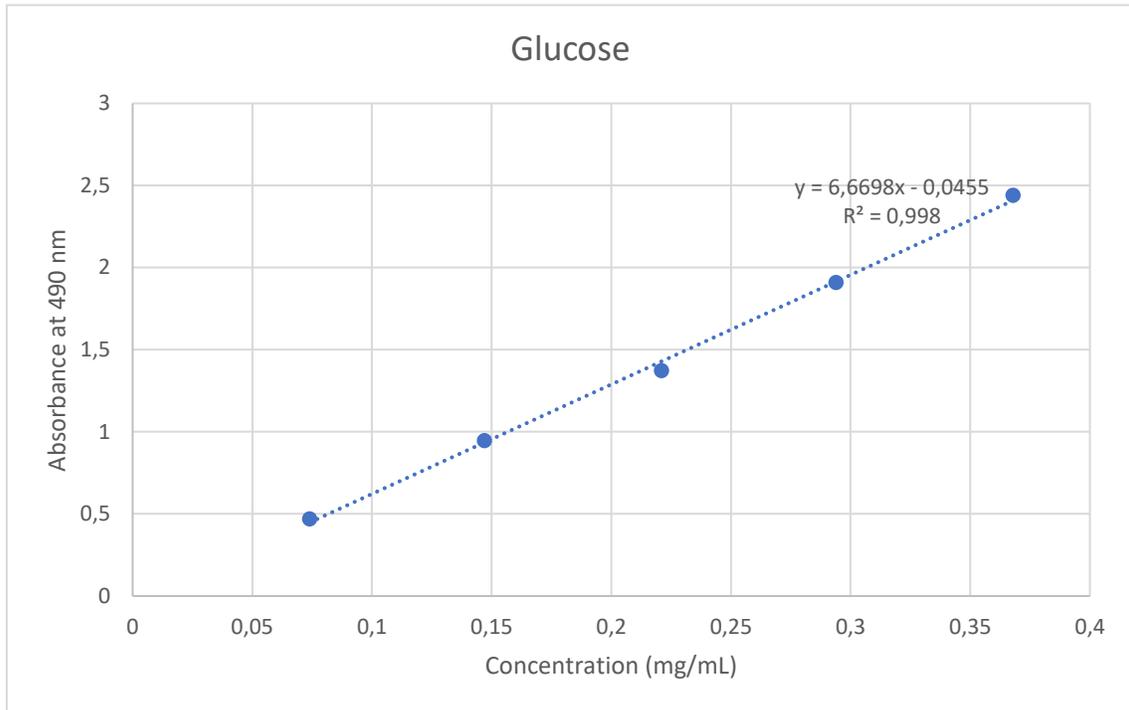


Figure 17. Glucose standard curve

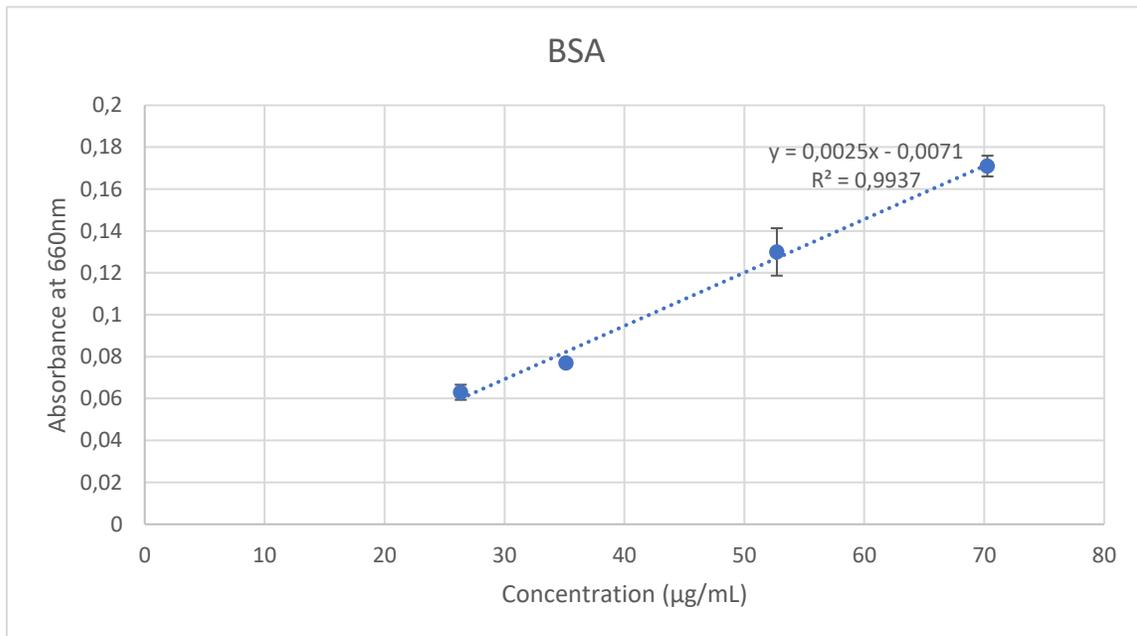


Figure 18. BSA standard curve

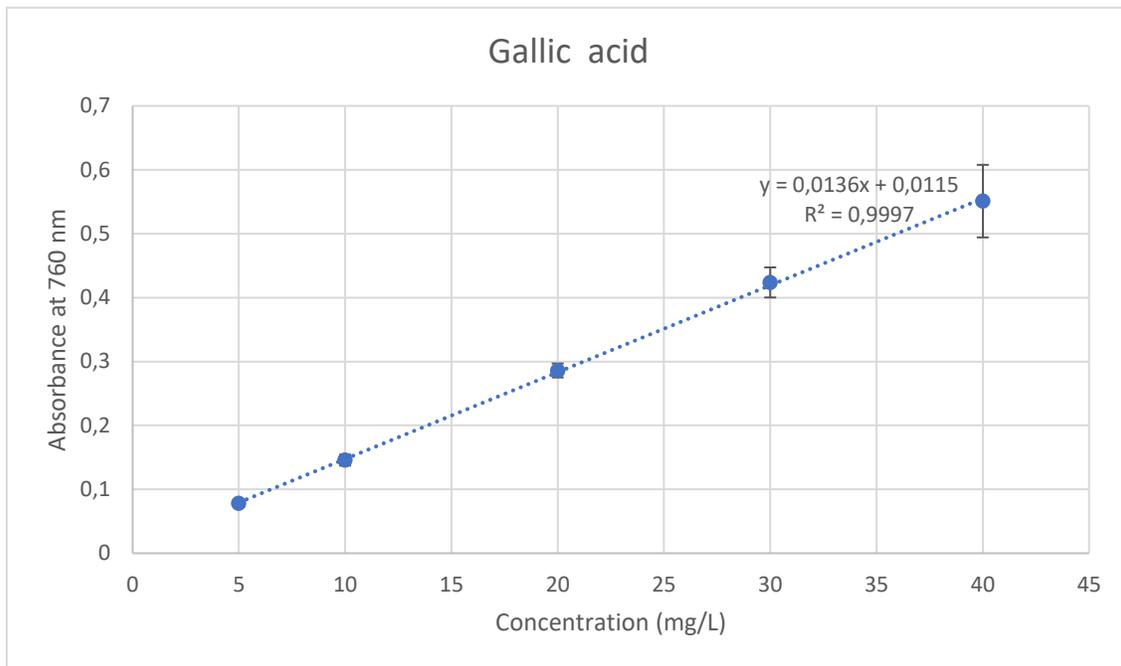


Figure 19. Gallic acid standard curve

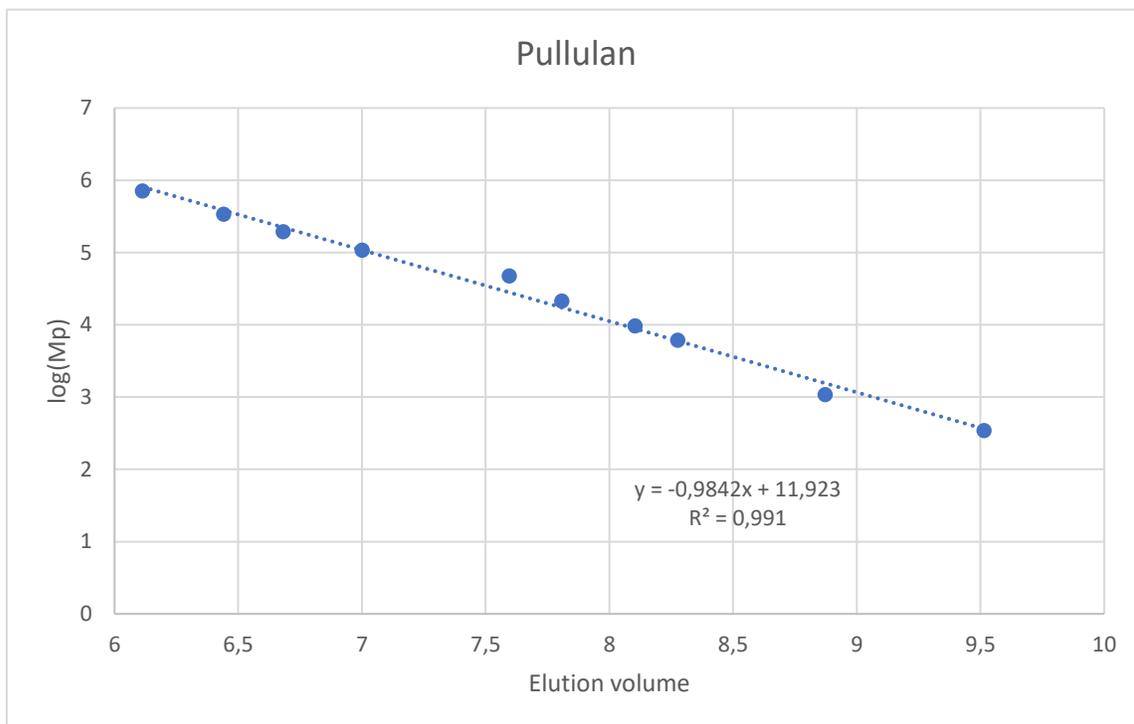


Figure 20. Pullulan standard curve

Table 6. One-way ANOVA paired with Tukey test for protein content

Multiple Comparisons						
Dependent Variable: Protein						
Tukey HSD						
(I) Samples	(J) Samples	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
(0+0.5)_H W	(0+0.5)_AL K	-10,09157706 0931908*	2,153043511 390010	,007	-16,98638026 6065463	-3,196773855 798352
	(1+2)_HW	1,718421052 631577	2,153043511 390010	,853	-5,176382152 501979	8,613224257 765133
	(1+2)_ALK	-10,19814814 8148146*	2,153043511 390010	,006	-17,09295135 3281702	-3,303344943 014590
