Structural characterization and biological activity of *Lactarius scrobiculatus*

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Ivana Tomic

Abstract

Mushrooms have been reported to possess certain compounds with antibacterial activity, against both G+ and G- bacteria. The aim of this thesis was to obtain different extracts from mushroom *L.scrobiculatus*, isolate polysaccharides, investigate their chemical composition and eventual biological effect on activity of membrane protein *MgtA*, present in *E.coli* cell wall.

Lactarius scrobiculatus, a wild non-edible mushroom, contains a milky-juice, which plays important role in protecting them from insects, animals and microorganisms. β-glucans from mushrooms cell wall are assigned as compounds which can activate macrophage. In addition, other secondary metabolites from mushrooms such as phenolic compounds, terpenoids and others showed activity against bacteria and fungi. *MgtA*, belongs to P-type ATP-ase, found in bacteria, *E.coli,* which is responsible for import Mg^{2+}ions into the cytoplasm of bacteria using energy from ATP. The possible inhibition of *MgtA* might hinder bacterial homeostasis.

Extraction and fractionations of *Lactarius scrobiculatus* were performed to obtain several crude extracts and fractions of the water extract, named LsW. Polysaccharides from *L.scrobiculatus* were isolated by sequential extraction, enzymatic treatment, SEC-HPLC and SEC preparative chromatography using three different preparative columns. The obtained fractions were analysed with respect to monosaccharide composition and glycosidic linkage determination by methylation and GC-MS.

The results from SEC-HPLC showed that LsW contained fractions with the molecular weight range from 2985,4 kDa to 2.4 kDa which may indicate that LsW contains a mixture of several polysaccharides. The results obtained by methanolysis and methylation suggested that the water extract LsW might contain a (1 → 3) -linked-D-glucan, a (1 → 6) -linked D-galactan. The terminal unidentified 6-deoxy hexose was also detected.

Determination of glycosidic linkage structure of alkali extract, named LsA suggested the presence of two different glucans mainly in the alkali extract LsA: a (1 → 3) -linked -D-glucan, a (1 → 6) -linked -D-glucan and a significant amount of amylose like (1 → 4) -linked α-D-glucan. The GC-MS and iodine-potassium iodide assay has indicated the presence of high amounts of (1 → 4)-α-D-glucan.

^{1}H NMR spectroscopy showed the presence of mannitol in ethanol extract precipitate, LsK.

The three of extracts, named LsK, LsDCM and LsEt gave about 30 % inhibition of activity of *MgtA*, while LsW and mannitol decreased *MgtA* activity by about 20 %. It was only screening test which should evaluate whether the inhibition of MgtA with extracts is possible in general. The further activity tests needed in the future.

Table of contents

1 Abberviations

Abs	Absorbance
Ac	Acetylated
ATP	Adenosin-5´-trifosfat
AUC	Areal under curve
CR-3	Complement receptor 3
DCM	Dichloromethane
DMSO	Dimethyl sulfoxide
ER	Endoplasmic reticulum
EtOH	Ethanol
Gal	Galactose
Gal A	Galacturonic acid
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
Glc	Glucose
HPLC	High Performance Liquid Chromatography
IL	Interleukin
INF	Interferon
LsA	Alkali extract of *Lactarius scrobiculatus*
LsDCM	Dichloromethane extract of *L.scrobiculatus*
LsEt	Ethanol extract of *L.scrobiculatus*
LsK	Ethanol extract precipitate of *L. scrobiculat.*
LsW	Water extract of *L.scrobiculatus*
LsW1a, LsW2a, LsW3a	Fractions of water extract after purification on Sephacryl S500 column

LsW1b, LsW2b	Fractions of water extract after purification on Sephacryl S500 HR column
LsW1c, LsW2c, LsW3c	Fractions of water extract after purification on Superdex 200 HR column
Man	Mannose
Me	Methylated
ml	Mililiter
MgtA	Magnesium transport protein
Mp	Peak molecular weight
MS	Mass spectrometry
Mw	Molecular weight
MWCO	Molecular Weight Cut Off
MQ-water	Milli-Q Water
NK	Natural Killer cells
NMR	Nuclear Magnetic Resonance Spectroscopy
NO	Nitrogen oxide
RI	Refractive index
RID	Refractive index detector
ROS	Reactive oxygen species
Rt	Retention time
SEC	Size exclusion chromatography
SERCA	Sarcoendoplasmic reticulum
SR	Sarcoplasmic reticulum
TFA-α	Trifluoroacetic acid
TLR	Toll like receptor
TMS	Trimethylsilyl
TNF	Tumour necroes factor

Vo	Void volume
Vt	Total column volume
Xyl	Xylose

2. Introduction

2.1. MUSHROOMS

Mushrooms have been collected and consumed in oriental countries for more than 2000 years. About 140.000 species are known worldwide and it is accepted that there may be around 1.5 million fungal species on Earth, including both micro and macrofungi of which about 2.000 are edible and 700 show significant medicinal properties(S.-t. Chang & Miles, 2004).

Mushrooms are appreciated for their special flavor, economic and ecological values and medicinal properties. The edible macrofungi have been traditionally used in many countries, not only as a source of food, but as a medicinal resource (S.-t. Chang & Miles, 2004) (Stachowiak & Reguła, 2012; Wasser, 2002). Many kinds of mushrooms which are not edible, can also possess medical qualities (S.-t. Chang & Miles, 2004). On the other side, many mushrooms produce mycotoxins, such as alkaloids and polyketides which are toxic to animals and humans (Charya, 2015).

2.1.1 Classification of mushrooms

The Kingdom Fungi comprises a large group of organisms, which are neither plants nor animals. Fungi are a group of eukaryotic organisms and include both microfungi (yeast and molds) and macrofungi (mushrooms)(Charya, 2015).

By definition, mushrooms are" macrofungi with a fruiting body of sufficient size, large enough to be seen with the naked eye and to be picked by hand"(S.-t. Chang & Miles, 2004). Mushrooms may be categorized into two major categories: the true Fungi (Eumycota) and ″pseudofungi″. Oomycota is one of the most important pseudofungi. The traditional feature to distinguish the Oomycota from the Eumycota has been the absence of the chitin in the Oomycota cell walls(Webster & Weber, 2007).

The traditional taxonomic scheme classifies the true fungi- Eumycota into four divisions which are based on variations in sexual reproduction: Basidiomycota, Ascomycota, Zygomycota and Chytridiomycota (see Figure 1)(Webster & Weber, 2007). Mushrooms may belong to the Ascomycota or the Basidiomycota. The Ascomycota include more than 32.000 described species, while the Basidiomycota include over 30 000 species(S.-t. Chang & Miles, 2004);(Webster & Weber, 2007). If the spores of fungi are located in a special structure, which called basidium, they belong to Basidiomycota. In the Ascomycota, sexual spores are contained in cylindrical cells called ascus (Puri, 2017).

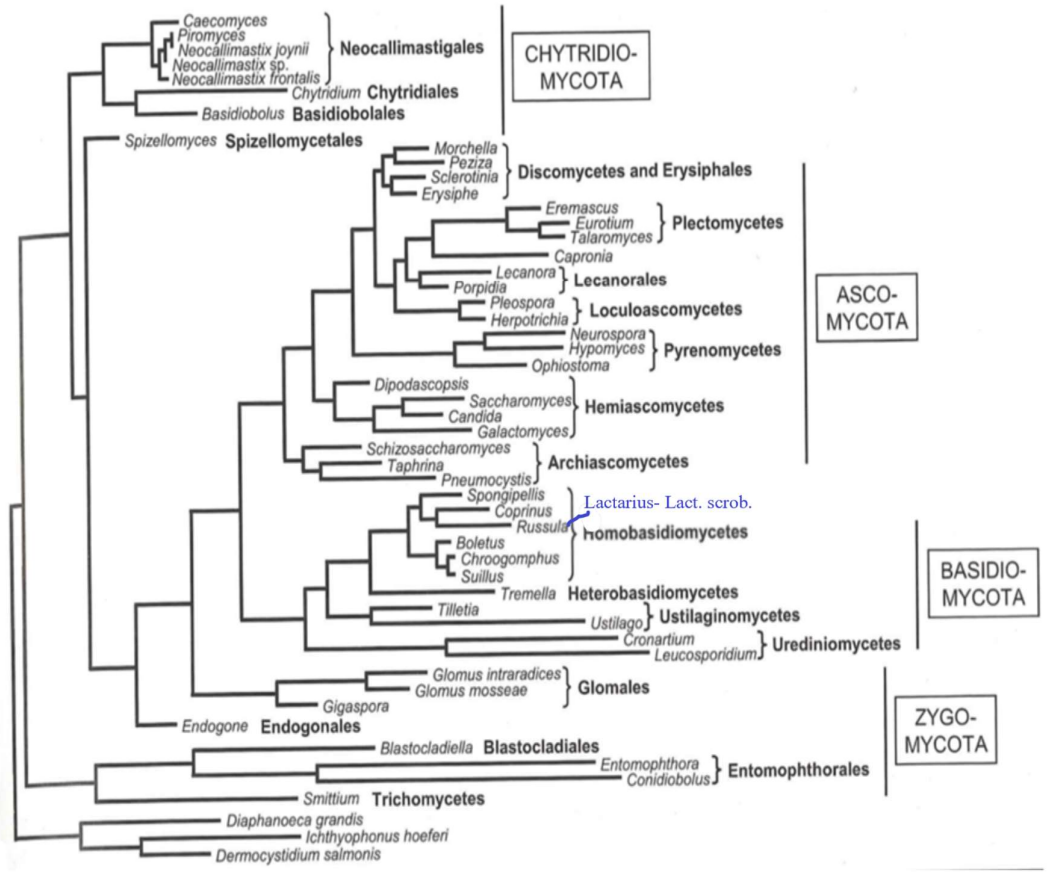


Figure 1. The classification of the Kingdom fungi (Eumycota) (Figure is based on (Webster & Weber, 2007). *Lactarius scrobiculatus* belong to order *Russula*, familie *Russulaceae*.

The Basidiomycota are a large group of fungi (referred to as higher fungi), estimated to include 34 % of described fungi and comprise four subphyla. They include many mushrooms: puffballs, earth balls, false truffles jelly fungi and other familiar forms which belongs two main classes: Homobasidiomycota that includes true mushrooms and Heterobasidiomycota which includes the jelly fungi (Webster & Weber, 2007).

The vegetative state, reproduction, composition of the cell wall and nutrition of four main groups of the large Kingdom Fungi are represented in Table 1.

Table 1: The characteristics of 4 divisions in the Kingdom Fungi: Basidiomycota, Ascomycota, Zygomycota and Chytridiomycota. Table is based on (Webster & Weber, 2007)

	Vegetative state	Reproduction	Cell wall composition	Nutrition
Basidiomycota	septate mycelium or yeast; grow as hyphae and yeast cells, divides by fission	asexual spores absent, or sexual spores are formed in a basidium	highly branched glucans and chitin	usually saprotrophic and some of them are pathogens of trees
Ascomycota	septate mycelium or yeasts; grow as hyphae and yeast cells divides by fission	asexual spores which are not formed in sporangium and sexual spores are formed in ascus	highly branched glucans and chitin	some of them are saprotrophs, other parasites of plants, animals and humans
Zygomycota	aseptate mycelia	nonmotile asexual spores formed in a sporangium-often large, thick-walled structures with lipid reserves-zygospores	glucans and chitin; chitin fibres are modified to produce chitosan	usually saprotrophs, only a few are parasitic on plants and animals
Chytridiomycota	the thallus has sporangia-organ for reproduction which arises from vegetative part and often consist of a system of the branching rhizoids	zoospores, sexually produced spores	chitin and cellulose	many are saprotrophs, and some are parasites of algae

2.1.2 Mushrooms: structure, growth and nutrition

Mushrooms consist of **the fruiting body** -the reproductive part of the fungus and **the mycelium**- the vegetative part of the fungus. The fruiting body consist of an umbrella-shaped pileus (cap) and a stipe (stem). Spores are usually in lamellae- structures on the underside of the pileus (see Figure 2) (Puri, 2017).

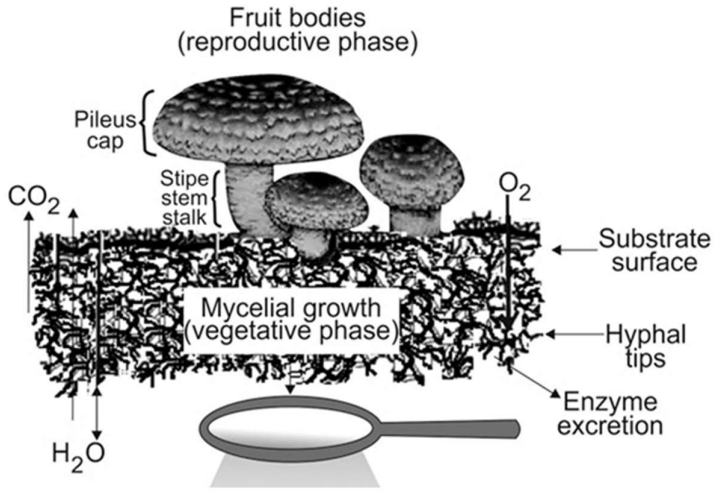


Figure 2. Schematic image of mushroom phases of growth and basic mycological terms (Puri, 2017)

The mycelium consists of a mass of branching tubes, called **hyphae** and cordlike strands that branch through soil and wood log (Peter C. K. Cheung, 2008). Each hypha is a tube consisting of rigid wall, protoplasm and septa (cross walls) (see Figure 3) (Charya, 2015; Puri, 2017; Sánchez, 2017)

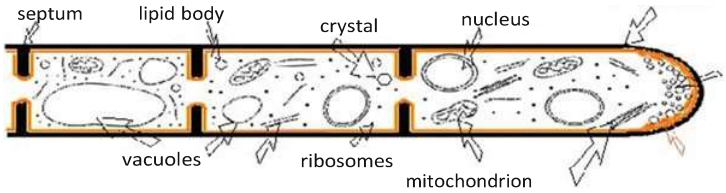


Figure 3. Structure of hypha (Charya, 2015)

Mushrooms can appear either above ground (**epigeous**) or below ground (**hypogeous**)(Charya, 2015). Hyphae exhibit usually apical growth (see Figure 4) and branching leads to form a network of hyphae or mycelia (mycelial growth) (Puri, 2017; Sánchez, 2017).

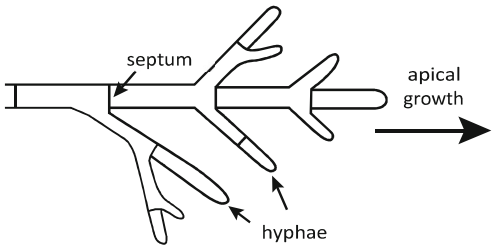


Figure 4. Apical growth of hypha (Charya, 2015)

Mushrooms can be divided into three groups: saprophytes, parasites and mycorrhiza. Mycorrhiza mushrooms live in a close partnership with plants and animals while saprophytes get their nutrients from dead organic materials. Parasites take food nutrients from living plants, animals and humans (Peter C. K. Cheung, 2008)

The fruiting body of mushrooms contains in general:

- **Proteins, peptides** - the protein content of edible mushrooms is high (15- 35% of dry weight) (Rathore, Prasad, & Sharma, 2017) and amino acids as a part of protein, range from 34-47% of dry weight (DW) include leucine, valine, glutamic acid, aspartic acid and arginine (Peter C. K. Cheung, 2008; Reis, Martins, Vasconcelos, Morales, & Ferreira, 2017).

- **Fat** -unsaturated fatty acids are dominant over saturated; especially palmitic acid, monounsaturated oleic acid and polyunsaturated linoleic acid- (2-6%)(Kalač, 2009; Rathore et al., 2017; Wasser, 2002).

- **Carbohydrates** varies with species and ranges from 35 to 70% of dry weight, including digestible and non-digestible carbohydrates (P. C. K. Cheung, 2010; Erjavec, Kos, Ravnikar, Dreo, & Sabotič, 2012; Rathee, Rathee, Kumar, & Rathee, 2012). Glucose and mannitol are the major monosaccharides in mushrooms, belonging to digestible carbohydrates (Kalač, 2009). The content of mannitol in mushrooms ranges from 0.2% to 13.9 %. Non-digestible carbohydrates include oligosaccharides such as trehalose and polysaccharides such as β-glucans and mannans (P. C. K. Cheung, 2010). Dietary fibers are components of fungal cell walls- 100g of fresh mushrooms generally provide between 10-40% (Peter C. K. Cheung, 2008).

- **Secondary metabolites**: polyphenolic compounds (Barros, Cruz, Baptista, Estevinho, & Ferreira, 2008), phenolic acids, flavonoids, tannins, terpenoids (mono-, sesqui- and triterpenoids) and ergosterol (a terpenoid, precursor of vitamin D) (Peter C. K. Cheung, 2008; Sánchez, 2017) anthraquinones, quinolines and primary metabolites such as oxalic acid (Alves et al., 2012; Taofiq, Martins, Barreiro, & Ferreira, 2016). Some mushrooms contain alkaloids mainly indole alkaloids such as: psilocybin and psilocin from *Psilocybe semilanceata* and laccarin A from *L. subplinthogalus* (Charya, 2015) (Aniszewski, 2015; Y. Wang, Yang, Wu, & Yue, 2004) (Brosse & Brossi, 1991).

- **Vitamins** –mushrooms are rich in vitamins such as folic acid, niacin, ascorbic acid, thiamine and contain low amounts of β-carotene, riboflavin and vitamin E (Muszyńska, Grzywacz-Kisielewska, Kała, & Gdula-Argasińska, 2018).

- In general the water content is about 85-95% **water** (Stachowiak & Reguła, 2012).

- **Ash** content in mushroom is 5-12% of dry weight. Mushrooms contain a wide variety of **minerals**: selenium, zinc, copper, iron, magnesium, zinc, calcium and phosphorus (Peter C. K. Cheung, 2008; Kalač, 2009).

2.1.3 Polysaccharides and chitin in the fungal cell wall

Mushroom polysaccharides are highly complex molecules, which are divided into: **homoglycans** and **heteroglycans**. The glycans are macromolecules which may have linkages with α or β configuration and different degrees of polymerization They can be further differentiate on the basis of glycosidic bond position, distribution of specific glycosidic bonds along the chain, branching and molecular mass (Synytsya & Novák, 2013).

Homoglycans contain residues of only one type of monosaccharide molecules, typically α-, β-D-glucans and chitin (Enshasy & Hatti-Kaul, 2013). **Heteroglycans** contain residues of two or more types of monosaccharide molecules (Enshasy & Hatti-Kaul, 2013; Andrea C. Ruthes, Smiderle, & Iacomini, 2016). Heteroglycans such as **xyloglucans**, **xylomannans** and heterogalactans, which may contain a variety of side chains including **mannogalactans**, **fucogalactans** **fucomannogalactans** are isolated from mushrooms (Andrea C. Ruthes et al., 2016).

α-D-glucans

Linear α-D-glucans have been found in many yeasts and higher fungi. The glycosidic linkages are variable in different fungi and both (1→3)-, -(1→4)- and (1→6)-linked α-D-glucans have been identified (Synytsya & Novák, 2013) (see Figure 5).

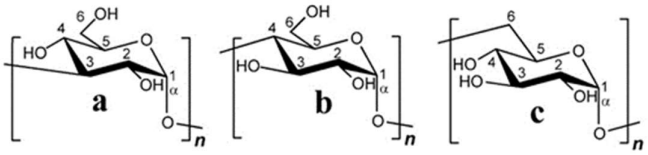


Figure 5. Structure of fungal glucans: (a) (1→3)- α-D-glucan; (b) (1→4)- α-D-(glucan; (c) (1→6)- α-D-glucan; (Synytsya & Novák, 2013).

The reserve polysaccharide in mushrooms, **glycogen** is branched (1→4), (1→6)- α-D-glucans (Figure 6 e) and usual content is 5-10 % of dry matter (see Figure 6 d) (Kalač, 2009; Wasser, 2002) (Synytsya & Novák, 2013).

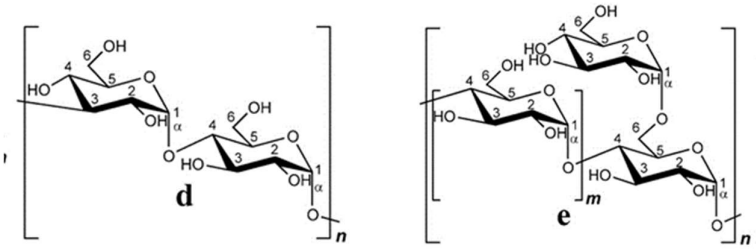


Figure 6. (d) mixed-linkage (1→3), (1→4)- α-D-glucan; (e) branched (1→4),(1→6)- α-D-glucan (glycogen); (Synytsya & Novák, 2013)

2.1.4 β-glucans

Mushrooms β-glucans consist structurally of (1→3)- and/or (1→6)- β-D-linked backbones (see Figure 7 f, g) with side chains attached to O-3 or O-6 which can form tertiary structures (Zhu, Du, Bian, & Xu, 2015). There are many reports about isolation, structure and biological effect of these polysaccharides.