(0+0.5)_AL K	(0+0.5)_H W	10,09157706 0931908*	2,153043511 390010	,007	3,196773855 798352	16,98638026 6065463
	(1+2)_HW	11,80999811 3563484*	2,153043511 390010	,003	4,915194908 429928	18,70480131 8697040
	(1+2)_ALK	-1,1065710872 16238	2,153043511 390010	1,000	-7,001374292 349794	6,788232117 917318

(1+2)_HW	(0+0.5)_H W	- 1,718421052 631577	2,153043511 390010	,853	- 8,613224257 765133	5,176382152 501979
	(0+0.5)_AL K	- 11,80999811 3563484*	2,153043511 390010	,003	- 18,70480131 8697040	- 4,915194908 429928
	(1+2)_ALK	- 11,91656920 0779723*	2,153043511 390010	,002	- 18,81137240 5913280	- 5,021765995 646167
(1+2)_ALK	(0+0.5)_H W	10,19814814 8148146*	2,153043511 390010	,006	3,303344943 014590	17,09295135 3281702
	(0+0.5)_AL K	,1065710872 16238	2,153043511 390010	1,000	- 6,788232117 917318	7,001374292 349794
	(1+2)_HW	11,91656920 0779723*	2,153043511 390010	,002	5,021765995 646167	18,81137240 5913280
*. The mean difference is significant at the 0.05 level.						

Protein			
Tukey HSD ^a			
Samples	N	Subset for alpha = 0.05	
		1	2
(1+2)_HW	3	22,49824561 4035090	
(0+0.5)_H W	3	24,21666666 6666665	
(0+0.5)_AL K	3		34,30824372 7598570

(1+2)_ALK	3		34,41481481 4814810
Sig.		,853	1,000
Means for groups in homogeneous subsets are displayed.			
a. Uses Harmonic Mean Sample Size = 3,000.			

Table 7. P-value obtained from Kruskal Wallis test for the monomers of mycelium extracts

Monosaccharide	P value (Extract)	P value (Fraction)
Xylose	0.004	1.000
Galactose	1.000	0.004
Rhamnose	0.149	0.004
Glucose	0.004	1.000
Mannose	0.004	1.000
Arabinose	0.149	0.004
Fucose	0.004	1.000

Table 8. Multiple comparison results (adjusted p-value) of mycelium extracts obtained from Dunn Kruskal Wallis

Multiple comparison		Xyl	Gal	Rha	Glc	Man	Ara	Fuc
(0+0.5) 2%	(0+0.5) HW	1.000	1.000	1.000	1.000	1.000	1.000	1.000