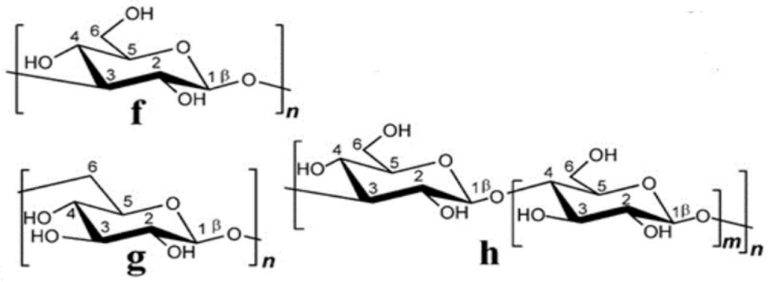


Figure 7. (f) (1→3)- β-D-glucan; (g) (1→6)- β-D-glucan; (h) mixed-linkage (1→3), (1→4)- β-D-(glucan; (Zhu et al., 2015)

A wide range of β-glucans of different structure have been identified for example cereal β-glucan (see Figure 7 h). Branched glucans contain various side chains, with one or more monosaccharide units, attached to the backbone at different positions. Branched (1→3), (1→6)- β-D-glucans (Figure 8 i) have been isolated from *Pleurotus ostreatus*, *Pleurotus eryngii, S. cerevisiae* and *A. niger.* From medicinal mushroom *Agaricus blazei* has been identified water soluble (1→6), (1→3)- β-D-glucan (Figure 8 j) (Zhu et al., 2015).

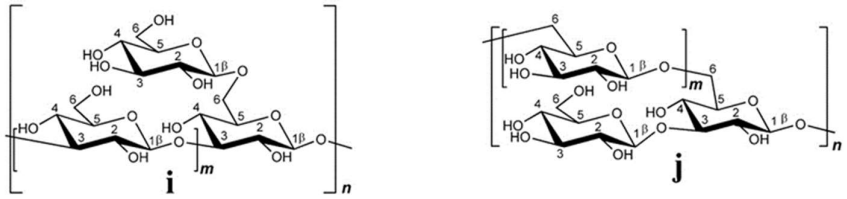


Figure 8. (i) branched (1→3), (1→6)- β-D-glucan; (j) branched (1→6), (1→3)- β-D-glucan (Synytsya & Novák, 2013)

Lentinan, see Figure 9 a, has been extracted from edible mushroom *Lentinus edodes*, known as shiitake mushroom. It is composed of a main chain of (1→3)- β-D-Glc*p* residues to with branching in O-6 for every second unit along the main chain (Khan, Gani, Khanday, & Masoodi, 2017) (Wasser, 2002).

Also, grifolan, schizophylan and scleroglucan are glucans derived from medicinal mushrooms (Khan et al., 2017; Synytsya & Novák, 2013; Wasser, 2002).

The polysaccharide, **schizophyllan**, is shown in Figure 9 b, is obtained from the inedible mushroom *Schizophyllum commune*. It has a (1→3)- β-D-Glc*p*-linked backbone, with branching in O-6 and substitution on every third unit of backbone (Zhu et al., 2015).

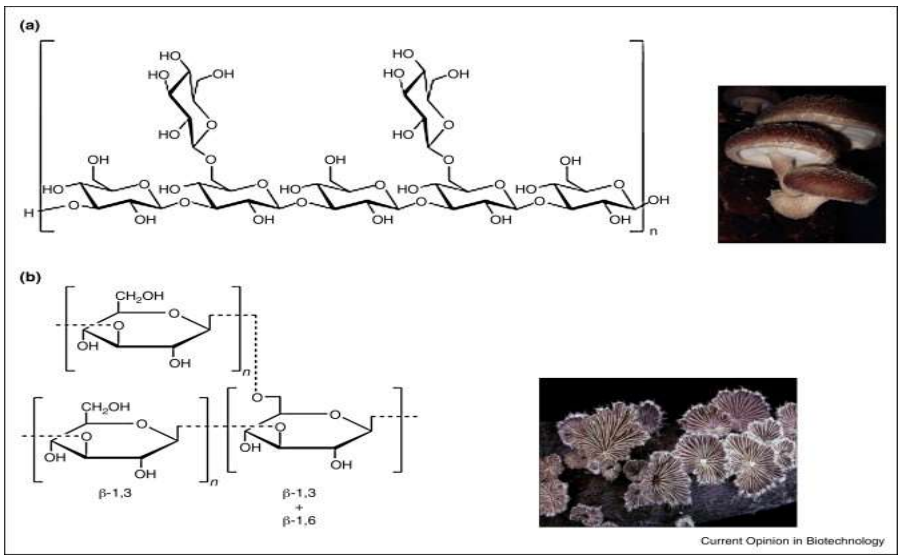


Figure 9. Fruiting bodies (right) and chemical structures(left) of (a) Lentinan and (b) shizophyllan (Giavasis, 2014)

Fungal cell wall is composed of glucans, chitin, heteroglycans and glycoproteins (Free, 2013). The cell wall becomes rigid after microfibrils have been fixed by cross-linking, consisting of highly branched glucans. Microfibrils are made of chitin (Bartnicki-Garcia, Bracker, Reyes, & Ruiz-Herrera, 1978). Both (1→3)- α-glucans and (1→4)- α-linked branched or unbranched chains have function as part of the matrix of the wall. (1→3)- and (1→6)- β- D-glucan provides rigidity to the cell wall (Fig.10) (Free, 2013; Webster & Weber, 2007; Zivanovic, Buescher, & Kim, 2003).

Chitin is important for cell wall integrity. It forms the innermost layer of the fungal cell wall, next to the cell membrane (as shown in Figure 10). it is a linear water insoluble polymer of β-(1→4)-linked-N-acetylglucosamine, accounting for up to 80-90 % of dry matter in mushroom cell walls (Kalač, 2009; Webster & Weber, 2007). In the Ascomycota and Basidiomycota, the fibres are chitin microfibrils, synthesized at the plasma membrane. Chitin is a primary constituent of shells, insect cuticles, and fungal cell walls (Kalač, 2009; Webster & Weber, 2007).
The structural proteins in the cell wall are **glycoproteins** with N- and O- linked carbohydrates. Most of the proteins in the cell wall are glycosylated and contain mannose and/or both mannose and galactose residues. Because of this, these proteins are called **mannoproteins** or **mannans** (see Figure 10) (Watkinson, Boddy, & Money, 2016). Furthermore, these structural proteins often contain a glycosylphosphatidylinositol by which they are attached to the lumen of endoplasmic reticulum (ER) and further to the external plasma membrane surface. They can be also modified and covalently binds to (1→6)- β-D-glucan fraction of the cell wall (Webster & Weber, 2007).

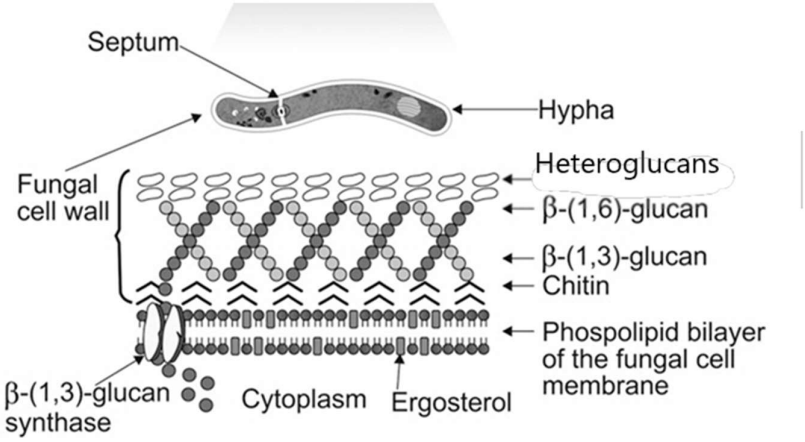


Figure 10. Schematic representation of fungal cell wall (Figure is based on (Puri, 2017))

The polysaccharide structure and composition of the cell wall can vary between fungal species. The structure of cell wall can also vary between morphotypes during the life cycle of the fungus. The content of β-glucan depends of the maturity of fruiting body: mushrooms exhibited the highest level of these compounds before the spores begin to mature (Stachowiak & Reguła, 2012). Also, the content of β-glucans is higher in stipes than in caps of the mushrooms (Sari, Prange, Lelley, & Hambitzer, 2017).

These mushroom cell wall components are non-digestible carbohydrates because of their resistance to human enzymes and can be consider as source of dietary fiber (Peter C. K. Cheung, 2013). Only, (1→4)-linked α-D- glucans that are prone to digestion by amylase (Stachowiak & Reguła, 2012).

2.2 β-glucan effect on the immune system

It is well known that the immune system protects against pathogens: bacteria, viruses and parasites. Epithelial barriers like skin, urinary tract, the linings of the gastrointestinal tract and lungs are first line of the innate immune system. **The innate immune system** is non-specific and is regulated by chemical-messengers and cytokines (Meng, Liang, & Luo, 2016). **The adaptive immune system** is specialized and induced by cells specialized in antigen production and recognition, namely B- and T- lymphocytes (cytotoxic and helper T-lymphocytes). Killer T-cells eliminate virus infected cells and tumor cells (see Figure 11) (Reis et al., 2017; Volman, Ramakers, & Plat, 2008).

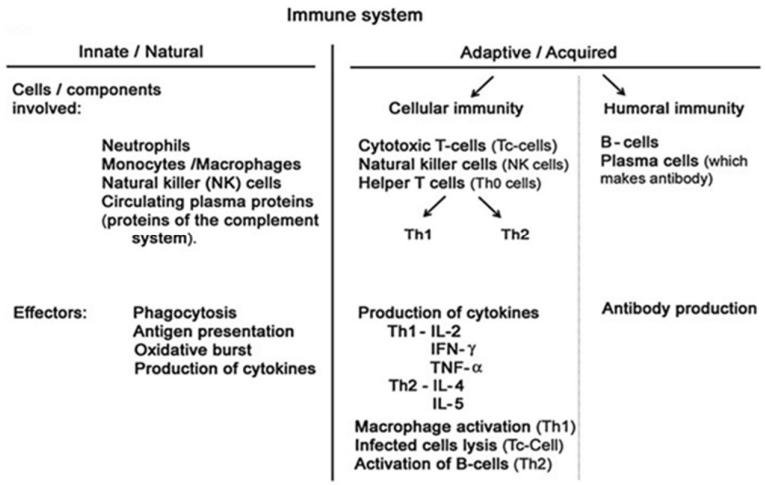


Figure 11. Immune system response (Banerjee, Parasramka, & Paruthy, 2015)

Macrophages and monocytes together with neutrophils contribute in the innate immune response and produce cytokines and local immunomodulators which act to activate adaptive immunity (Sari et al., 2017) and can activate leukocytes and macrophages and thereby stimulate the immune system (see Figure 12) (Brown & Gordon, 2003).

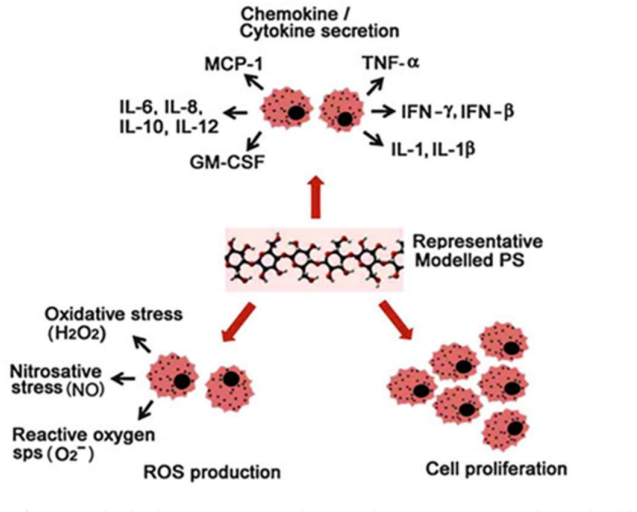


Figure 12. Macrophage response to polysaccharides (Banerjee et al., 2015)

Some of the mushrooms polysaccharides activate the innate immune system to produce various pro-inflammatory cytokines: TNF-α, INF-γ, IL-1β, IL-10 or IL-12 and NO (Bedirli et al., 2007; Meng et al., 2016). These cytokines activate the adaptive immune system through the promoting of B cells and stimulation of T cells and differentiation to T helper cells (Th-1 and Th-2) (see Figure 12) (Enshasy & Hatti-Kaul, 2013).

2.2.1 β-glucan receptors

β glucans are recognized by numerous receptors expressed on cell membranes of leucocytes, macrophages and dendritic cells, T and B lymphocytes, natural killer cells, endothelial cells, alveolar epithelial cells and fibroblasts (Brown et al., 2003): **CR3** (complement receptor 3**)**, **TLR** (toll-like receptors), **scavenger receptors** and **Dectin 1**, represented in Figure 13 (Ferreira, Passos, Madureira, Vilanova, & Coimbra, 2015; Stachowiak & Reguła, 2012).

Dectin-1, a type 2 glycoprotein is most important receptor in activating of innate immune response (Brown et al., 2003). Dectin-1 signal is activated by particulate β-glucans which leads to the release of cytokines such as IL-12, IL-10, IL-6 and TNF-α and activate both humoral and cell immunity (Ferreira et al., 2015).

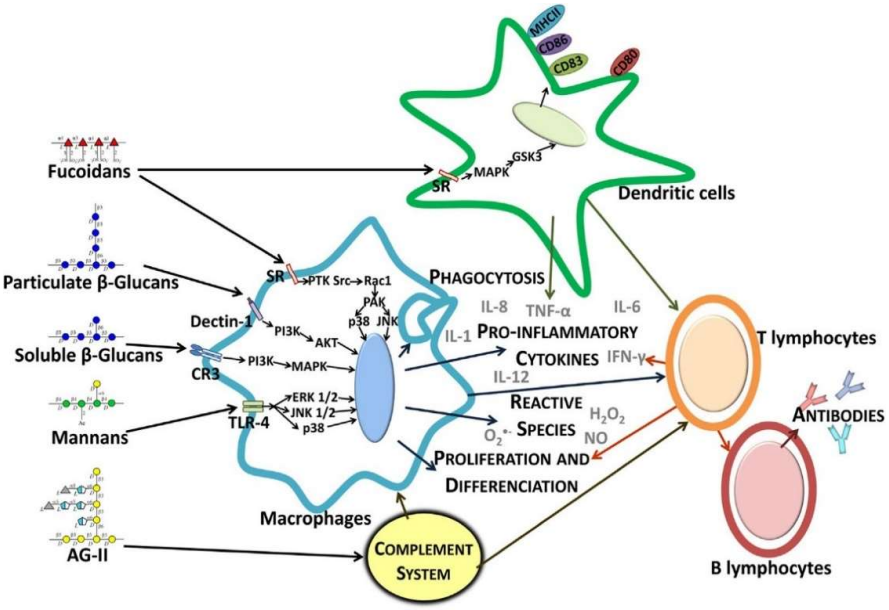


Figure 13. Overview of immune system activation by immunostimulatory polysaccharides after interaction of several molecules and signal pathways (Ferreira et al., 2015)

The CR3 receptor (also called Mac-1) comprises CD11b and CD18 domains and has ability to recognize many microbial cells and acts as an adhesion molecule. It is presented on neutrophils, monocytes and NK cells, but not on macrophages. Soluble β-glucans can bind to CR3, which result in tumor cytotoxicity (see Figure 13) (Ren, Perera, & Hemar, 2012).

The Toll-like receptors (TLRs) such as TLR-2, TLR-6, recognize β-glucans inducing the nuclear transcription factor κB (NF-κB) to produce cytokines such as interleukin 12 (IL-12) and tumor necrosis factor (TNF-α) (see Figure 13) (Dalonso, Goldman, & Gern, 2015).

2.3 Antitumor activity of mushroom β-glucans

Medicinal mushrooms have a long history of use in traditional ancient therapies and their derivatives were used as nutraceutical therapy and dietary supplements (Cheung, 2008). It has been reported that medicinal mushrooms can modulate the immune system and thereby inhibit tumors, inflammation and microbial growth (Elaine R. Carbonero et al., 2008; Kües & Liu, 2000; F. R. Smiderle et al., 2008) (A. Ruthes et al., 2013) (Fang et al., 2012).

Polysaccharides extracted from mushrooms may have a role in antitumor activity. They activate effector cells such as macrophages, T-lymphocytes, B-lymphocytes and natural killer cells and then they express cytokines: TNF-α, IL-1β and IFN. Cytokines cause apoptosis and differentiation in tumor cells (Meng et al., 2016). Polysaccharides such as **lentinan** from *Lentinus edodes*, **krestin** from *Trametes versicolor*, **maitake** from *Grifola frondosa***, schizophyllan** from *Schizophyllum commune* and **scleroglucan** have shown antitumor activity (S.-t. Chang & Miles, 2004; Peter C. K. Cheung, 2008; Khan et al., 2017; Wasser, 2002). The antitumor activity of polysaccharides is in correlation with their capabilities to bind to cell receptors, resulting in immune response by activating multiple signal pathways (Ren et al., 2012).

Lentinan showed that can improve life quality in patients with stomach cancer and colon cancer (Rathee et al., 2012). **Schizophyllan** is highly potent antitumor agent and increases immune responses. The similar β-glucan, **grifolan** has been extracted from *Griflola frondosa*, known as Maitake. Grifolan exhibits antitumor activity in gastrointestinal, lung, liver and breast cancers. (Meng et al., 2016) (Peter C. K. Cheung, 2013) (Stachowiak & Reguła, 2012). **Krestin**, isolated from *Trametes versicolor,* is inhibitor of the angiogenesis, which leads to development of metastasis (Peter C. K. Cheung, 2013; Meng et al., 2016) (Stachowiak & Reguła, 2012). A β-glucan, obtained from edible mushroom *Agaricus blazei*, showed also antitumor activity against human ovarian cancer *in vitro* (Peter C. K. Cheung, 2008). The two polysaccharides isolated from *G.lucidum* exhibited immunomodulatory activity and antitumor effect on human breast cancer (Meng et al., 2016).

Usually, larger polysaccharides with more repeating units and higher variability possess more connections and stronger affinity to receptors and proteins. They exhibit higher bioactivity and better antitumor effect in general (Meng et al., 2016). For example, Schizophyllan possess molecular weight of 450 kDa and lentinan owns molecular weight of 400-800 kDa and possess a strong antitumor activity (Meng et al., 2016).

The most bioactive mushroom polysaccharides have a triple helix structure which is usually more stable than the other conformations (Enshasy & Hatti-Kaul, 2013). Most polysaccharides in triple helical conformation possess stronger anticancer activity than any other which have not. For example, β-glucan, isolated from *A. blazei* possess a triple helix in water solution and acts against cancer cells. Ten mushroom polysaccharides with antitumor activity possess triple helix (Meng et al., 2016; S. Wasser, 2011).

A polysaccharide from *Lactarius deliciosus,* named **LDG-A** was identified and its structure is shown in Figure 14 (Ding, Hou, & Hou, 2012).

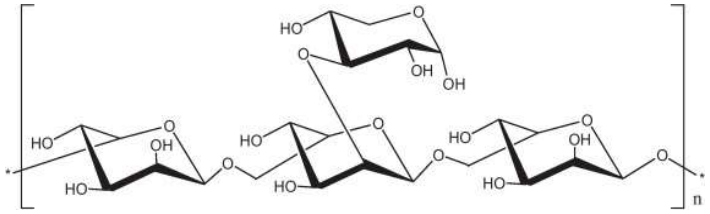


Figure 14. Chemical structure of polysaccharide LDG-A (Ding et al., 2012)

This β-glucan has showed significant antitumor activity in mice (Ding et al., 2012).

The immunomodulatory activity of mushroom β-glucans is their ability to activate leukocytes (Brown & Gordon, 2003) and can also stimulates the macrophages to produce NO through regulation of NO synthase (Meng et al., 2016). Polysaccharides isolated from *L.edodes*, *G. frondosa* and *T. versicolor* can stimulate the non-specific immune system (P. C. K. Cheung, 2010).

2.4 Antimicrobial activity of mushrooms

Today, it has become increasingly important to find new antibiotics due to bacterial resistance to existing ones. Mushrooms might be a source of new antibiotics. Considering that mushrooms need antibacterial and antifungal compounds to survive in their natural environment, they are potential sources of natural antibiotics (Peter C. K. Cheung, 2013). The mushroom hyphae contain antibacterial and antifungal substances, which help them to survive in their natural habitat(Peter C. K. Cheung, 2013)

2.4.1 Mushroom extracts with antimicrobial activity

Most studies on mushrooms that have reported antibacterial activity of its extracts are lacking identification of the active compounds. Examples are extracts from *Agaricus bisporus* (the champignon) and *Cantharellus cibarus* (the golden chanterelle) that have shown effect against G+ bacteria such as *Bacillus subtilis* and *Staphylococcus aureus* (Alves et al., 2012; Peter C. K. Cheung, 2013). In addition, *Agaricus bisporus*, the most cultivated mushroom in the world, showed antimicrobial activity against *Bacillus cereus*, *Micrococcus luteus* and *Staphylococcus epidermidis* (Peter C. K. Cheung, 2013).

The antimicrobial effect of the ethanolic extract of *Laetiporus sulphureus* was shown through inhibition of the growth of G+ bacteria such as *Bacillus subtilis*, *Bacillus cereus*, *Micrococcus luteus* and *Micrococcus flavus* (Alves et al., 2012).

The methanol extract obtained from *Lactarius* species have shown antimicrobial activity against G+, *Staphylococcus aureus and Bacillus cereus* and G- bacteria such as *E.coli*, *Klebsiella pneumonie* and *Pseudomonas aeruginosa* (Alves et al., 2012).

Both edible and non-edible mushrooms have shown antimicrobial activity, but higher activity against G+ than gram G- bacteria.

2.4.2 Antibacterial activity of mushrooms secondary metabolites

Many secondary metabolites and several compounds are isolated from mushrooms such as sesquiterpenes and other terpenes, steroids, anthraquinones, benzoic acid derivatives, and quinolines, but also primary metabolites such as oxalic acid and high molecular compounds (mainly proteins and peptides), possessed antifungal and antibacterial activity (Alves et al., 2012; Peter C. K. Cheung, 2013).

- Several mushroom compounds have been described to have antibacterial activity. Plenty of them are terpenes such as **confluentin**, **grifolin** and **neogrifolin** from *Albatrellus fletti* had activity against *Bacillus cereus* and *Enterococcus faecalis*. **Ganomycin A and B** from *Ganoderma pfeifferi* showed activity against *Bacillus subtilis*, *Micrococcus flavus* and *Staphylococcus aureus* (Alves et al., 2012).

- Anthraquinone derivates, isolated from *Cortinarius species*, were effective against Staphylococcus aureus. **2-aminoquinoline** from *Leucopaxillus albissimus* exhibits the highest antimicrobial activity against G- bacteria. The benzoic acid derivative, **coloratin A**, isolated from *Xylaria intracolorata*, has shown activity against *Staphylococcus aureus* (Alves et al., 2012).

- The peptide, **plectasin** from P*seudoplectania nigrella* showed high antimicrobial activity against G+ bacteria (Puri, 2017). The ribonuclease from *Pleurotus sajor-caju*, was also active against *Staphylococcus aureus* (Alves et al., 2012).

2.4.3 Antimicrobial activity of β-glucans- immunomodulating activity

Lentinus edodes is the most studied species and it seems to have an antimicrobial action against both G+ and G- bacteria. **Lentinan** has shown activity against tuberculosis and *Listeria monocytogenes* infection, as well as *Salmonella enteritidis* and *Staphylococcus aureus* infection (Alves et al., 2012). Lentinan and an acidic **proteoglycan** from *G. lucidum* and glucans from *G. frondosa* and *T. versicolor*, have been used to enhance resistance to HIV virus and limited the toxicity of anti-HIV drugs (Ramawat & Mérillon, 2015).

Insoluble β-glucan which was extracted from *Saccharomyces cerevisiae* (Zymosan) showed immunomodulating activity. The Toll-like receptors (TLRs) recognized by the β-glucan

particles inducing the nuclear transcription factor κB (NF-κB). This leaded production of antiinflammatory cytokines such as interleukin 12 (IL-12) and tumour necrosis factor (TNF-α) and increased the production of reactive oxygen species (ROS) that contribute to the death of invading microorganisms. This is an indirect antimicrobial effect through the stimulation of phagocytosis of microbes by macrophages (Ramawat & Mérillon, 2015).

Furthermore, parenteral administration of β-glucans has shown protection against pathogens and also β-glucans that was given enteral, had antimicrobial effect. For example, *Kournikakis et al.* found an increased survival in mice challenged with anthrax bacteria when was orally administrated β-glucan (Volman et al., 2008).

2.5 *Lactarius scrobiculatus*

Figure 15. *Lactarius scrobiculatus* (Nylén & Stordal, 1990)

The genus *Lactarius*, popularly known as the milk caps, belong to the ***Russulaceae*** family and includes about 400 species worldwide, both edible and non-edible mushrooms The *Lactarius* species are characterized by containing a milky fluid or latex which can taste mild or acrid and may be white or colored depending on the species (Vieira, Barros, Martins, & Ferreira, 2014).The generic name *Lactarius* means producing milk or lactating (Knudsen, Vesterholt, & Aaronsen, 2008).

In the Nordic countries there are registered 92 *Lactarius* species. Many *Lactarius species* are edible, especially those having orange and red milky latex e.g *L.determinus* and *L.delicious*. There is a long tradition in Finland of salting and consuming *L. trivalis* which has an acrid taste like *Lactarius scrobiculatus* (Knudsen et al., 2008)

Milky juice from the basidiocarps of fruiting bodies of *Lactarius genus* can protect them from attack from insects, animals and microorganisms. It is noticed that young mushrooms which contain a significant quantity of milky juice, are not slug and wormy and forest animals do not eat them.

These substances in milky juice are mainly higher fatty acids and their esters, ergosterol, sesquiterpenes, phthalates and quinoline derivates. They are very unstable and differ from substances present in dried mushrooms. It means that many labile substances of the milky

juice become more stable, but they have less biologically activity after drying. Moreover, they may possess bioactivity and may be used for medical purposes. Therefore, they are of big interest for biotechnology, agriculture and medicine (Tsivinska, Antonyuk, Panchak, Klyuchivska, & Stoika, 2015).

2.5.1 Description and habitat

Lactarius scrobiculatus (see Figure 15) contains a yellow milky-latex which changes to sulfur yellow up on exposure to air. The surface is wet and viscid, glossy and slimy especially in wet weather. The cap surface is yellow or yellow-orange with concentric rings and the cap is convex, 7 to 20 cm in diameter. The stem is yellow, 6-8 cm long and 2-3 in diameter. Slightly darker hollows on the stem are known as **scrobicules**, thereby the name scrobiculatus (Mossberg, Nilsson, Gulden, & Persson, 2000). The specific name ***scrobiculatus*** is derived from latin **scrobis** which means a trench (Vieira et al., 2014).

This mushroom grows in moist forest as shown in Figure 16, in groups and sometimes beside *Lactarius intermedius,* from august to october (Bosetti, Fronza, Vidari, & Vita-Finzi, 1989; Kunkele, 2007)

Figure 16. *L. scrobiculatus* grow in spruce forest, covered with big tujamoss (Thuidium tamariscinum)(Kunkele, 2007)

L. scrobiculatus occurs in countries such as in Britain, Scotland, Sweden, Finland and Norway. In Norway it appears along the south coast from Østfold to Sør-Trøndelag (Knudsen et al., 2008)

2.5.2 Taxonomic classification and different names

This milk cap was first described by Giovanni Antonio Scopoli in 1772. He named mushroom as *Agaricus scrobiculatus*. The Swedish mycologist Elias Magnus Fries transferred this species to genus *Lactarius* in 1838.

Synonyms of *L. scrobiculatus* are presented in Table 2.

Table 2. Synonyms of *L. scrobiculatus* ("Mycobank," 2018)

Synonyms of *L.scrobiculatus*	Year
Agaricus scrobiculatus Scop.	1772
Agaricus intermedius Fr.	1815
Galorrheus scrobiculatus (Scop.) P.Kumm	1871
Lactifluus scrobiculatus (Scop.) Kuntze	1891

Table 3. Taxonomic classification of *L.scrobiculatus* ("Dyntaxa," 2015)

Taxonomic classification	
Kingdom:	FUNGI
Division:	Basidiomycota
Underdivision:	Agaricomycotina
Class:	Agaricomycetes
Order:	Russulales
Family:	Russulaceae
Genus:	Lactarius
Species:	***L. scrobiculatus***

Table 4. Names of *L. scrobiculatus* in different countries (Ammirati, Traquair, & Horgen, 1985) ("Artsdatabanken," 2017)

English name	Spotted milkcap, Spotted stemmed lactarius, Pitted milky cap
Nordic name	Svovelriske
Danish name	Grubestokket mælkehat

Nowadays, *L. scrobiculatus* is considered as **non-edible** mushroom. It can cause stomach and intestinal pain- irritation of gastrointestinal tract and a burning sensation in the throat. Taste is very hot and acrid. Although in some parts of Europe these fungi are eaten after frequent boiling and throwing the water with toxins away (Knudsen et al., 2008).

2.5.3 Identified compounds in *L. scrobiculatus*

The several **furan and lactone sesquiterpenes** have been isolated and identified from fruiting bodies of *L. scrobitulatus.*

The intact fresh fruiting bodies of *L.scrobiculatus* originally contain a fatty acid ester of velutinal **(stearoyl-velutinal)** as the only sesquiterpenoid, which is probably stored in the lipid layers of the cell membranes. In this way, they are protected against enzymes. When the fruiting bodies are injured the velutinal esters are converted by enzymes into sesquiterpene furans, mono-and di-aldehydes and lactones which have been isolated (Figure 17) (De Bernardi, Garlaschelli, Toma, Vidari, & Vita-Finzi, 1993). Some **sesquiterpenes** have been isolated from different *L.scrobiculatus* extracts such as furanosesquiterpenes, namely: **furoscrobiculin**, **furanethers**, **lactaral**, **lactardial**, **blennin C** (see Figure 17 (2)) and **furandiol.**

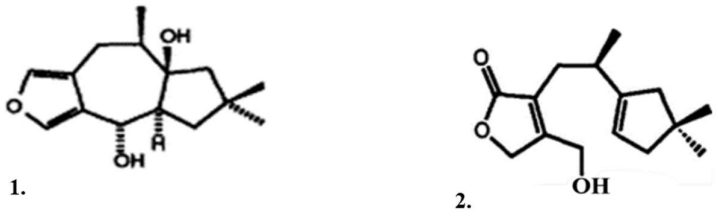


Figure 17. The two isolated lactaran sesquiterpenes are isolated as conversion products of stearoylvelutinal in fruiting bodies of *L. scrobiculatus:* (1) Furoscrobiculin D (2) Blennin C (Z. Pang, Bocchio, & Sterner, 1992).

The pungent taste of the fruiting bodies is caused by rapid enzymatic formation of **sesquiterpenoid dialdehydes** from fatty acid ester velutinal (Z. Pang et al., 1992). **Chrysorrhedial** (Figure 18 (2)) is extremely pungent and with **lactardial** (Figure 18 (1)) appear to be responsible for the pungency of these mushrooms (Z. Pang et al., 1992).

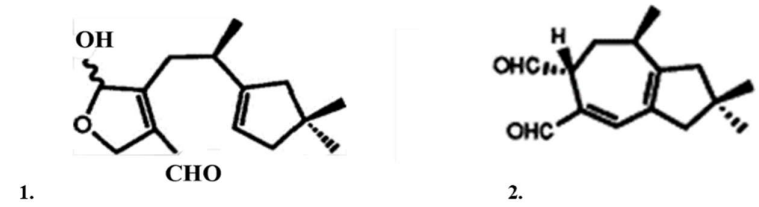


Figure 18 , Lactardial (1) and chrysorrhedial (2) (De Bernardi et al., 1993)

Also, sesquiterpene lactone **lactaro-scrobiculide A** and **chysorrhelactone** have been isolated (De Bernardi et al., 1993). **Lactaroscrobiculide A** (Figure 19 (1)) and **chrysorrheal** (Figure 19(2)) are bitter and slightly astringent, but not acrid (De Bernardi et al., 1993).

Figure 19: Sesquiterpenes-lactones 1. Lactaro-scrobiculide A 2. chrysorrheal are involved in the hot-taste and yellowing of the mushrooms (De Bernardi et al., 1993)

In addition, **Lactaroscrobiculide A** (Figure 19 (1)) has not shown antibacterial activity, but both **chrysorrhedial** (Figure 18 (2)) and **chrysorrheal** (Figure 19 (2)) were active against *Bacillus subtilis* and *Staphylococcus aureus* (De Bernardi et al., 1993).

The polysaccharides and lipids in *L. scrobiculatus* have not been investigated previously.

2.6 Biological activity-antimicrobial activity

2.6.1 Magnesium transport protein (*MgtA*)

MgtA -the magnesium transporter A, is found in bacteria, fungi and plants. It belongs to the P-type ATP-ase that allows import of Mg^{2+} into the cytoplasm of bacteria. P-type ATPases are ion pumps that carry out many fundamental processes in biology. They use the energy stored in ATP and to transport specific ions across the cell membrane against a concentration gradient. P-type pumps are a large, varied family of membrane proteins that are involved in many transport processes in all living organisms. Homeostasis of Mg^{2+} ions is studied in

gram-negative bacteria such as *Escherichia coli*. *MgtA* is highly depended on phospholipids such as cardiolipin. Both *MgtA* and cardiolipin are found together in the membrane of *E.coli* (Subramani, Perdreau-Dahl, & Morth, 2016).

Two different conformations of Mg-type ATP-ase, called E1 and E2 (enzyme-1 and enzyme-2) exist, with different affinity for the nucleotide and the transported ions. Besides, the pumping cycle involves several intermediate states. P-type ATP-ase pumps ions using ATP to maintain an ion gradient across the cell membrane. It is a reversible process. P-type ATPases can use a membrane potential to produce ATP. *MgtA* alternate between the E1 and E2 states during the transport cycle. The E1 state is the high-affinity binding site and bind the Mg^{2+} ions from the cytoplasm (see position 1- Figure 20). In *E. coli* occurs first phosphorylation of aspartate (position 2, Figure 20) which induces further that the cytoplasmic domain shifts and rotates, leading to the E2P state (position 3, Figure 20). E2P state has low affinity to bond the ions. But it has high affinity to the counter ions such as Mg^{2+} ions. This exchange leads to dephosphorylation of the enzyme and E2 state (see position 4, Figure 20). Further, the conformational changes lead to E1 state and Mg^{2+} ions will be released into the cytoplasm. In this way, the cycle will be completed (Subramani et al., 2016; Werner, 2004).

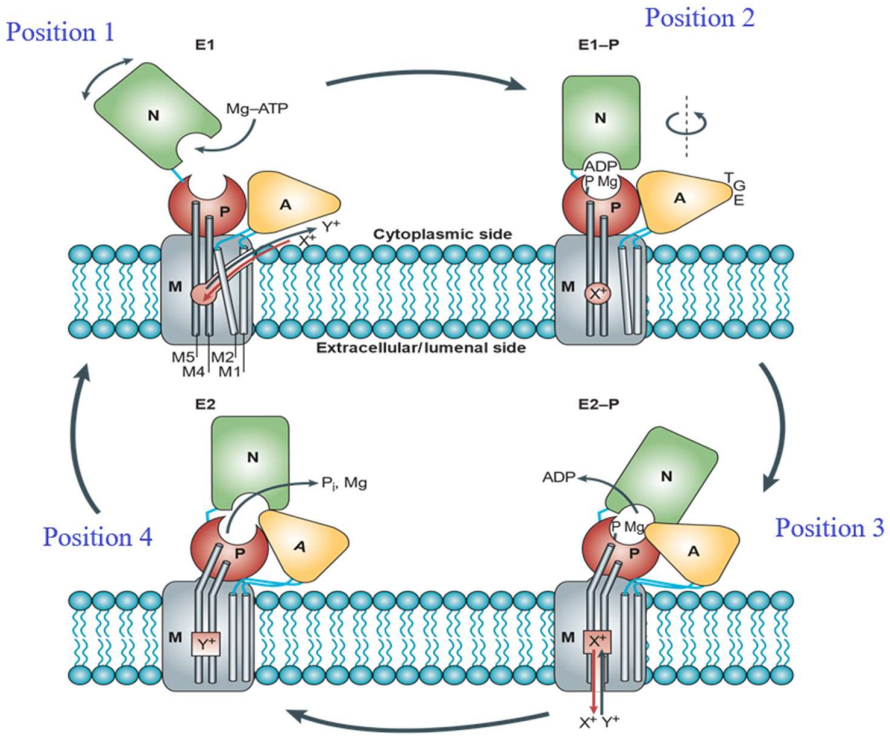


Figure 20 Schematic diagram of the P-ATP-ase cycle: E1 bind the Mg^{2+} ions (position 1) Phosphorilation of aspartate (Asp) leads to E1-P state (position 2) and further to conformational changes and forming the E2P state (position 3). Hydrolysis of the phosphorylated Asp results in the E2 state (position 4). Mg^{2+} and inorganic phosphate (Pi) dissociate and the enzyme reverts to the E1 state, in which Mg^{2+} is released into the cell, and another cycle begin (Werner, 2004)

Cardiolipin is very important for *in vitro* activation of MgtA, because of MgtA and cardiolipin were found together in the membrane of living *E.coli* indicating that the two work together. It suggested that the membrane transporter that pumps ions needs cardiolipin to work by changing the levels of specific lipids in their membranes (Subramani et al., 2016).

MgtA protein can be target for antibacterial effect. Probably, inhibition of activity of *MgtA* could lead to inhibition of transport of Mg^{2+}into the cytoplasm of bacteria which is a cofactor and essential element for all living cells, from bacteria to humans.

3. AIMS OF THE STUDY

The general aim of this thesis was to obtain different extracts from mushroom *L. scrobiculatus* for further investigation, to isolate the polysaccharides from water, alkali extracts, determinate monosaccharide composition, and linkage structure in obtained fractions.

The main objectives were:

- To obtain different extracts of *L.scrobiculatus* in order to further investigation
- To find out the optimal column for purification of polysaccharides from *L.scrobiculatus* by SEC preparative chromatography
- To characterize polysaccharides from *L.scrobiculatus* relative to molecular weight, monosaccharide composition and linkage structure.
- To perform a screening test (the biological activity test of the obtained extracts) with a focus on possible inhibition of *MgtA*, the membrane protein, present in G- bacteria, *E.coli*

4. Methods

4.1 List of reagents

Chemicals	Producer
1-Metylimidazol	Sigma Aldrich
6-deoxy-D-glucose	Sigma Aldrich
Acetic acid	Merck
Acetic anhydride	Merck
Adenoside 5´- triphosphate disodium salt hydrate (ATP)	Sigma Aldrich
Ammonia (NH_3)	VWR Chemicals
Ammonium hydroxide (NH_4OH)	Merck
Ammonium molybdate [$(NH_4)_2MoO_4$]	Merck
Argon	Praxair, VXR Chemicals
Ascorbic acid	Sigma Aldrich
Bismuth citrate	Sigma Aldrich
Cardiolipin	Avanti Polar Lipids, Alabaster, Alabama
Chloroform	Sigma Aldrich
Chloroform-d	Aldrich
D-Fucose	Sigma Aldrich
Dichloromethane	Merck
Dimethyl sulphoxide (DMSO)	Merck
Endo-1.3 β-glucanase	Megazyme
Ethanol 96%	Sigma Aldrich
Hexamethyldisylizane (HMDS)	Fluka
Hexane	Merck
HEPES	Sigma Aldrich
Hydrochloric acid 1 M (HCl)	Sigma Aldrich
Hydrochloric acid 37 %(HCl)	VWR Chemicals, Prolabo
Iodine	Merck
Iodomethane (CH_3I)	Sigma Aldrich
Magnesium transport protein (MgtA) of E.coli	NCMM
Magnesium chloride ($MgCl_2$)	Sigma Aldrich
Mannitol	Sigma Aldrich
Methanol	VWR Chemicals, Prolabo
Methanol (anhydrous)	Merck
Methanol-d_4	Aldrich
Metyljodid (CH_3I)	Sigma Aldrich
Nitrogen 50l	Praxair, VWR Chemicals

Octaethylene glycol monododecyl ether ($C_{12}E_8$)	Nikko Chemicals, Tokyo, Japan
Octanol	Merck
Pancreatin	Sigma Aldrich
Phenol 4%	Merck
Potassium chloride (KCl)	Sigma Aldrich
Potassium iodide (KI)	Fluka Honeywell
Potassium nitrate (KNO_3)	Sigma Aldrich
Pullulan standards	Polymer Laboratories LTD
Pyridine (anhydrous)	Merck
Silver nitrate 1 % ($AgNO_3$)	Merck
Sodium azide (NaN_3)	Merck
Sodium borodeuteride ($NaBD_4$)	Sigma Aldrich
Sodium borohydride	Sigma Aldrich
Sodium chloride (NaCl)	VWR Chemicals, Prolabo
Sodium citrate	Sigma Aldrich
Sodium dodecyl sulphate	Sigma Aldrich
Sodium hydroxide (NaOH)	VWR Chemicals, Prolabo
Sodium hydroxide (pellets)	Merck
Sodium molybdate (Na_2MoO_4)	Sigma Aldrich
Sodium sulphate (Na_2SO_4)	Merck
Sodium thiosulphate-5-hydrat	Merck
Starch (soluble GC for analysis)	Merck
Sulphuric acid 95%	VWR chemicals
Toluene	Sigma Aldrich
Triflouroacetic anhydride (TFA)	Sigma Aldrich
Trimethylchlorosilane (TMCS)	Fluka

4.2 General methods

4.2.1. Weighing

Two types of weighing equipment were used: an analytical balance and precision balance.
Equipment:

-Analytical balance: Sartorius Extend ED224S (Sartorius mechatronics)
Weighing range: 0.1 mg - 220 g
-Precision balance: Mettler BB2400 (Mettler Toledo)
Weighing range: 0,01g - 12kg

4.2.2. Water quality

Purified water was used in all methods.
Tap water was purified by reverse osmosis followed by deionization and UV-irradiation in an Elix® 10 Essential Water Purification System (EMD Millipore) with Progard® NP2 filter.

4.2.3 Measurement of pH

The pH paper was used to measure and control pH values, for example the acid washing of glass equipment.
pH paper: pH-Fix 0-14 Fisher Brand (Fisher Scientific)

4.2.4 Filtration

Filtration was performed to remove particles from solutions.