	(1+2) 2%	1.000	0.013	0.249	1.000	1.000	0.249	1.000
	(1+2) HW	0.249	0.249	1.000	0.249	0.249	1.000	0.249
(0+0.5) HW	(1+2) 2%	0.249	0.249	0.013	0.249	0.249	0.013	0.249
	(1+2) HW	1.000	1.000	0.249	1.000	1.000	0.249	1.000
(1+2) 2%	(1+2) HW	0.013	1.000	1.000	0.013	0.013	1.000	0.013

Table 9. One-way ANOVA paired with Tukey test for sugar content

Multiple Comparisons						
Dependent Variable: Sugar						
Tukey HSD						
(I) Fraction	(J) Fraction	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
(0+0.5) HW	(0+0.5) 2%	13,9609035 44648488	9,70197826 2684241	,512	- 17,1082459 95439056	45,0300530 84736030
	(1+2) HW	8,33507345 7082729	9,70197826 2684241	,825	- 22,7340760 83004815	39,4042229 97170270
	(1+2) 2%	27,6436278 33151662	9,70197826 2684241	,082	- 3,42552170 6935882	58,7127773 73239206
(0+0.5) 2%	(0+0.5) HW	- 13,9609035 44648488	9,70197826 2684241	,512	- 45,0300530 84736030	17,1082459 95439056
	(1+2) HW	- 5,62583008 7565760	9,70197826 2684241	,935	- 36,6949796 27653304	25,4433194 52521784

	(1+2) 2%	13,6827242 88503174	9,70197826 2684241	,527	- 17,3864252 51584370	44,7518738 28590720
(1+2) HW	(0+0.5) HW	- 8,33507345 7082729	9,70197826 2684241	,825	- 39,4042229 97170270	22,7340760 83004815
	(0+0.5) 2%	5,62583008 7565760	9,70197826 2684241	,935	- 25,4433194 52521784	36,6949796 27653304
	(1+2) 2%	19,3085543 76068933	9,70197826 2684241	,267	- 11,7605951 64018610	50,3777039 16156480
(1+2) 2%	(0+0.5) HW	- 27,6436278 33151662	9,70197826 2684241	,082	- 58,7127773 73239206	3,42552170 6935882
	(0+0.5) 2%	- 13,6827242 88503174	9,70197826 2684241	,527	- 44,7518738 28590720	17,3864252 51584370
	(1+2) HW	- 19,3085543 76068933	9,70197826 2684241	,267	- 50,3777039 16156480	11,7605951 64018610

Table 10. Univariate analysis paired with Bonferroni for Galacturonic acid content of the extracts

Multiple Comparisons						
Dependent Variable: GalA						
Bonferroni						
(I) Variable	(J) Variable	Mean Difference (I- J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0_0.5_HW	0_0.5_AL K	,2359082080 00000*	,0426105020 48076	,003	,0876714191 50981	,3841449968 49019
	1_2_HW	- 1,544618223 000000*	,0426105020 48076	,000	- 1,692855011 849019	- 1,396381434 150981

	1_2_ALK	- ,7532652230 00000*	,0426105020 48076	,000	- ,9015020118 49019	- ,6050284341 50981
0_0.5_AL K	0_0.5_HW	- ,2359082080 00000*	,0426105020 48076	,003	- ,3841449968 49019	- ,0876714191 50981
	1_2_HW	- 1,780526431 000000*	,0426105020 48076	,000	- 1,928763219 849019	- 1,632289642 150981
	1_2_ALK	- ,9891734310 00000*	,0426105020 48076	,000	- 1,137410219 849019	- ,8409366421 50981
1_2_HW	0_0.5_HW	1,544618223 000000*	,0426105020 48076	,000	1,396381434 150981	1,692855011 849019
	0_0.5_AL K	1,780526431 000000*	,0426105020 48076	,000	1,632289642 150981	1,928763219 849019
	1_2_ALK	,7913530000 00000*	,0426105020 48076	,000	,6431162111 50981	,9395897888 49019
1_2_ALK	0_0.5_HW	,7532652230 00000*	,0426105020 48076	,000	,6050284341 50981	,9015020118 49019
	0_0.5_AL K	,9891734310 00000*	,0426105020 48076	,000	,8409366421 50981	1,137410219 849019
	1_2_HW	- ,7913530000 00000*	,0426105020 48076	,000	- ,9395897888 49019	- ,6431162111 50981
Based on observed means.						
The error term is Mean Square(Error) = ,003.						
*. The mean difference is significant at the 0,05 level.						