1. Small volumes (1-5 ml) were filtrated using syringe filters. There were typically used membrane filters:

-PALL, Life Sciences, Acrodisc® 37 mm Syringe Filter with 1 μm Glass Fiber Membrane
-PALL, Acrodisc® 32 mm Syringe Filter with 0,45 μm Supor® Membrane

2. Filtration of large amounts of liquid was performed with Büchner funnel and vacuum pump using:
- membrane filter: Nylon membrane 0,45 μm HNWP from Millipore.
-membrane filter: Whatman® filter paper 46 x 57 cm, S&S Paper filter

4.2.5. Freeze drying (lyophilization)

Principle:
Freeze drying is a dehydration method performed by freezing of substances and then removing the ice by sublimation. This is achieved by cooling under vacuum at reduced pressure and at low temperature. This method is used to increase durability, solubility and storage of temperature sensitive substances. The final product will be dehydrated with a good quality after freeze-drying (see Figure 21) (Christ, 2013).

Equipment:
-Methanol bath: Hetofring (Heto Birkerød, Denmark)
-Freeze-Dryer: Freeze Dryers Alpha 1-4 LD plus (Christ) (see Figure 23)
-Vacuum pump: Edwards RV5 Vacuum Pump
-Parafilm
-Cannula
-Glass wool
-Round glass flask

Procedure:
The samples were frozen in round glass flasks in a methanol bath (- 40 ° C) before freeze-drying. Then, they were attached to the outside of the freeze-dryer under vacuum and low temperature. Transition with glass wool was used to fasten them.
When all water had been removed, the lyophilized material were weighed.

Figure 21. Freeze dryer Alpha 1-4 LD plus

4.2.6. Centrifugation

Different types of laboratory centrifuges were used for the isolation and separation of suspensions. They vary widely in speed, capacity, and temperature control. There were used:

- Multifuge® 4 KR Heraeus® 72 (VWR) for samples of large volume (1.5 to 1000 ml).

-Minicentrifuge (LABNET International Inc) was used for separating samples with small volume (0.4- 9 ml) in Eppendorf tubes.

Procedure:
Time and relative centrifugal force (g) varies in different experiments.

4.2.7. Evaporation with rotary evaporator

A rotary evaporator is a specially designed instrument which was used to evaporate solvents under vacuum using rotation and heat. The vacuum pump is used to create a low pressure so that the solution is evaporated at a temperature lower than the normal boiling point.

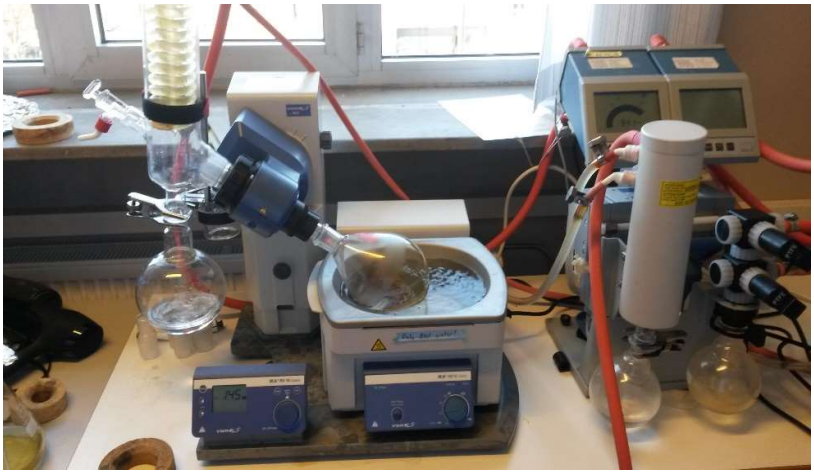


Figure 22. Rotary evaporator for organics solvents with water bath: IKA® RV 10 basic, IKA® HB basic and vacuum pump- Vaccubrand CVC2

Large volumes of liquids were reduced using a rotary evaporator with vacuum pump and a water bath (see Figure 22) under reduces pressure at 40 °C.

Equipment:

- Rotary evaporator: IKA® RV 10 basic, IKA® HB 10 basic with vacuum pump- PC 101NT 230 V
- Rotary evaporator for organics solvents: IKA® RV 10 basic, IKA® HB basic, vacuum pump- Vaccubrand CVC2

4.2.8. Degassing of solutions

Solutions were bubbled with helium gas for 10-15 minutes to remove air in the solutions.

4.2.9 Mixing of solutions

Mini shaker: Mini Shaker MS3 Basic (IKA)
Shaker: Vibrax-VXR Basic (IKA)
Vortex: Lab Dancer (IKA)
Magnet stirrer: RCT basic IKA® laborteknikk (IKA)

4.2.10 Storage of aqueous solutions of polysaccharides

Aqueous solutions of carbohydrates are susceptible to bacterial growth and degradation. Therefore, the samples were stored at low temperature (+ 4 ºC) overnight and some drops of toluene were added.
If solutions must be kept during the night, they were frozen (-20 ºC).

4.2.11 Acid washing of glass equipment

Principle:
Glass equipment may contain trace of cellulose during storage in paper boxes. Cellulose is a polysaccharide and therefore can affect analysis results.
In order to remove contamination from cellulose all glassware, prior for methanolysis or methylation analysis, were washed with concentrated hydrochloric acid (HCl).

Reagents:
Hydrochloric acid (37% HCl)
Tap water
Purified water
Methanol

Equipment:
- Glass tubes with teflon screw cap (large)
- Glass tubes with teflon screw cap (small)
-Supelco tubes (Clear vials with Teflon screw cap, 4 ml)
Oven: Heraeus Function line (Thermo Scientific)

Procedure:
The glass tubes were covered with concentrated hydrochloric acid for 30 minutes. Afterwards, all the hydrochloric acid was discarded. All equipment was rinsed thoroughly in tap water until pH 5.0 and then with purified water and methanol. Clean equipment was put to dry in the oven at 80 ºC overnight. Dried glass equipment was covered with aluminum foil and stored in plastic boxes.

4.2.12 Phenol-sulfuric acid test

(Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) (Cui, 2005)

Principle:

The phenol-sulphuric acid assay is a classic colorimetric method for determining total content of carbohydrates. Carbohydrates form furfural aldehydes with concentrated sulphuric acid. The furfural aldehydes condense with phenol forming a yellow coloured complex. The intensity of the yellow colour at constant phenol concentrations is proportional to the amount of carbohydrates in the sample (see Figure 23).

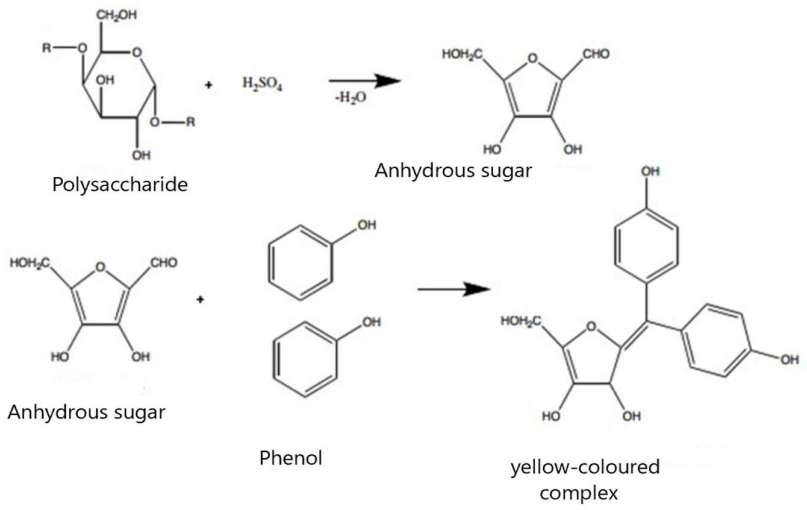


Figure 23. The yellow-coloured complex was formed in the reaction between the polysaccharide, phenol and sulfuric acid. This can be measured in Absorbance reader

Reagents:
- 4% phenol
- Concentrated H_2SO_4

Equipment:
- Glass tubes
- Finn pipette® 200µl

-Vortex-described in section 4.2.9
- Acid resistant pipette Gilson Microman M250
- Microplate Absorbance Reader Model 3350 BIO-RAD (400-750 nm)

Procedure:
1. Each collection tube (100 µl) was transferred to glass tubes and 200µL 4% phenol and 1 ml concentrated H_2SO_4 were added. The solution was mixed on vortex.
2. The glass tubes were left for 30 minutes at room temperature.
3. 100 µl from each tube was transferred to a microtiter plate. Water was added to 4 wells as a blank sample. UV absorbance was measured at 490 nm.

The amount of colour at a constant phenol concentration was proportional to the amount of present carbohydrates. The standard curves obtained by plotting the absorbance and number of fractionation tubes. Based on the carbohydrate profile, determined by this test, the fractions were collected and combined.

4.2.13 Dialysis

Principle:

Dialysis is a simple process in which low-molecular substances diffuse from a solution of high concentration to a solution of low concentration across a semipermeable membrane until it reaches equilibrium.

The porous membrane selectively allows smaller solutes to pass while retaining larger molecules. Dialysis can effectively be used as a separation process based on size rejection. In this way, a solution with high molecular weight compounds (e.g. polysaccharides) is purified while undesired low molecular weight substances such as salts are removed (see Figure 26) ("Spectrum Laboratories Inc ", 2010).

The dialysis membrane consists of a matrix of crosslinked polymers and the pore rating is referred to as Molecular Weight Cut Off (MWCO) which is an indirect measure of the retention performance. Using dialysis tubing with MWCO, for example 12- 14 000 Da, molecules of molecular weight 12-14 kDa or more are retained in the tubes, while smaller molecules diffuses into the dialysis water (see Figure 24) ("Spectrum Laboratories Inc ", 2010)

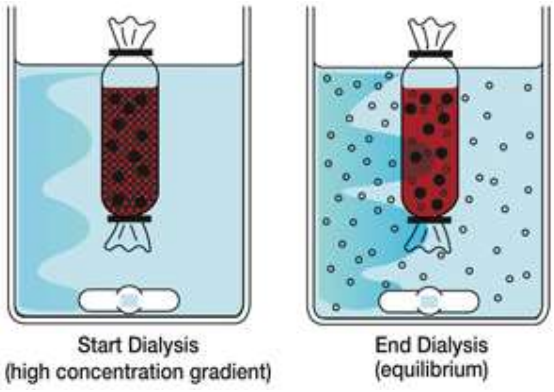


Figure 24. The principle of the dialysis. Dialysis of the sample solution starts from the left where the concentration is high to reach equilibrium (to right) ("Spectrum Laboratories Inc ", 2010)

Reagents:

-0.1% NaOH-solution (aq) -
-0.1% sodium azide solution (NaN_3 (aq))
-1% $AgNO_3$ solution
-toluene
- purified water

Equipment:

-Dialysis tubes: -Spectra/Por® Dialysis Membrane, MWCO: 12-14 000 Da, pH 5-9, d: 29 mm, nominal flat width: 45 mm (Spectrum Laboratories Inc)

-Spectra/Por® Dialysis Membrane, MWCO: 3500 Da, nominal flat width: 45 mm, d: 29 mm
-Hotplate (Elektra)
-Large beaker (5 l)
-Plastic pot
-Magnet stirrer: described in section 4.2.9
-Dialysis clamps
-Glass rods

Procedure:

The dialysis tubes were cut off to the desired length (30-40 cm) and washed inside and outside with purified water. Then, they were boiled in 1l purified water, added 100 µl 0.1 M NaOH solution for 10 minutes. After boiling, they were washed well both inside and outside with purified water. The tubes were boiled again in purified water for 10 minutes, then they were rinsed again with purified water. The dialysis tubes were stored in a refrigerator in 0.1% NaN_3 (aq) to avoid bacterial growth and they were then washed well with purified water before use in order to remove residual sodium azide solution. Before the start of dialysis, it was needed first to fill in the dialysis tubes. They were closed at the one end by clamps and filled with a small volume of purified water to ensure that there were no leakages. The tubes were filled ½- ¾ of volume with a funnel and 2-3 drops toluene were added in the dialyse tubes. The residual air was removed from the tubes and closed with the clamps. The tubes were transferred into a plastic pot with purified water and left in the pot on stirring with magnet overnight. Dialysis performed during two or three days at 4 °C on stirring. Dialysis water was changed every 4 hours.The dialyse water was tested by phenol sulphuric test or test on the presence of the chloride ions. Dialysis was finished until dialyse water under phenol sulphuric test become colourless. This indicates that the carbohydrates with low Mw will not present in dialyse water. If the water become colourless by addition of few drops of $AgNO_3$ solution, the dialysis was ended.

5. Isolation of polysaccharides from *Lactarius scrobiculatus*

Cell wall polysaccharides were extracted from mushroom cell walls using step wise extraction and size exclusion chromatography (SEC) as shown in Figure 25. After isolation and purification of polysaccharides, the monosaccharide composition was determined by methanolysis and gas chromatography (GC). Linkage analysis was performed by methylation and GC-MS.

5.1 Extraction

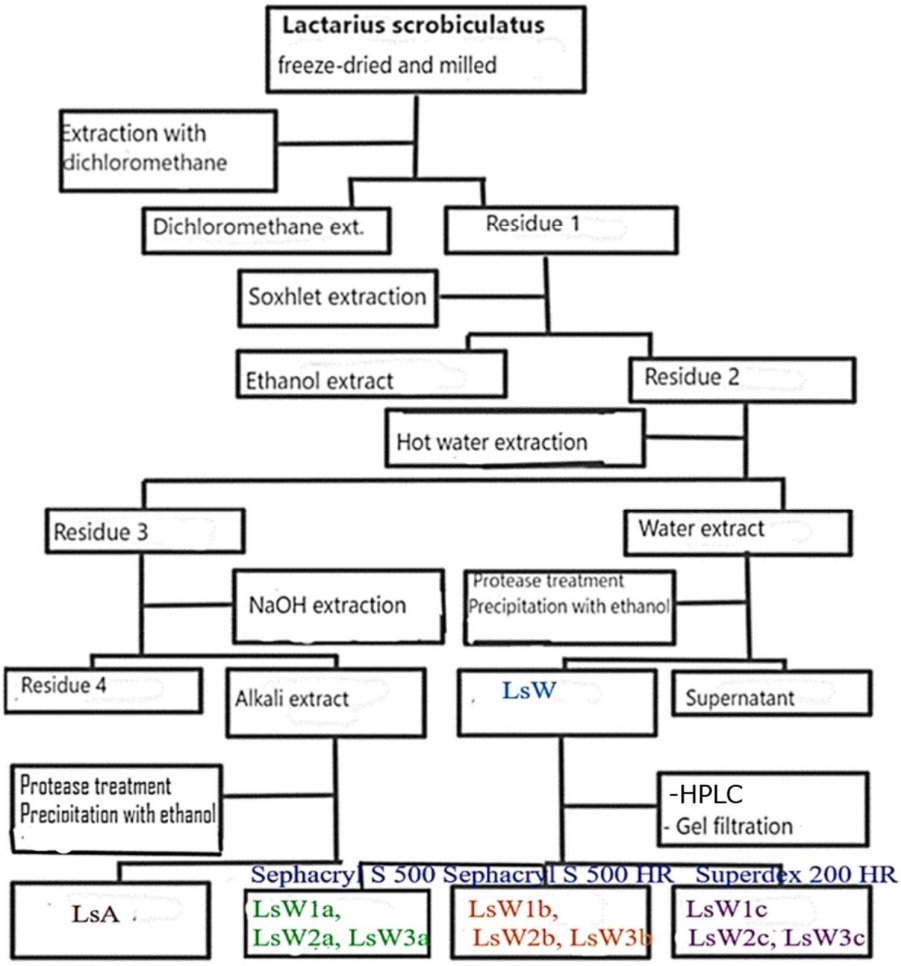


Figure 25. Schematic overview of extraction of the mushroom ***L.scrobiculatus***. This work was based on obtaining of the water extract LsW, its polysaccharide fractions and the alkali extract (LsA).

5.2 SEQUENTIONAL SOLVENT EXTRACTION

Water-soluble polysaccharides were obtained by extraction with boiling water, while the extraction with alkali solution yielded the water-insoluble (M. Zhang, Cui, Cheung, & Wang, 2007). The two extraction steps with dichloromethane and ethanol were performed in order to remove lipid soluble and low molecular weight compounds and to obtain organic extracts for further investigations.

5.2.1 Extraction with dichloromethane

Reagents:

-DCM: dichloromethane

Equipment:

- Mixer- Commercial blender (RAW PRO X 1500)
- Beaker (3l)
- Precision balance- section 4.2.1
- Büchner funnel
- Büchner flask
- Magnet stirrer: RCT basic IKA® laborteknikk (IKA)
- S&S Paper filter (Whatman)
- Aluminium foil
- Centrifuge: Multifuge® 4 KR Heraeus® 72 (VWR)
- Rotary evaporator for organics solvents: described in section 4.2.7
- Vacuum pump: ILM Vac, p< 8 mbar/ 230V

Procedure:

Lyophilized fruiting bodies of *L. scrobiculatus* was milled in the blender, weighed (50.01 g) and transferred to a beaker (3l). They were treated three times with 500 ml dichloromethane (DCM), covered with aluminium foil under gentle stirring on magnet stirrer in the fume hood at room temperature overnight (18-24 hours).

The obtained weak-yellow coloured extracts were combined, centrifuged at 1000 rpm for 15 minutes and filtered through Büchner funnel and S&S filter paper using vacuum pump.

DCM extracts were further concentrated on rotary evaporator for organics solvents in pear-shaped flask to be concentrated. The residue was scraped into glass beaker.

The mushrooms residue was spread out over aluminium foil in the fume hood for 2 days, to be dried at room temperature.

5.2.2 Extraction with 96% ethanol -Soxhlet extraction

Principle:

The mushroom residue was extracted with 96 % ethanol to remove medium polar compounds. Lipids and most triterpenoids are removed by DCM.

In Soxhlet extraction, the round bottom flask is heated and the solvent (ethanol) evaporates and moves up into the condenser. The extracted compounds will be collected in the boiling flask below while the solvent has been condensed into the extraction chamber (see Figure 26). Mushroom residue was extracted until colourless extraction solvent and was spread over an aluminium foil and dried in a fume hood in 24 hours.

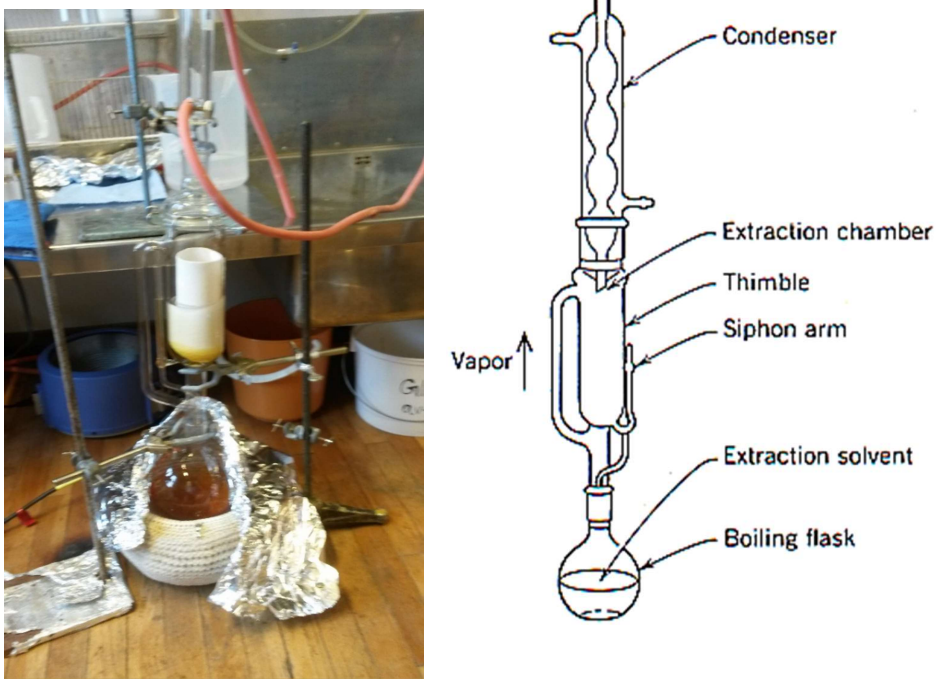


Figure 26. Soxhlet extractor consist of the extraction chamber, the condenser and round bottom boiling flask that heats on heating mantle

Reagents: -Ethanol 96% -solvent

Equipment:

-Soxhlet extractor: the thimble, the extraction chamber, reflux condenser and a round bottom boiling flask (3l)
-Heating Mantle: BI Barnstead Electrothermal
-Precision balance- described in section 4.2.1
-Filter paper 46 x 57 cm (Whatman ®)
-Oven (Theraeus, Thermo scientific)
-Centrifuge- described in section 4.2.6

Procedure:
The dried mushroom residue (45g) was treated with 96 % ethanol. The mushroom residue was put in a Soxhlet extraction thimble, made of strong filter paper, which is placed in the Soxhlet chamber of the Soxhlet extractor. Total 2.5 litres 96 % ethanol was added in 3l round bottom boiling flask and heated with a heating mantle.
It was left staying in ethanol during the night, without heating. Then extraction was performed until the solvent was colourless. The ethanol extract was filtered with Büchner funnel and S&S filter paper, up-concentrated on rotary evaporator for organic solvents and left to stay at 4 ºC overnight. The crystals appeared in the ethanol extract and were isolated by centrifugation at 1000 rpm for 10 minutes. The mushroom residue was dried at 40 ºC in the oven.

5.2.3 Hot water extraction (see Figure 27)

Principle:
Hot water extraction is the most common extraction method. All water-soluble components will be present in the resulting extract. For example, water-soluble polysaccharides will be presented in the water extract.

Reagents:

-Toluene

-Purified water

Equipment:

-Large round flask (2L)
-Hot Plate (Wilfa CP-1)
-Reflux condenser
-Vacuum pump: ILM Vac, p< 8 mbar/ 230V
-Cooking pot
-Rotary evaporator, described in section 4.2.7
-Centrifuge: equipment and method - described in section 4.2.6
-Magnet stirrer- described in section 4.2.9
-Filter paper GF/F 150 mm Whatman- glass microfibre filter

Procedure:
The mushroom residue after evaporation of ethanol (70 g), was transferred into a 2l round bottom flask, connected to a reflux condenser. Purified water (500 ml) was added in the flask with mushroom residue.

Figure 27. Water extraction: Large bottom flask with mushroom material and purified water in cooking pot as a water bath and reflux condenser

Then, it was extracted on a boiling water bath (see Figure 29) for 6 hours two times. The water extracts were subsequently centrifuged at 1500 rpm for 15 minutes and filtered with a Büchner funnel and with a filter paper with the aid of a vacuum pump. The collected aqueous

supernatants were combined and concentrated on a rotary evaporator at 40 ºC before treatment with pancreatin.

5.2.4 Alkaline extraction

Principle:

The remaining water insoluble residue from hot water extraction was extracted with aqueous basic solution (1 M NaOH) at 100 ºC. Carbohydrates residue must undergo β-elimination reactions on treatment with a base. The extraction with base can lead to undesirable disruption of the regular organization of the structure β-glucan chain. Therefore, NH_4OH was used to undergo rapid ″alkaline peeling″ or β elimination reaction from any unprotected reducing ends $NaBH_4$ will be used as a reducing agent in this reaction which reduce end-units, avoiding degradation of polysaccharide chains ((1→3)-linkages in this case) (Cui, 2005).

Reagents:

-sodium hydroxide (NaOH)
-sodium borohydride ($NaBH_4$)
-octanol
-96% ethanol
-70 % ethanol

Equipment:

The same equipment was used as in water extraction.

Procedure:

The mushroom residue was transferred to a 2 l flask and treated twice with 500 ml 1M NaOH and 0.135 M $NaBH_4$ under reflux condenser at 100 ºC for 6 h. 10 ml octanol was added as a defoaming agent.

Extracts were cooled down, combined and isolated by centrifugation (1500 rpm, 15 minutes). Supernatants were filtered with Büchner funnel and a filter. The NaOH extract (640 ml) was poured into a 3l beaker and precipitated with three volume of 96% ethanol (1920 ml) at 4 ºC for two days.

NaOH extract was centrifuged at 3500 rpm for 15 minutes. Supernatants were discarded, and the precipitates were combined and then washed with approximately 200 ml of 70% ethanol and centrifuged again (3500 rpm, 15 minutes) Extraction has performed for 3 times. Precipitates were dissolved in water, dialyzed at MWCO 12-14 kDa and freeze-dried.

5.3 Treatment with enzymes

Enzymatic hydrolysis is a specific and gentle method for fragmentation of many polysaccharides.

5.3.1 Protease treatment

Principle:

Pancreatin is a mixture of several digestive enzymes, composed of amylase, lipase and protease. The water extract LsW was treated with a pancreatin in order to remove interfering proteins.

Reagents:

-Pancreatin (Porcine pancreas)
-Toluene

Equipment:

- Oven: Heraeus Function line (Thermo Scientific)
-Centrifuge- Multifuge® 4 KR Heraeus® 72 (VWR)
-Magnet stirrer: RCT basic IKA® laborteknikk (IKA)

Procedure:

The water extract was transferred into a 3 l flask. Pancreatin (200 mg) and 3 drops of toluene (to prevent bacterial growth) were added and incubated for 3 hours at 40 ºC. Enzyme activity was terminated by boiling for 10 minutes and left to cool down on the bench. Three volumes of 96 % ethanol were added and left to precipitate for 24 h at 4 ºC. The precipitate was collected after centrifugation at 3500 rpm for 15 minutes and then washed with 70 % ethanol three times, followed by centrifugation. The supernatants were thrown. Then the solutions were dialyzed at Mw cut-off 12-14 000 Da against water at 4 ºC for 60 hours and freeze-dried.

5.3.2 Enzyme treatment of β-glucan with endo-(1→3)-β-glucanase

Principle:

The endo-glycosidase are class of enzymes that specifically cleaves (1→3)-glycosidic linkage between the two (1→3)-β-glucose in polysaccharide. An enzyme endo-(1→3)-β-glucanase catalyse the hydrolysis of (1→3)-β-glucose residue. LsW2b and LsW2c were chosen to examine because they contained higher amount of galactose then glucose in these fractions. The purpose of this method was to break down (1→3)-β-glucose-linkages and thus, to get more purified fractions with more galactose and less glucose.

Reagents:

-Endo-(1→3)-β-glucanase, Cat.no E-lamse 55 U/ml in 3.2 ammonium sulphate (Megazyme®)

-Toluene

Equipment:

- Oven: Heraeus Function line (Thermo Scientific)
-Centrifuge- described in section 4.2.6
-Magnet stirrer- RCT basic IKA® laborteknikk (IKA)

Procedure:

The polysaccharide fractions of mushroom obtained after gel filtration, LsW2b and LsW2c were weighed in glass container and dissolved in water to concentration 1 mg/ml. Enzyme, 2 µl (0.1 U) per mg polysaccharide was added. Toluene (2 drops) was added to prevent bacterial growth during incubation at 40 ºC for 24 hours. Enzyme activity was terminated by boiling for 10 minutes in water bath and left to cool down on the bench. Then the solutions were dialyzed at MWCO cut-off 3500 Da against water at 4 ºC for 2 days and freeze-dried. Before freeze-drying, dialyse water had to be checked on carbohydrates with phenol-sulfuric acid test.

5.3.3 Iodine- potassium-iodide assay

(Tomasik & Schilling, 1998)

Principle:

Starch is carbohydrate which consist of amylose and amylopectin. Amylose, a polysaccharide, is composed of several thousand α-D-glucose units, linked together by (1→4)-linkages. Amylopectin is highly branched. The main backbone is built in a similar manner as in amylose, but with a higher proportion of branching points which located at C6 of glucose units. Both amylose and amylopectin of the starch form complexes with iodine-potassium iodide and form dark blue-coloured complex. The amylose has a six glucose units per turn, organized in helical structure. Since amylose made a helical structure, iodine complex occupies the central cavity of the helical molecule, causing an intense blue colour. The colour of the amylose-iodine complex varies with amylose chain length. This assay indicates that samples contain (1→4)-α-D-glucans (Cui, 2005).

Equipment:

-Glass pipette
-Porcelain plate with 12 wells

Reagents:

-3% iodine in 6.7 % potassium iodide
-purified water

Procedure:

A small amount (ca. 1 mg) of the samples (water extract LsW and alkali extract LsA) were placed in wells on porcelain plate and dissolved with small amount of water. Subsequently, two drops of iodine-iodine potassium solution were added and mixed well with the sample. Starch was used as a positive control in the assay. Positive control registers by forming of blue colour. Orange coloured solution indicates that samples does not contain α-(1→4)-glucans. Water was used as negative control. Change of the colour was registered.

6 Size exclusion chromatography (SEC)

Principle:

SEC (*Size exclusion chromatography*) is used to separate different compounds according to their molecular size or more correctly their hydrodynamic volume (Zhu et al., 2015).

SEC is used in **group separation mode** to remove small molecules from a group of larger molecules. Small molecules such as salt are easily separated from larger molecules and samples can be prepared for other chromatography techniques and assays

SEC column is packed with porous matrix of spherical particles with specific pore size, chemical and physical stability and inertness. Separation take place in that the mobile phase is pumped at a constant rate through the column. Large molecules are excluded from the pores and are transported through the column with the same speed as the mobile phase. **Exclusion volume** is the volume of mobile phase transporting them through the column. This allows that the smaller molecules will take a long time to get through the column and thereby is eluted out later than the large molecules (see Figure 28) (Pedersen-Bjergaard & Rasmussen, 2010).

Molecules that do not enter the matrix are eluted together in the **void volume**, $\mathbf{V_o}$ and they pass through the column at the same speed as the flow of buffer. Molecules elute from the column in order of decreasing size. Small molecules such as salts move down the column, but do not separate from each other. These molecules usually elute just before one **total column volume**, $\mathbf{V_t}$, of buffer has passed through the column ("GE Healthcare," 2014)

The electronic response of the substances will be detected by **the refractive detector (RI)** and processes further through the software to chromatogram (see Figure 30). Molecular weight distribution of the various fractions was determined using the standard curves of the standards with known molecular weight.

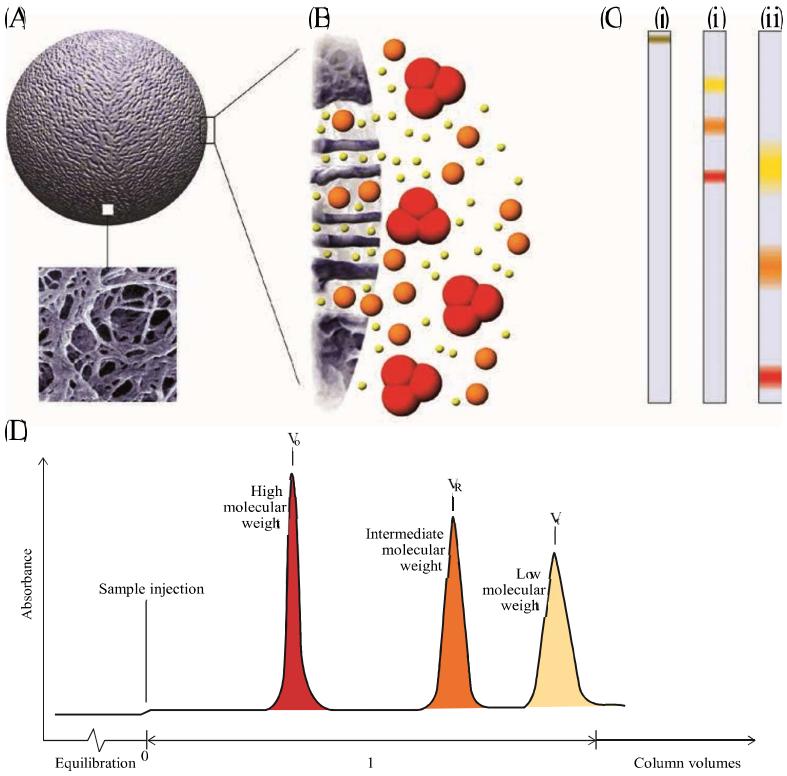


Figure 28. SEC: (A) Schematic picture of a bead (B) Sample molecules diffuse into bead pores. (C) Separation (graphical description) (i) sample is applied on the column; (ii) the smallest molecule (yellow) has shorter way than the largest molecule (red); (iii) the largest molecule is eluted first from the column. (D) Schematic overview of chromatogram ("GE Healthcare," 2014)

6.1 SEC-HPLC (*High Performance Liquid Chromatography*)

High-performance liquid chromatography (HPLC) is the most frequently used liquid-chromatographic technique. HPLC is used for separation a mixture of compounds and for determination of molecular weight distributions of for example polysaccharides. Separation is carried out using **a liquid mobile phase** which pumps through the column at constant rate under high pressure and **a solid stationary phase** filled in columns(Polymer Molecular Weight Measurement, 2013).

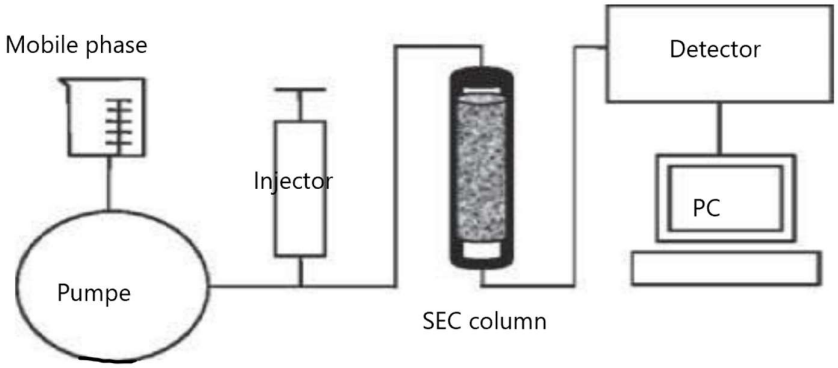


Figure 29. Structure of an HPLC system (Polymer Molecular Weight Measurement, 2013)

The interaction between each component from the sample and solid adsorbent material, causes difference in flow rates of each component and further due to separation of the components. The time at which a specific analyte elutes, is called **the retention time**.

HPLC systems can be divided into **two main** different **parts**: one part is for **separation**, and the other part is for **detection**, including RI detector (refractive index detector) and a signal output device (see Figure 29). There are many different stationary and mobile phases that can be used in HPLC and for this reason there is variety of separations.

Equipment:

-HPLC: Hitachi LaChrom Elite Chrom with pump, autosampler and Software (Hitachi High Technologies America, Inc.)

-Pump: PUMP L-2130 with (Hitachi High Technologies America, Inc)
-Autosampler: AUTO SAMPLER L-2200 (Hitachi High Technologies America, Inc.)
-Detector: RI Detector L-2490 EZ
-Software: EZ Chrom Elite;
-Precolumn: TSK Gel PW_{XL} Guard Column (6mm x 4 cm), (Bioscience)
-Column:
TSK Gel G5000PW_{XL} (Tosoh Bioscience LLC)
Column:

- column material: hydrophilic polymethacrylate spheres
- 7.8 mm x 30 cm (D x L)
- Volume: 14.3 ml
- Max column pressure: 20 bar
- Separation Area: dextran of molecular weight 50-7000 kDa
- Max flow: 1 ml /min, standard flow: 0.3-0.6 ml/min
- pH: 2-12

-Mobile phase: 0.05M Na_2SO_4 filtered with 0.45µm, degassed with helium
-Vortex: Lab Dancer (IKA)
-Water bath
-Eppendorf tubes
-Falcon ED Heating Immersion Circulator (JULABO GmbH)
-HPLC vials
-Filter: 0,45 µm (EMD Millipore)
-PALL, Acrodisc® 32 mm Syringe Filter with 0,45 µm Supor® Membrane
- Büchner funnel
- Büchner flask
-Vacuum pump: ILM Vac, p< 8 mbar/ 230V

All solutions were filtered through Büchner funnel and degassed with helium.

Reagents:

Mobile phase:
-Purified Water
-0.05M Na_2SO_4
-EtOH 20%

Procedure:

Equilibration of the column:
The column was stored in 20 % ethanol. Before starting the analysis, the column was washed with 1 column volume of water at 0.25 ml /min and afterwards with water 0.5 ml /min 1 column volume to wash out 20% ethanol.

Application and elution of the sample:

Polysaccharide samples (2.4 mg LsW ;1.8 mg LsA) were weighed into Eppendorf tubes and dissolved in 1ml 0.05M Na_2SO_4 to 2 mg/ml concentration. First, they were mixed well on vortex and heated in a water bath (80 ºC) to be completely dissolved. Prior to injection into the column all the sample solutions were filtered through 0.45 µm membrane filter in open vials, injected and eluted at 0.5 ml/min. The analysis of each sample took 30 minutes.

Washing the column after use:
After HPLC analysis, the column was washed with water at 0.5 ml/min for 1 hour and then with 20% EtOH at 0.25 ml/min for 2 hours.
The column was stored in 20% EtOH.

Standard calibration curves:

Different molecular weight population of extracts are estimated using a standard calibration curve with pullulan standards whose peaks best overlap with those obtained for the samples. Pullulan standards (8 standards) of known molecular weight (5.8-853 kDa) are used to calibrate the column.

Standards:

Pullulan is a water-soluble polysaccharide with repeated units of maltotriose condensed through a (1 → 6)-α- linkage. It is nontoxic, non-immunogenic, non-mutagenic, and noncarcinogenic in nature.

Pullulan standards (Polymer Laboratories LTD): 5.8, 12.2, 23.7, 48, 100, 186, 380 and 853 kDa (Mp) were used to determine a linear standard curve.

Linear standard curve was constructed by plotting the logarithm Mp to the retention time of selected pullulan standards.
The molecular weight of all the polysaccharide extracts was determined using a linear regression model:

$Y = aX + b$
Y: Retention time

X: Ln_Mp
The analysis of standards was performed under the same conditions as the sample.

6.1.2 Preparative size exclusion chromatography (SEC)

SEC or preparative gel permeation chromatography was carried out on the different columns: Sephadex S-500, Sephacryl® S-500 HR and Superdex 200 prep grade column. Three different columns were used in order to get purified fractions.

6.1.2.1 Sephacryl S-500 -gel filtration column

Principle:

Sephacryl S-500 column which was packed in our lab, at the Departement of Pharmacognosy, was used the column. The column consists of a composite gel prepared by covalently cross-linking allyl dextran with N, N – methylene bisacrylamide to form a hydrophylic matrix. Sephacryl S-500 separates substances between 40 - 20 000 kDa, measured dextran and thus is well suited for separation of polysaccharides ("GE Healthcare," 2014).

Reagents:
0.1 M sodium chloride
20% Ethanol for storage the column.
All solutions were filtered with 0.45 μm filter and degassed with helium gas.

Equipment:
Column: Sephacryl S-500
Column volume: 635 mL (H: 90 cm, D: 1.5 cm)
Injector: Valve IV-7
Pump: Pump P-50 (Pharmacia Biotech)
Detector: RID-6A detector (SHIMADZU)
Fraction collector: Pharmacia LKB Super Frac (Pharmacia)
Software: Chrome Leon Version 7
Filter: PALL® Acrodisc® Syringe Filter with 0, 45-micron Supor®
Membrane (PALL)
Collection tubes

Conditions:

Superloop: 50 ml
Eluent: 0.1 M NaCl
Flow: 2.5 ml/min
Fraction Size: 3.5 ml per collection tube, a total of 180 fractions
Injected volume: 25 ml
Acrodisc Syringe Filter 37 mm with1 μm Glass Fiber Membrane
Acrodisc Syringe Filter 32 mm with 0.45 μm Supor Membrane
Vacuum pump: ILM Vac, p< 8 mbar/ 230V

Procedure:

• **washing the column at startup:** The column was washed with water at flow rate 0.5 ml/min for 20 hours then with 0.1 M NaCl at flow rate 2.5 ml/min for 4 hours

• **Application and elution of the sample:** The sample (51.2 mg) was dissolved on a water bath at 100 ºC in 30 ml 0.1 M NaCl so that the concentration was 1.8 mg/ ml. The sample was filtered through two membrane filters with1 µm and 0.45 µm glass membrane. This was done just before injection. The sample was then pumped onto the column through the loop and was eluted through the column at flow rate 2.5 ml/min for 9 hours. The elution profile was determined by the signals from the RI detector. An elution profile was obtained from RI signals or by phenol-sulphuric acid method. The obtained fractions LsW1a, LsW2a, LsW3a were collected, combined and then dialyzed and lyophilized.

• **washing the column after use:** The column was washed with water at a flow rate 0.5 ml/min for 1 hour (2 column volume) at room temperature, then with 20% ethanol for 2 hours at a flow rate 0.25 ml/min.

6.1.2.2 Sephacryl® S-500 HR (Figure 30)

Principle:

Gel filtration column for preparative separation of biomolecules, Sephacryl® S-500 was used. It uses for separating polysaccharides, macromolecules with extended structures, and even small plasmids. It is a cross-linked copolymer of allyl dextran and N, N-methylene bisacrylamide that gives the matrix good rigidity and chemical stability. HR (high resolution) of this column contribute to covering a wide fractionation range. The fractionation range defines the range of molecular weights that have access to the pores to the matrix. This high-resolution column is most suitable for samples that originally contain few components or for samples that have been partially purified by other chromatography techniques. This ensures that most of the unwanted molecules of similar size are eliminated ("GE Healthcare instruc 16/60," 2011).

Figure 30. HiPrep™ 16/60 Sephacryl® S-500 HR, bed length 600 mm, i.d. 16 mm

Table 5. Column data- HiPrep™ 16/60 Sephacryl® S-500 HR, ("GE Healthcare instruc 16/60," 2011)

Mean particle size:	47 μm (25-75 μm)
Separation range (Mr) -dextran	4 x 10^4-2 x 10^7
Column volume:	120 ml
Sample volume:	up to 5 ml
Flow rate:	0.5 ml/min
Max flow rate:	1 ml/min
pH stability:	3-11 (long term and working range)
Storage:	4 ºC to 30 ºC in 20 % ethanol

The porosity of the gel is controlled by the dextran component to give five types with different fractionation ranges. To avoid breaking the column, post-column pressure must never exceed 3.5 bar.

Reagents:

0.1 M sodium chloride

All solutions were filtered with 0.45 μm filter and gassed with helium gas.

Equipment:
Column: HiPrep™ 16/60 Sephacryl® S-500 HR
Injector: Valve IV-7
Pump: Pump P-50 (Pharmacia Biotech)
Detector: RID-6A detector (SHIMADZU)

There were used the same fraction collector, computer, filter, collection tubes and syringe as in gel filtration with Sephacryl S-500 column.

Conditions:

Loop: 4 ml
flow rate: 0.3 ml/min
Eluent: 0.1 M NaCl
Fraction Size: 0.66 ml per collection tube, a total of 180 fractions
Injected volume: 4.0 ml

Procedure:

Before connecting the column to a chromatography system, it was necessary to start the pump to remove all air from the system, in tubing and valves.

• Calibration of the column: washing the column at startup: The column was washed with 1.5 column volume of distilled water at flow rate 0.5 ml/min and then with 0.1 M NaCl at flow rate of 0.5 ml / min

• Application and elution of the sample: the sample (24.2 mg) was dissolved in 10 ml 0.1 M NaCl in water bath, so that the concentration was 3 mg / ml. The solution was filtered with 0.45 µm filter. This was done just before injection. The sample was eluted through the column. The elution profile was determined, and the fractions were obtained: LsW1b, LsW2b, LsW3b. Then fractions were dialyzed and lyophilized.
• Washing the column after use: The column was washed with ½ column volume of 0.2 M NaOH at flow rate 0.5 ml/min to remove most aggregates non-specifically bound to the medium. After cleaning was column equilibrated with 2 column volumes of 0.1 M NaCl. The next sample was injected onto the column when the baseline had stabilized.

• Finally, the column was preserved with 4 column volumes of distilled water and then followed by 4 column volumes of 20% ethanol and kept in cold storage. Washing of the column was performed at a flow rate of 0.5 ml / min.

6.1.2.3 Superdex 200 prep grade column (see Figure 31)

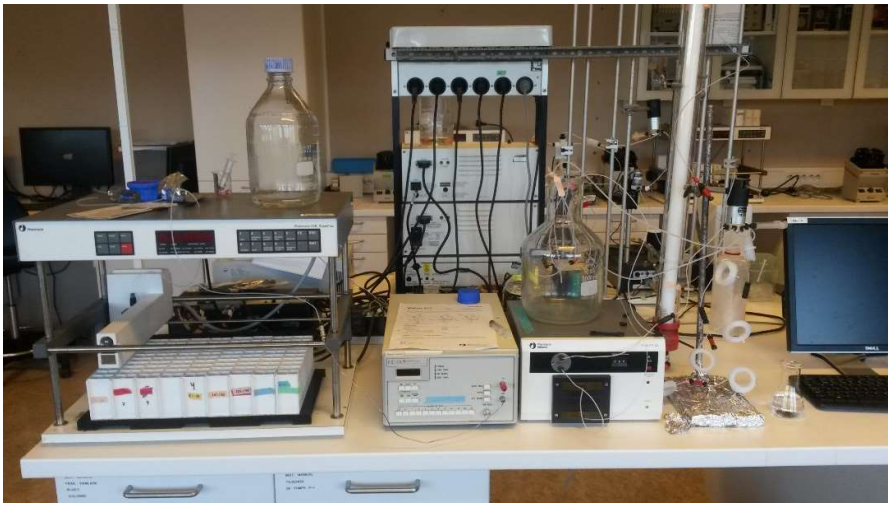


Figure 31. Gel filtration chromatography (SEC): a. fraction collector, b. detector, c. injector med pump, d.column Superdex 200 HR and computer med appropriate software

Principle:

It is SEC media where the dextran chains are covalently linked to a highly cross-linked porous agarose beads.The dextran chains are stabilized with highly cross-linked agarose. This medium has god selectivity, high resolution and low nonspecific interaction (see Figure 32) ("GE Healthcare Hi Load 200," 2011).

Superdex 200 has a broad fractionation range, M 1 x10^3- 1 x10^5 Da, suitable for separation of dextrans.

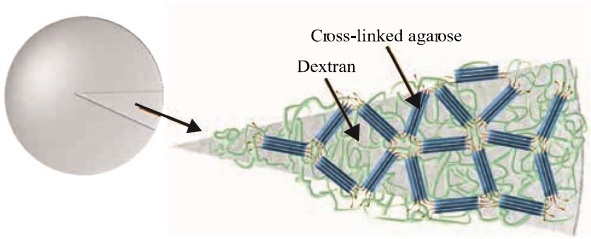


Figure 32. A schematic section of a Superdex particle, where the dextran chains are covalently linked to a highly cross-linked agarose matrix ("GE Healthcare," 2014)

Table 6. Column data- HiLoad 16/60 Superdex 200 prep grade, ("GE Healthcare Hi Load 200," 2011)

Mean particle size:	34 µm
Separation range (Mr) -dextran	1 x 10^3-1 x 10^5
Column volume:	120-124 ml
Sample volume:	up to 5 ml
Flow rate:	0.3-1.6 ml/min
Max pressure:	3 bar, 0.3 MPa
pH stability:	3-12 (long term and working range)
Storage:	20 % ethanol

Reagents:

0.1 M sodium chloride
20% Ethanol for storing column.
All solutions were filtered with 0.45 µm filter and gassed with helium gas.

Equipment:
Column: HiLoad 16/60 Superdex 200 prep grade

There were used the same equipment as in gel filtration with Sephacryl S-500 column and HiPrep™ 16/60 Sephacryl® S-500 HR column.

Conditions:

Loop: 2 ml
flow rate: 0.5 ml/min
Eluent: 0.1 M NaCl
Fraction Size: 0.66 ml per collection tube, a total of 180 fractions
Injected volume: 2 ml

Procedure:

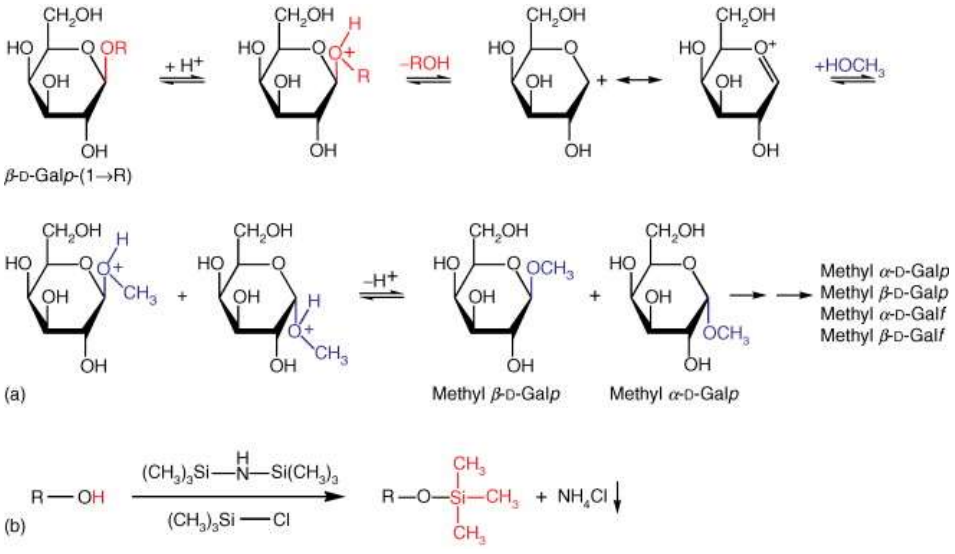
It was performed the same procedure, as well as with HiPrep™ 16/60 Sephacryl® S-500 HR. LsW (61.0 mg) was dissolved in 10 ml 0.1 M NaCl in water bath to concentration of 6 mg/ml. The solution was filtered with 0.45 µm filter. The elution profile was determined by the signals from the RI detector. If profile was not good or impossible to read, phenol sulphuric acid test was used to obtain elution profile.
Then, the obtained tree fractions: LsW1c, LsW2c and LsW3c were collected, dialyzed and lyophilized.

7 QUANTITATIVE AND QUALITATIVE DETERMINATION OF MONOSACCHARIDES

7.1 Methanolysis and TMS derivatization

(Chambers & Clamp, 1971)

Principle:
Monosaccharide composition of the polysaccharides was determined by methanolysis. The methanolysis involves cleaving polysaccharides to individual monosaccharides by methanolic HCl.
In an anhydrous acidic environment, the polysaccharides are converted to methyl glycosides on the OH group at the C_1. Protonation of the glycosidic O atom, followed by cleavage of the glycosidic linkage and reaction with methanol gives the mixture of 1-O-methylated α, β-anomers of the original ring form. During the glycosidation process, new glycosidations can occur and there were finally obtained the mixture of methyl derivatives of the α- and β-pyranose and furanose forms for each sugar (Figure 33).



Figur 33. Reaction mechanism of the methanolysis of a model β-(1-4)- galactan (a) and trimethylsilylation of a hydroxyl group (b) (Kamerling & Gerwig, 2007)

Reagents:

-3M HCl in anhydrous methanol

-Internal standard (1 mg/ml mannitol in anhydrous methanol)

-Anhydrous methanol

Equipment:

-Supelco tubes (Clear vials with Teflon screw cap, 4 ml)
-parafilm and cannula
-vacuum desiccator with P_2O_5
-Oven- Function line (Heraeus Instruments)
-SMI pipette 100 µl
-glass pipettes
-N_2 gas setup: Pierce, Reacti-Therm III TM, Heating Module (Thermo Scientific)

All glass equipment was acid washed.

Procedure:

Lyophilized polysaccharides (1mg) of *L. scrobiculatus* were weighed into supelco tubes. The tubes were covered with parafilm, perforated by a cannula. The samples were dried in a vacuum desiccator over P_2O_5 for about 24 hours prior to methanolysis. Then, 3M HCl in anhydrous methanol (l ml) and mannitol 1 mg/ml (100 µl) as internal standard were added. The tubes were kept in a dry oven at 80 ° C for 20-24 hours (overnight). After 10 min standing at 80 ˚C, tubes were re-sealed to prevent evaporation. The samples were dried with N_2 gas and washed with anhydrous methanol (250 µl) to remove the solvent. The samples were again evaporated with N_2 gas. This procedure was repeated twice. The samples were covered with perforated parafilm and finally dried in a vacuum desiccator over P_2O_5 for at least 1 hour.

7.2 TMS derivatization

Principle:

Methyl glycosides and methyl esters must be converted to thermally stable and volatile substances to be analysed on the gas chromatography column. They were converted to their TMS derivatives by trimethylchlorosilane (TMC). The free hydroxyl groups of the monosaccharides were reacted with hexamethyldisilazane (HMDS), trimethylchlorosilane (TMCS) dissolved in pyridine. In this way, the carbohydrate derivatives as final products were volatile and thermally stable (Figure 34).

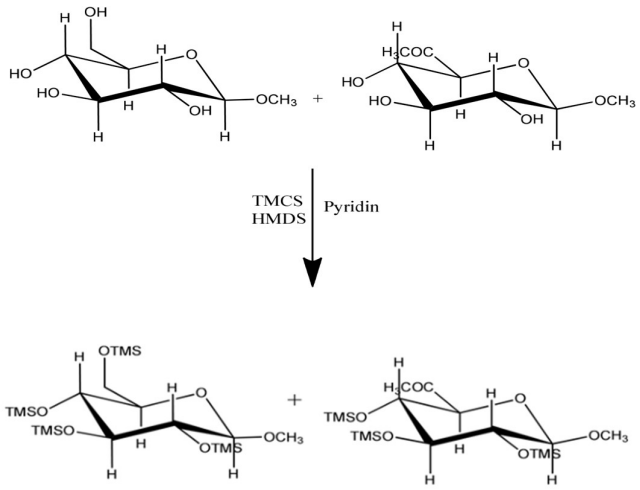


Figure 34. Derivatization a methyl ester and a methyl glycoside with TMS reagent to make them volatile

Reagents:
-TMS reagent: Trimethylchlorosilane (TMCS) 1 ml
Heksamethyldisilizane (HMDS) 2 ml
Pyridine (anhydrous) 5 ml

Equipment:
-SMI pipette 5 ml, 200µl
-Mini Shaker MS3 Basic (IKA)
- Spectrafuge mini (labna International Inc)
-Eppendorf tubes

Procedure:
TMS reagent (160 µl) was added to the dried samples and mixed well on vortex for about 30-40 sec. Before the gas chromatographic analysis, the samples were left standing at least 30 minutes at room temperature.
Afterwards, the samples were transferred to Eppendorf tubes and centrifuged to remove precipitated material. Supernatants were transferred in GC vials and analyzed by capillary GC.

7.3 Gas Chromatography (GC)

Principle:
GC is commonly used analytic technique, a column chromatographic method, that can separate and analyse volatile compounds in gas phase.

It was used to identify monosaccharides. Monosaccharides can exist both in the pyranose or furanose form. In addition, they can exist in α and β configuration and in open or closed ring form. The different conformations of a specific monosaccharide allocated different between the carrier gas and the stationary phase. This provides a characteristic pattern of peaks with characteristic retention times and area ratio, which were used to identify the monosaccharides in the sample. Internal standard was added to the sample to correct any loss and variations in sample preparation and analysis (Pedersen-Bjergaard & Rasmussen, 2010).

High temperatures in injector rapidly vaporize a liquid sample. Gas stream moved through a tube and carried substances through the column to the detector. The substances migrated through the column at different speeds, which were determined by their volatility and interaction with the stationary phase. The difference of speeds caused that monosaccharide derivatives were separated in the GC column.

GC equipment consist of a carrier gas system, injector, gas chromatographic column, detector and data-processing unit (Pedersen-Bjergaard & Rasmussen, 2010).

Reagents:
-Methanol
-Hexane

Equipment:

Gas chromatograph instrument model: Focus^{TM} GC (Thermo Scientific)
Detector: Flame Ionization Detector (FID)
Injector: Split/ splitless
Autosampler: Fisons AS-800
Steering software Chrome Leon 7.2 SR4
Column: RXI 5MS (Restek); "Fused silica" capillary column.
Length: 30 m; Inside diameter: 0.32 mm
Film Thickness: 0.25µm
Injection volume: 1 µl (Manuel)

Carrier Gas: Helium

Conditions:

Flow mode: Constant pressure (0.7 bar)

Injection mod: Split (ratio 1/10)

Injection volume: 1.0 µl

The injector temperature: 250 ºC

The detector temperature: 300 ºC

Temperature program: 140 ºC(initial)
140-170 ºC at rate 1ºC/min
170-250 ºC at rate 6 ºC/min

Procedure:

The derivative samples (1µl) were injected by an autoinjector and introduced into a heated injector where they were vaporized. The syringe was washed with hexane.

Monosaccharides can exist both in the pyranose or furanose form. In addition, they can exist in α and β configuration and in open or closed ring form. The different conformations of a specific monosaccharide allocated different between the carrier gas and the stationary phase. This provides a characteristic pattern of peaks with characteristic retention times and area ratio, which were used to identify the monosaccharides in the sample. Internal standard was added to the sample to correct any loss and variations in sample preparation and analysis (Pedersen-Bjergaard & Rasmussen, 2010).

Monosaccharides in each sample were identified by comparing the relative retention times of each individual monosaccharide with relative retention times of standards which were run before under the same conditions.

The relative retention time for each monosaccharide was calculated as the ratio Rt of each monosaccharide top and Rt internal standard with known quantity and concentration.

Calculations (Identification):
Retention times: Rt

Relative Rt = Rt (peak of each monosaccharide) / Rt (internal standard peak)

Calibration curves for quantification were also constructed by plotting the areal under curve (AUC) for each peak in the chromatogram relative to the AUC of the largest peak.

A (rel) = A (peak of each monosaccharide) /A (the largest peak)

GC analyzes were performed by Hoai Thi Nguyen and Cristian Winther Wold at the Department Pharmaceutical Chemistry, Pharmacy, University of Oslo.

7.4. DETERMINATION OF THE STRUCTURE

7.4.1. Linkage analysis by methylation and identification by GC-MS.

The analysis is complicated and consists of some steps: methylation, hydrolysis, reduction, acetylation and identification and quantification by GC-MS. The method involves complete methylation of a polysaccharide, hydrolysis to a mixture of partially methylated monosaccharides, reduction of the methylated monosaccharides to alditols, acetylation of the alditols and identification of the partially methylated alditol acetates by GC-MS (see Figure 35) (Filomena, Cherie, Geoffrey, & Antony, 2012).

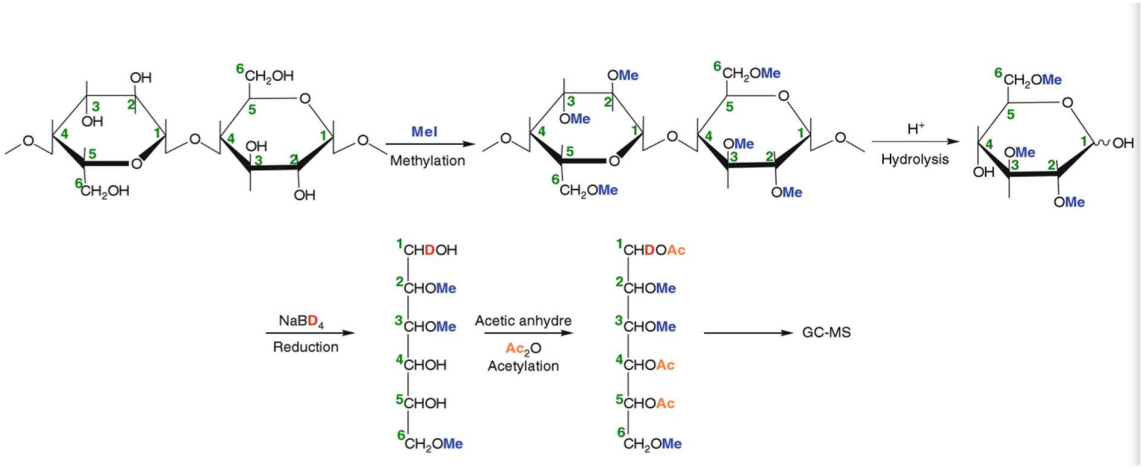


Figure 35. Methylation reactions: Partially methylated alditol acetates were obtained after methylation and acid hydrolysis. They are further reduced with $NaBD_4$ which opens the sugar ring, forms the alditol and tags C1 atom with a deuterium atom. Partially methylated alditols are acetylated with acetic anhydride and analyzed on GC (Filomena et al., 2012).

7.4.2. Methylation

(Ciucanu & Kerek, 1984)

Principle:

Methylation is a method for determination of the linkage position between the monomers in a polysaccharide. This method helps to distinguish between hydroxyl groups which are not in the glycosidic bond and hydroxyl groups that are included in the glycoside bonds. In this procedure, all free hydroxyl groups of the polysaccharide are methylated.

Dimethylsulfoxide (DMSO) in the basic environment, NaOH form DMSO carbanion which together with the OH ions, deprotonate the free hydroxyl groups. The deprotonated OH groups as strong nucleophiles react with methyl iodide (CH_3 I) and form O-methylated polysaccharides (Figure 36).

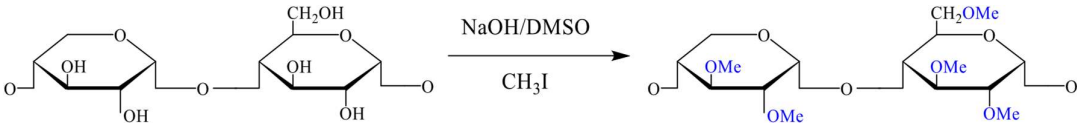


Figure 36. Methylation of the hydroxyl groups in polysaccharides (Filomena et al., 2012)

Reagents:

-Dimethyl sulfoxide (DMSO) solution
-NaOH pellets, anhydrous, dried in a desiccator
-Methyl iodide solution (CH_3I)

-Anhydrous methanol
-Dichloromethane
-Sodium thiosulfate-5-hydrat
-Purified water

Equipment:

- Glass tubes with teflon screw cap (large)
- Glass tubes with teflon screw cap (small)

-Speed Vacuum Dryer Savant® SPD121P (Thermo Scientific)
-Vapornet Savant® VN100 (Thermo Scientific)
Refrigerated Vapor Trap: RVT4104
-Vacuum pump: OFP4000 OIL-FREE (Thermo Scientific)
-Shaker: Vibrax-VXR Basic (IKA)
-Pipette: 500 µl- 5 ml
-Centrifuge Multifuge 4 KR Heraeus 72 (VWR)
-N_2 gas setup: Pierce, Reacti-Therm III TM, Heating Module (Thermo Scientific)
-SMI pipette 100 µl
-Vacuum desiccator with P_2O_5
-Mortar and pestle
-Glass pipets
All gas equipment was acid washed.

Procedure:
Freeze-dried polysaccharide samples (ca.1mg) were weighed into acid washed glass tubes. Then, the samples were covered with perforated parafilm and dried overnight in a vacuum desiccator over P_2O_5. DMSO (500 µl) was added to each sample and then shacked for 20 minutes at 200 rpm. NaOH / DMSO suspension was made (2 pellets NaOH per 1 ml DMSO to reach concentrations 120 mg/ml NaOH) with stone mortar and pestle. The suspension of DMSO / NaOH (500 µl) was added directly to the polysaccharide solution with SMI-pipette without coming into contact with methylation tubes. All samples were flushed with N_2 gas to remove eventual interfering gases and then were mixed on shaker for 40 minutes at 200 rpm. The samples were heated in water bath at 80 ºC ca. 30 minutes.
Methyl iodide was added in three portions (100, 100 and 200 µl) in sample in a fume hood. Between each addition, the mixture was shaken for 10 minutes at 200 rpm. After the third addition, the mixture was shaken for 20 minutes at 200 rpm. Then, 10 ml freshly prepared solution of 100 mg /ml sodium thiosulfate and 2.5 ml dichloromethane (DCM) were added. This step was done to remove iodide and NaOH by extraction. The samples were mixed well (40 seconds) on a mini shaker and separated by centrifugation at 1000 rpm for 3 minutes. After centrifugation the upper aqueous phase was removed and discarded by using of acid-washed glass pipette. The lower DCM phase was washed four times with 5 mL purified water.
Each time before the water phase has been removed, the samples were mixed well (40 seconds) on vortex and centrifuged at 1000 rpm for 3 minutes. The DCM phases were transferred with glass pipette to small tubes and evaporated to dryness on Speed Vacuum Dryer.

7.4.3 Hydrolysis

(Cui, 2005; Filomena et al., 2012)

Principle:

Acid-catalyzed hydrolysis results in cleavage of the glycosidic bonds between monomers in the methylated polysaccharide and formed partially methylated M monosaccharides (Figure 37).

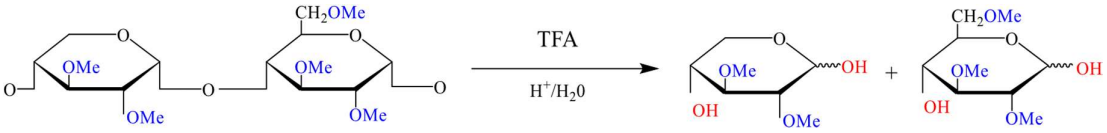


Figure 37. The methylated polysaccharides are hydrolysed to the partially methylated monosaccharides using TFA (trifluoroacetic acid) (Filomena et al., 2012)

Reagents:

2.5M trifluoroacetic acid (TFA)

Equipment:

-Pipette 100µl-1 ml
-Oven: Function line (Heraeus Instruments)
- N_2 gas setup: Pierce, Reacti-Therm III TM, Heating Module (Thermo Scientific)
-Speed Vacuum Dryer:
Vapornet Savant® VN100 (Thermo Scientific)
Refrigerated Vapor Trap: RVT4104
Speed Vacuum Dryer Savant® SPD121P (Thermo Scientific)
Vacuum pump: OFP4000 OIL-FREE (Thermo Scientific)

Procedure:

The methylated polysaccharides were hydrolysed to monosaccharides using TFA (trifluoroacetic acid). Trifluoroacetic acid 2.5 M (500 µl) was added to the methylated polysaccharide samples. The samples were flushed with N_2 gas and kept in an oven (100 ºC) for 2 hours. The hydrolyzed samples were cooled and evaporated to dryness on Speed Vacuum Dryer until formation of yellow oily liquid.

7.4.4. Reduction

(Cui, 2005; Filomena et al., 2012)

Principle:

After acid hydrolysis, the partially methylated monosaccharides are released and reduced with $NaBD_4$ under basic conditions. This process opens the sugar ring, form the alditol and tags the anomeric C atom (C_1) with a deuterium atom. In this way, C_1 atom can be distinguished from carbon C_6. However, information on the anomeric configuration (α/β) of the glycosyl residue is lost during this reduction step (Figure 38).

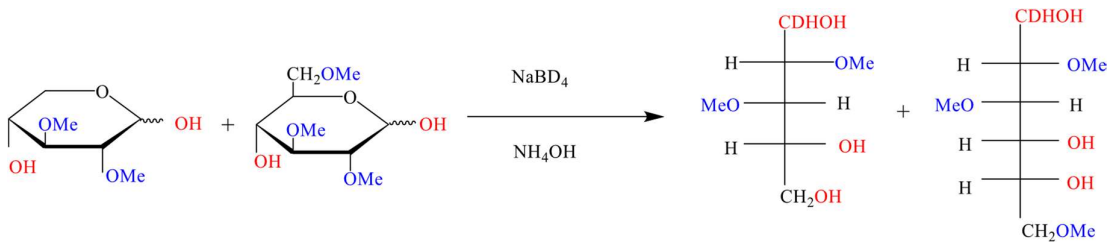


Figure 38. Opening the ring follow reduction of the aldehyde group with $NaBD_4$, forming partially methylated alditol and tags the C1 atom by deuterium (Filomena et al., 2012)

Reagents:
-2M NH_4OH
-1M $NaBD_4$, fresh
-Glacial acetic acid
-5% acetic acid in methanol
-Anhydrous methanol

Equipment:
Pipette 100µl-1 ml
Pipette 10µl-100ml
-Mini Shaker MS3 Basic (IKA)
- N_2 gas setup: Pierce, Reacti-Therm III TM, Heating Module (Thermo Scientific)
-Speed vacuum dryer:
Vapornet Savant® VN100 (Thermo Scientific)
Refrigerated Vapor Trap: RVT4104
Speed Vacuum Dryer Savant® SPD121P (Thermo Scientific)
Vacuum pump: OFP4000 OIL-FREE (Thermo Scientific)

Procedure:
The residue of samples (yellow oily residue) were dissolved in 2M NH_4OH (500 µl). Freshly prepared solution: 1M $NaBD_4$ (500 µl) in 2M NH_4OH was added to each sample, then incubated in an oven for 60 minutes at 60 º C. The excess of reductant in the sample was destroyed by carefully adding glacial acetic acid (50 µl) 3 times. After each addition of acetic acid, the samples were mixed well. Active bubbling indicated that the reduction was complete.
Reduced samples were dried in Speed Vacuum Dryer for about 3 hours (2.5 hours with the heat). This step due to the formation of borate complexes. Complexes with borate was broken and the excess of reagent was removed with 2.5 ml 5% acetic acid in methanol two times. After each addition, the samples were dried in Speed Vacuum Dryer, until dryness.
Methanol (2.5 ml) was added to the samples and then they were dried 60 min without heat. This process was repeated.

7.4.5 Acetylation

(Cui, 2005; Filomena et al., 2012)

Principle:

Acetylation was performed to make the partially methylated alditols more volatile for GC-MS analysis. The hydroxyl groups were acetylated with acetic anhydride in pyridine (Figure 39).

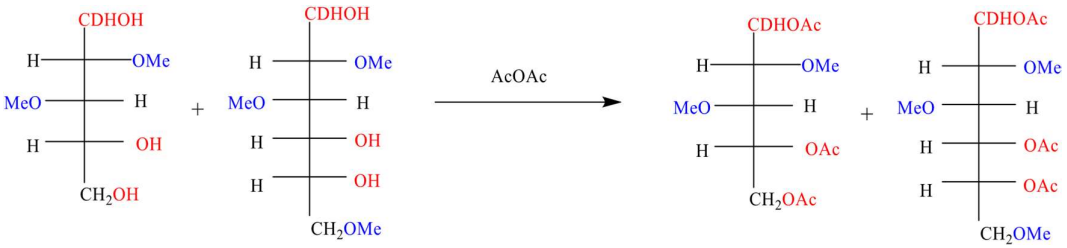


Figure 39. Acetylation of partially methylated alditoler with acetic acid anhydride (Filomena et al., 2012)

Reagents:

-1-methylimidazole
-Acetic anhydride in pyridine
-Dichloromethane
-Purified water
-Anhydrous methanol

Equipment:

-Glass tubes with teflon screw cap (large)
-Glass tubes with teflon screw cap (small)
-Supelco tubes (Clear vials with Teflon screw cap, 4 ml)
-Pipette 100µL-1 mL
-Pipette 500µL-5 mL
-Glass pipettes
-Mini Shaker MS3 Basic (IKA)
-Spin Multi joint 4 KR Heraeus 72 (VWR)
- N_2 gas setup: Pierce, Reacti-Therm III TM, Heating Module (Thermo Scientific)
-Vials GS-MS : Chromacol 03-FISV (300µl) (Thermo Scientific)
-Screw caps to vials: Solid Cap with PTFE, 13 mm, 4 ml vials (Supelco)

Procedure:

The residues of the samples were dissolved in 1-methylimidazole (200µl) following by acetic acid anhydride (2 ml). The solutions were mixed well on vortex and then they were left for 10 minutes at room temperature. Excess acetic anhydride was destroyed by adding purified water (10 ml), mixed well and left at room temperature for 10 minutes. The partially methylated alditol acetates were extracted with dichloromethane (1ml), mixed well (>30 seconds) and centrifuged at 1000 rpm for 4 minutes. The lower DCM phase was collected with a glass pipette and transferred to new small tubes. The procedure was repeated once. Then DCM phases were combined. Another phase which was over DCM phase, was washed by adding purified water (5 ml), mixed well (>30 seconds) and centrifuged at 1000 rpm for 4 minutes. The first aqueous phase was destroyed, and the DCM phase was washed again under the same

conditions. The DCM was collected with glass pipette and transferred to supelco tubes. Samples were dried under stream of nitrogen. The dried samples were dissolved in 250 µl DCM and mixed well (60 seconds) on vortex prior to GC-MS analyze. The mixture was left at room temperature for 30 minutes before the supernatants were transferred to GC-MS vials, injected and analyzed.

7.4.6 GC-MS

(Pedersen-Bjergaard & Rasmussen, 2010)

Principle:

Gas chromatography mass spectrometry (GC-MS) combines two techniques for separation, identifying and quantification compounds in the sample.

GC can separate volatile and semi-volatile compounds with great resolution while MS can provide detailed structural information on most compounds.

In Gas chromatography (GC), the substances are first separated by passing through a chromatographic column. Compounds in a sample are separated from each other because some substances take longer time to pass through the column to a detector. This time which can be used for identification, called a retention time. The detector provides an electronic response of the substances and the detector for the GC-MS is the Mass Spectrometer (MS).

Equipment:

gas chromatograph mass spectrometer: GC-2010 with GCMS-QP2010 (Shimadzu Corporation)
Software: GCMS Analysis
Carrier gas: Helium
Column:

Procedure:

GC-MS analyzes were performed by Hoai Thi Nguyen at the Department Pharmaceutical Chemistry, Pharmacy, University of Oslo.

GC used a mobile phase (carrier gas such as helium) which transported the sample through a stationary phase, fixed in the column. Capillary GC column was several meters long (10-120 m). GC oven had the temperature range from 5 º C to 400 ºC.

Eluted substances went into the mass spectrometer and they were ionized (fragmented). Fragments were accelerated into a mass analyzer where they were separated and sorted by mass to form a fragmentation pattern. It was used to identify molecules because the fragmentation pattern for a given compound is unique, like the retention time (RT).

The spectra were saved on the computer and analyzed (see Figure 40).

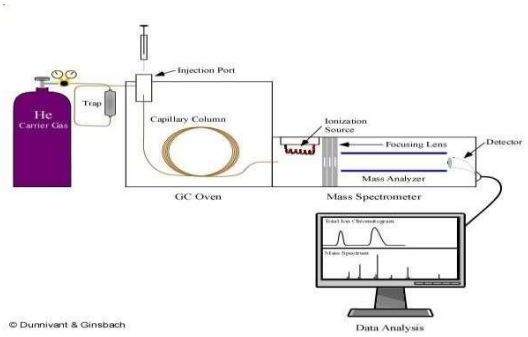


Figure 40. Schematic diagram of GC-MS instrument (M.Dunnivant, 2008)

Conditions:

Initial flow: 1 mL / min
the ion source temperature: 200 ° C
Injection System split (1:10)
Injection volume: 1 µl (automatic)
Flow mode: Constant pressure (77 kPa)
Injector temperature: 280 ° C
Interface Detector temperature: 280 ° C; Restek RTX-5 Length: 30 m, inner diameter: 0.25 mm, film thickness: 0.25 microns

30 ° C/min 0,5 °C/min 30 °C/min

Temperature program: 80 °C → 170 °C → 200 °C → 280 °C

8 Nuclear Magnetic Resonance Spectroscopy (NMR)

Nuclear Magnetic Resonance Spectroscopy (NMR) was used in further structural identification of the compounds in *L.scrobiculatus*.

Nuclear magnetic resonance spectroscopy (NMR) is based on phenomenon that large number of nuclei of atoms possess a magnetic property (spin). Spin is a property of electron and nucleus like mass, electric charge and magnetism (L.D.Field, 2015).

NMR spectrometer requires a Rf transmitter which can be adjusted to the appropriate frequency for the nucleus one wishes to detect. Rf detector observes Rf radiation which is absorbed and emitted by the sample. Rf detector and Rf transmitter are controlled by computer which process and present data for analysis.

Samples for NMR are liquids (solutions) or solids which must be placed in a strong magnetic field. The spectrum shows the magnetic fingerprint of the molecule, and provides also information for the molecules which are in the sample (L.D.Field, 2015).

This method requires deuterated solvents and high soluble sample on the chosen deuterated solvent. D-glucans are not sometimes soluble in water, because they can form gel solutions.

Reagents:

-Methanol-d_4, > 99.8 atom % D (MeOD)
-Chloroform-d, 99.8 atom % D ($CDCl_3$)

Equipment:

-NMR tubes for automatic sampling, with corks (Norell), long 4 cm
-NMR spectrometer: Bruker BioSpin Avance 400 MHz (GmbH), used ^1H spectra

Procedure:

Freeze-dried polysaccharide samples (ca. 25 mg) of different extracts of *L scrobiculatus* were dissolved in appropriate solutions before NMR analyses.

LsK, ethanol extract precipitate, was dissolved in methanol deuterium (MeOD).

Dichloromethane extract of *L. scrobiculatus* was dissolved in chloroform deuterium ($CDCl_3$).

Ethanol extract of *L.scrobiculatus* was dissolved in methanol deuterium (MeOD).

Sample solutions were transferred to NMR tubes and analysed on a 400 MHz User spectrometer. ^1H NMR analysis was performed by Karl Malterud at the Department of Pharmaceutical Chemistry, Pharmacy, University of Oslo.

9 BIOLOGICAL ACTIVITY ASSAY

9.1 *MgtA* activity test

Extracts of *L.scrobiculatus:* LsDCM, LsW, LsEt, LsA, LsK and mannitol were subjected to *MgtA* activity assay. The test was studied if the extracts can inhibit the activity of *MgtA*. *MgtA* is a bacterial transport protein for Mg^{2+} and important for the bacterial homeostasis.

Principle:

In this assay, purified *MgtA* from *E. coli* was used. The activity of *MgtA* and possible inhibition of activity provided by mushroom extracts were measured by the amount of the phosphate released from ATP. The ATP-ase activity of *MgtA* is dependent of the cardiolipin. It occurs the reaction between inorganic phosphate and acidified ammonium molybdate. The samples, containing the phosphate is mixed with an acid solution of Mo^{6+}- ammonium molybdate to produce PMo12O3-40. This reaction was terminated by reduction with ascorbic acid. The blue coloured complex with different intensity of colour was formed. The number of released inorganic phosphate are proportional the number of ATP molecules which were hydrolysed. The absorption can be measured using a colorimeter to determine the amount of phosphorus (Subramani et al., 2016).

Reagents:

-detergent $C_{12}E_8$ (Octaethylene glycol monododecyl ether)
-chloroform

--Argon
-purified *MgtA* 0.1 mg/ml – final 0.25 µg *MgtA*, stored at -80 ºC
-$MgCl_2$
- HEPES -
-KCl -
-Na_2MoO_4
-KNO_3
-NaN_3
-Cardiolipin 6.82 mM- final 116 µM Cardiolipin (Lipids from Avanti Polar Lipids, Alabama)
-ATP
- Milli-Q-Water (MQ-water)
- Ascorbic acid
- Ammonium molybdate
- Sodium dodecyl sulphate
-DMSO
-sodium citrate
- bismuth citrate
- 1M HCl

Equipment:

-96-well PVC microtiter plate with flat bottom
-Balance Denver instrument max 60/210 g, min 0.001 g
-Pipette 5-50 µl, 1-10 µl, 0.2-2 µl
-MS2 Minishaker IKA
-Absorbance reader: BioTek Instruments Inc
-Oven: Thermo scientific Heraeus Incubator
-Shaker: VWR pulsing Vortex Mixer
-pH meter: JenWay 3505
-IKA RCT magnet stirrer

Preparation of the reaction buffer

Buffer was prepared by mixing the following reagents: 50mM HEPES, pH 7, 400 mM KCl, 0.25 mM Na_2MoO_4, 5mM NaN_3 and 20 mM KNO_3. It was necessary to adjust pH of solution to 7 with 1M KOH.

Preparation of the lipid stock

It was necessary to prepare fresh solution of lipids for each experiment.
Cardiolipin was dissolved in 10 mg/ml chloroform in glass tube and dried under argon stream for 30 minutes. Then, dried lipid film was solubilized in 20 mM $C_{12}E_8$ (stock 10 %, 185.5 mM) to concentration of 10 mg/ml with shaking for 30 minutes.

Preparation of Solution I

Solution I was used to terminate the reaction and it had to be freshly prepared. The ascorbic acid (0.3 g) was dissolved in 3.5 ml MQ-water and added 5 ml HCl. Ammonium molybdate was prepared fresh (0.5 g dissolved in 1 ml water). It was added 0.5 ml of 10 % ammonium molybdate and 1.5 ml of 20 % sodium dodecyl sulphate and mixed on magnet stirrer. Solution had to be yellow coloured. The green solution indicates that it is contaminated with phosphate.

Preparing of the Solution II

Solution II was added to increase intensity of the colour. In glass flask was mixed: 3.5 % sodium citrate, 3.5 % bismuth citrate and 1 M HCl.

Dissolving of the extracts

The extracts of *L.scrobiculatus* were dissolved to dilution of 2 mg/ml in water (LsW and LsK) or 1 mg/ml in 50 %DMSO in water (LsEt and LsA) or 2 mg/ml in 50 % DMSO and water (LsDCM) in water bath at 80 ºC. LsA and LsEt have not been completely dissolved. For each one of the extracts three samples were used and all the assays were carried out in triplicate in order to obtain precision results.

LsA and LsEt were not completely dissolved.

For each one of the extracts three samples were used and all the assays were carried out in triplicate in order to obtain precision results.

The solutions were added to 96-well PVC microtiter plate, respectively:

Composition	Negative control: blank (without protein and extract)	Positive control (protein, without extracts)	Sample (LsDCM, LsW, LsEt, LsA, LsK)
MQ-water	21.8 µl	19.3 µl	18.3 µl
2x reaction buffer	30 µl	30 µl	30 µl
MgtA	-	2.5 µl	2.5 µl
$MgCl_2$	3.6 µl	3.6 µl	3.6 µl
Cardiolipin	1 µl	1 µl	1 µl
Extracts	-	-	1 µl
ATP	3.6 µl	3.6 µl	3.6 µl
Total	60 µl	60 µl	60 µl

The reaction started when 3 mM ATP-Mg^{2+} was added and incubated at 37 º C for 15 minutes. Furthermore, the reaction was terminated by adding 75 µl Solution I and incubated 10 minutes at room temperature. Then, 125 µl of Solution II was added to increase the colour and incubated at room temperature for 1 minute. Absorbance was measured at 690 nm (see Figure 41).

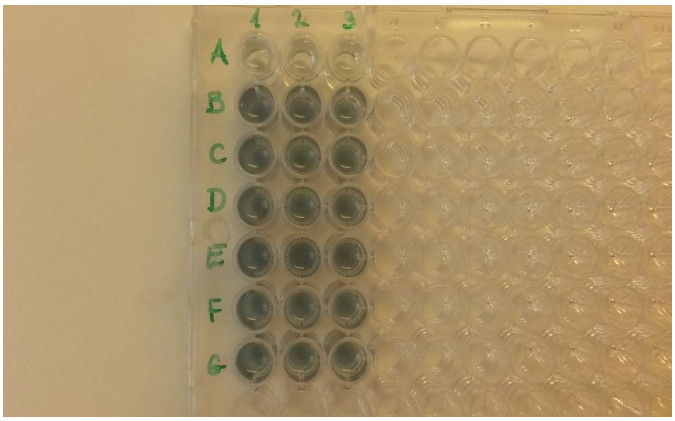


Figure 41. The microtiter plate with added all solutions in triplicate, before measurement of absorbance: Column A: All solutions without MgtA and extracts- negative control, Column B: All solutions with MgtA but without added extracts-positive control. Column C; D;E;F;G- added all solutions, MgtA and different extracts

Preparation of stock solutions: Exact amount of LsDCM and LsW were dissolved with DMSO and water to concentrations of 1.5 mg/mL; 1 mg/mL; 0.75 mg/mL; 0.5 mg/mL and 0.25 mg/mL;

10 Results and discussion

Lactarius scrobiculatus was extracted using a stepwise procedure with different solvents as shown in the flowing diagram Figure 25. The table 7.: An overview of the different fractions which were obtained.

Table 7: Overview of the crude extracts and fractions that were obtained and analysed

Name of the extract	**Explanation**
LsW	Water extract of *L.scrobiculatus*
LsA	Alkali extract of *L.scrobiculatus*
LsDCM	Dichloromethane extract of *L.scrobiculatus*
LsEt	Ethanol extract of *L.scrobiculatus*
LsK	Ethanol extract precipitate of *L. scrobiculat.*
LsW1a, LsW2a and LsW3a	Fractions 1, 2 and 3 after purification on Sephacryl S 500 column
LsW1b, LsW2b	Fractions 1, 2 and 3 after purification on Sephacryl S 500 HR column
LsW1c, LsW2c and LsW3c	Fractions 1, 2 and 3 after purification on Superdex 200 HR column
LsW2bE, LsW2cE	Fractions after treatment with enzyme endo-(1 → 3)-β-glucanase

10.1 Extraction yields of the *L. scrobiculatus*

The lyophilized fruiting bodies of *L.scrobiculatus* were milled and extracted first with dichloromethane and then with 96% ethanol by the Soxhlet extraction to remove lipids and low molecular compounds. The mushroom residue was further extracted with boiling water and aqueous basic solution (NaOH). Different extracts gave different yields.

The yield was calculated by the following formula:

yield (%) =[Ls(extracts)(g)/ Ls(g)] x 100%,

where the starting weight of Ls was 50 g.

The weights of dry extracts which were obtained using different solvents and yields were shown in Table 8.

Table 8. The yields of *L. scrobiculatus* extracts that were isolated by different solvents: **LsDCM**-dichloromethane extract, **LsEt**- ethanol extract, **LsK**- ethanol extract precipitate, obtained in Soxhlet extraction, **LsW**-water extract, **LsA**- alkali extract. Yield in % was calculated from starting the amount of *L.scrobiculatus* (50g)

	LsDCM	**LsEt**	**LsK**	**LsW**	**LsA**
Yield (g)	4.7	5.6	5.1	1.4	9.0
Yield (%)	9.4	11.2	10.2	2.8	18.0

The highest total yield was obtained from alkali extract LsA (18 %) and the lowest from LsW (2,8 %). There were obtained almost the same yield (about 10 %) of LsDCM, LsEt and LsK.

10.2 Monosaccharide composition in LsW extract

The monosaccharide composition of LsW was determined by methanolysis and GC.

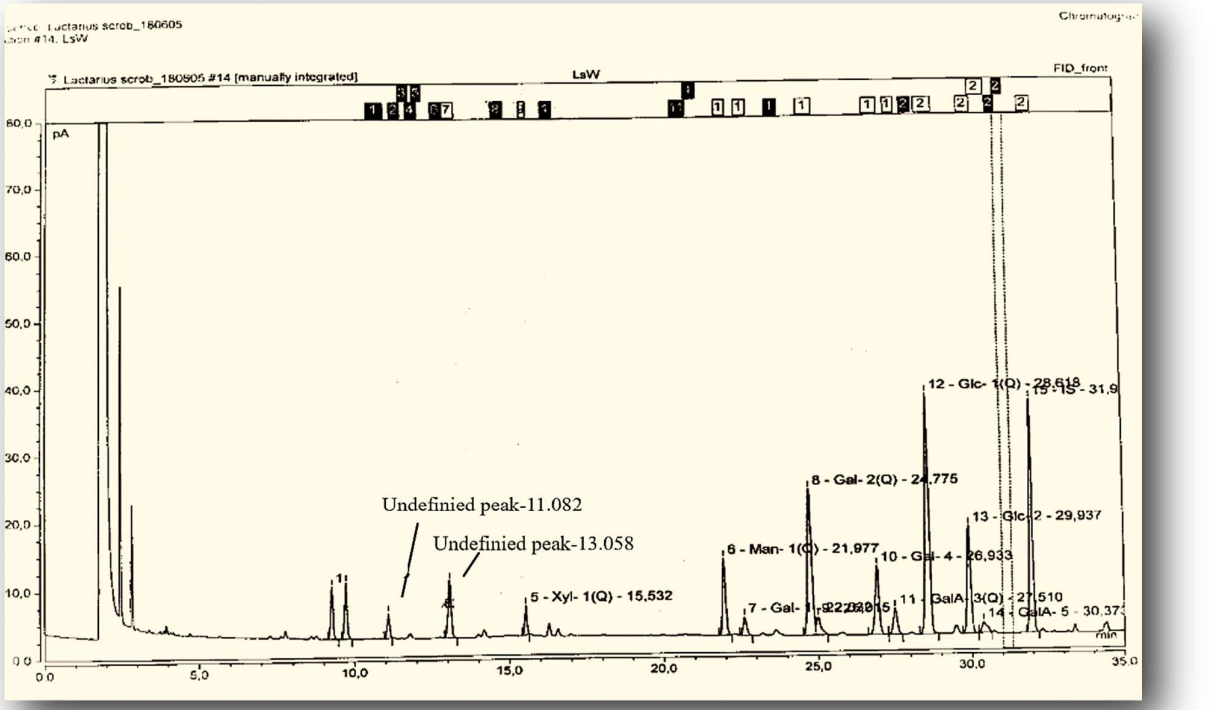


Figure 42. GC chromatogram of LsW after metanolysis and TMS derivatisation. Mannitol was used as internal standard (IS); The two unidentified peaks at Rt positions 11.082 and 13.058 min were registrered

Each monosaccharide of LsW gave two or more different peaks in the GC chromatogram which were used for quantification of monosaccharides, see Figure 42. Mannitol was used as internal standard. The methanolysis and GC of monosaccharides of internal standard were performed carefully in advance and they were used to integrate the obtained GC chromatogram of LsW. The peak size corresponds to its concentration (amount) in the sample. For example, the highest peak, came at Rt 28.618. After integration of GC chromatogram and comparing with the standards, the peak at Rt 29.937 overlapped with Rt of glucose standard and this high peak at Rt 28.618 also overlapped with glucose standards. They were used for quantification of glucose. Also, the baseline of the areal under curve was drawn manuel for both peaks and compared to areal under curven of glucose standard. In this way, glucose was obtained as a dominant monosaccharide in water extract LsW. The identification of each monosaccharides was performed comparing with the standard. All the peaks in the chromatogram of LsW were identified except the two peaks at retention time 11,082 and 13,058 min. The water extract

(LsW) contained mainly glucose (38 % of total carbohydrate content). Furthermore, the content of galactose was 32 %, glucuronic acid 10%. In addition, small amounts of xylose (3%) and mannose (8%) were found (see Table 9). According to methanolysis data, The total amount of carbohydrates was 55.3 % in the crude water extract (LsW) according to methanolysis data.

Table 9: The monosaccharide composition of the crude water extract **LsW,** determined by methanolysis and GC. The values are given in percentage (%) relative to total carbohydrate content.

Sample	LsW
Unidentified monosaccharide	9.6
Xylose	2.9
Mannose	7.6
Galactose	31.7
Glucose	37.9
Galacturonic acid	10.4

The types of glycosidic linkages between the monosaccharides was determined by methylation analysis. According to the retention time in the GC chromatogram and m/z of fragments in the mass spectra, the types of linkages were determined. The amount of each binding type is calculated from the area under the curve (AUC) for each peak in the chromatogram relative to the total AUC of certain monosaccharides in each sample. This ratio was multiplied by the amount of each monosaccharide found after methanolysis and GC.

Table 10: Semi quantitative linkage distribution of crude extract **LsW** and determined by methylation and GC-MS. The values are given in percentage (%) relative to total carbohydrate content

Sample:	LsW
6-deoxy hexose	8.6
T-Xyl*p*	0.6
(1 → 2)-Xyl*p*	2.0
T- Man*p*	4.5
(1 → 3,6)-Manp	2.3
T-Glc*p*	11.8
(1 → 3)-Glc*p*	14.6
(1 → 6)-Glc*p*	2.2
(1 → 3,6)-Glcp	5.3
T- Gal*p*	0.3
(1 → 6)-Gal*p*	16.1
(1 → 2,6)-Gal*p*	12.0

According to methylation data, see Table 10, it may be suggested that (1 → 6)-linked Gal*p*

(16.1%), (1 → 2,6)-linked Gal*p* (12 %) and T-Gal*p* (0.3%) might be part of a one type polysaccharide. Furthermore, the crude water extract LsW contains high amount of (1 → 3)-linked Glc*p* (14.6%), T-Glc*p* (11.8 %), (1→3,6)-linked Glc*p* (5.3%) and (1→ 6)-Glc*p* (16.1%).. This indicates presence of glucan in extract, which is excepted to find in mushrooms (Synytsya & Novák, 2013). Methylation analysis revealed that all monosaccharides were in the pyranose form (*p*).

Based on the results from the GC chromatogram, where two unidentified peaks appeared, and methylation and GC-MS analysis, it was attempted to identify the nature of this monosaccharide.

10.3 Determination of the structure of the unidentified monosaccharide

The monosaccharides of internal standard (mannitol) were analysed carefully in advance and this method was used for integration of GC chromatogram. Both retention times and mass spectral data were required for identification of the monosaccharide. There were registered two unidentified peaks in GC chromatogram at Rt 11.082 and 13.058 min. They could not be identified by integration of the monosaccharides of internal standard. The Rt of unidentified peaks were registered in region of Rt of L-fucose standard and eventual L-rhamnose, but they did not overlap with peaks of these standards. In order to find identity of two unknown peaks, the methylation and GC-MS were performed. Partially methylated alditol acetates, released from the partially methylated sugars in methylation process, were separated, identified and quantified by GC-MS. GC-MS registered peak at 13.7 min and masses of primary fragments (m/z) were found. The masses of primary fragments which were found in GC-MS chromatogram were: 118,131,162 and 175 (see Figure 43).

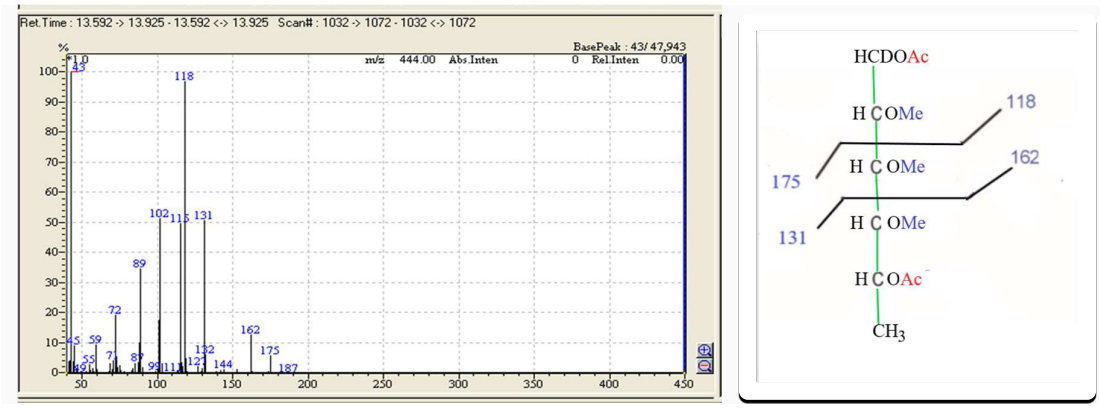


Figure 43: MS spectrum and structure of unidentified 6-deoxy-hexose with following obtained primary mass fragments: 118, 131, 162 and 175

Based on the obtained masses, characteristic for primary fragmentation in the MS spectrum, the possible monosaccharide and its linkage positions was created. The free hydroxyl groups which are not included in the glycosidic linkage were methylated and assigned as Me. The OH groups which were included in the glycosidic linkage were acetylated and assigned as Ac (Figure 45).

After drawing the primary fragments from mass spectrum, the primary fragment 118, which is characteristic for monosaccharides was found. It contained the anomeric C-atom (C1) with deuterium atom and one acetylated OH group, bonded to C1 and one methylated OH group at C2 atom (see Figure 45). The next primary fragment which was found, was fragment 162. It contained anomeric C atom (C1) with deuterium atom to which was bonded one acetylated OH group and C2 and C3 atoms with two methylated OH-groups. The fragments 175 and 131 which were found, had lower mass than fragments which are characteristics for hexose (for example in hexose, the fragment 161 are characteristics instead for 131). Therefore, it was drawn -CH_3 at position C6 instead of -CH_2OH and 6-deoxy hexose was obtained. The fragment 175 contained 2C-atoms with 2 methylated OH groups and 1C atom with acetylated OH group and one -CH_3 group (see Figure 45). The fragment 131 contained methyl group, one C-atom with acetylated OH group and one C-atom with methylated -OH group. 2 acetylated OH groups at C1 and C5 showed that glycosidic bond was present and that monosaccharide was in pyranose form. On this way, 6-deoxy hexose was determined.

In order to identify 6-deoxy hexose, the relative Rt of two peaks of 6-deoxy hexose were compared to the available standards. The two standards: 6-deoxy D-glucose and D-fucose were subjected to methanolysis and GC analysis. Determination of L-rhamnose and L-fucose standard were performed previously. The relative retention times of the two unidentified peaks were compared to the relative retention times of the standards: 6-deoxy-D glucose and D-fucose, L-rhamnose and L-fucose in order to identify unknown 6-deoxy-hexose (see table 11).

Table 11. The relative retention times of the different standards, according to methanolysis and GC, compared to the relative Rt of the two unidentified peaks in LsW

	Relative Rt-peak 1	**Relative Rt-peak 2**
6-deoxy-hexose	0.35	0,41
L-Rhamnose	0.38	0.40
L-fucose	0.41	0.46
D-Fucose	0.43	0.46
6-deoxy-D-glucose	0.55	0.57

The relative retention times of two unidentified peaks do not overlap the standards that were analysed. So, 6-deoxy-hexose from LsW is different type of monosaccharide from standards we compared to.

10.4 Estimation of the molecular weight distribution of LsW

Polysaccharides are polydisperse molecules. The molecular weight distribution of LsW was determined by SEC HPLC on a TSK Gel G5000 PWxl column with refractive index detection. This is an analytical technique that is useful for the study of polysaccharides. The retention time of a fraction provides a measure of the molecular size. The resulting chromatogram represents a molecular size distribution.

SEC-HPLC requires calibration using standards of known molecular weight. Fraction standards of known Mw such as pullulan standards are often used to calibrate the column. Different molecular population of mushroom extracts (LsW, LsA) are estimated using a standard calibration curve with pullulan standards whose peaks best overlap with those obtained for the samples.

The column separates pullulans in the region 5.8-853 kDa and they were used as a standard (see Figure 44).

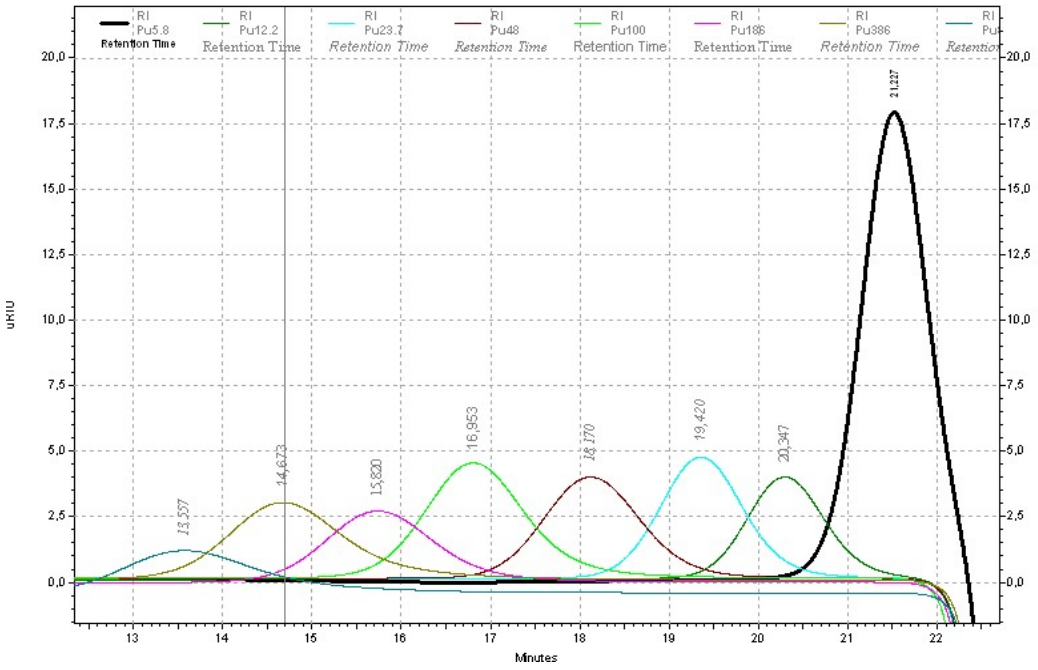


Figure 44. HPLC chromatogram of pullulan standards: Mp: 5.8 kDa, 12.2 kDa, 23,7 kDa, 48 kDa, 100 kDa,186 kDa, 386 kDa and 853 kDa. after analysis on the TSK-Gel® G5000PW$_{XL}$ column

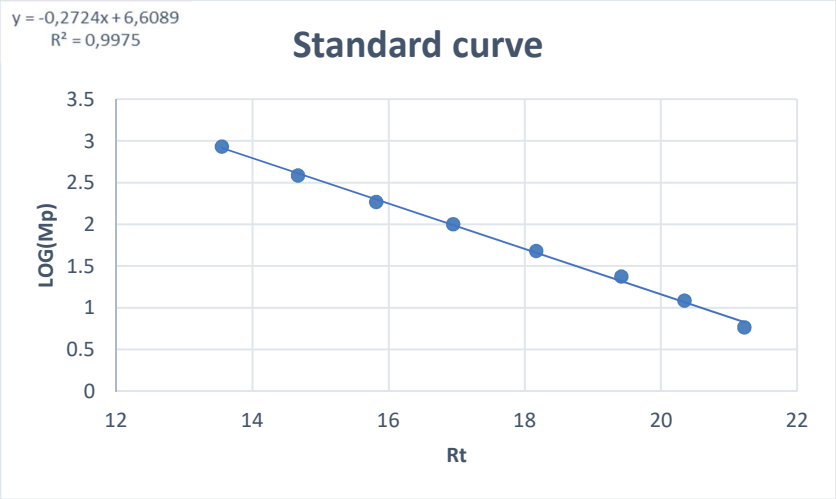


Figure 45. The standard curve for pullulan standards that were analysed on a TSK-Gel® G5000PW$_{XL}$ column by HPLC

The correlation coefficient for pullulan standards was close to 1, R^2 = 0,9975. This indicated that there was a good linear relation between Mw of pullulan standards and their Rt.

The calibration curve of log Mp of standards is plotted and used for determination of Mp of polysaccharides. The retention times of the different fractions of LsW were read from the elution profile and Mp – peak molecular weight distribution was calculated using formula:

y= - 0.2724x+6.6089

y= Ln_Mp, x= Rt (see Figure 45)

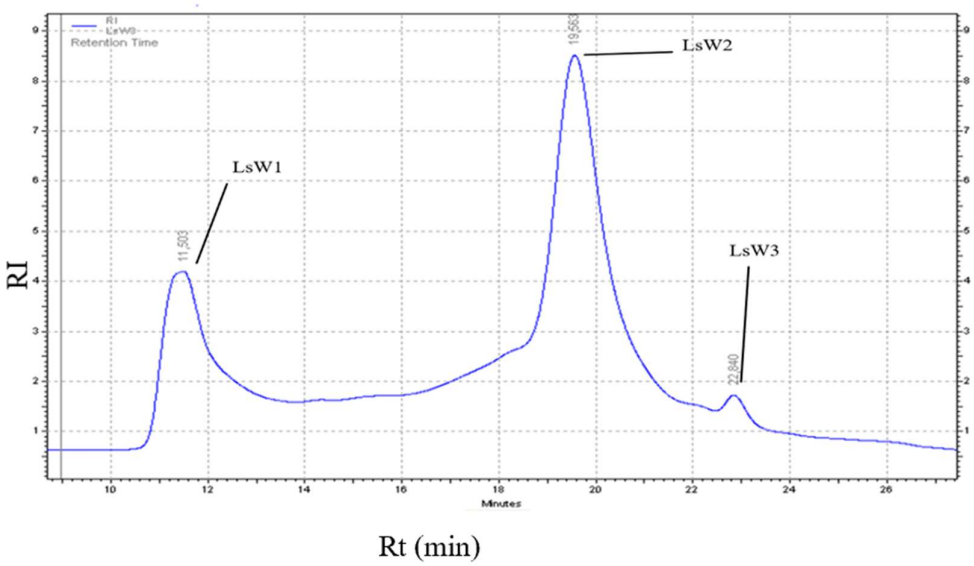


Figure 46. SEC-HPLC chromatogram after analysis on the TSK-Gel® G5000PW$_{XL}$ column: **LsW1**: Rt= 11.503, Mp=2985.4 kDa; **LsW2**: Rt=19.563, Mp= 19kDa; **LsW3**: Rt=22.840, Mp=2.4 kDa

Table 12: Estimated peak molecular weight (Mp) of the fractions LsW1, LsW2 and LsW3 compared to pullulan standards

LsW sample	Mp (kDa)
LsW1	2985.4
LsW2	19.0
LsW3	2.4

According to the peaks on the chromatogram, the water extract of *L.scrobiculatus* (LsW) contained three polysaccharide fractions with different molecular weights (see table 12). The obtained peaks were sharp and symmetrical (see Figure 46). The first molecular weight population at 11.5 min, represented the highest molecular weight fraction, Mp=2985.4 kDa, LsW1.The second came at 19.6 min, Mp=19 kDa, represented low molecular weight population, LsW2. The third population came at 22.8 min, represented the very low molecular weight fraction, Mp=2.4 kDa, LsW3 and might not be reproducible. The first molecular weight population is outside of the pullulans standard curve and therefore the calculated value is quite uncertain. The figure also shows that LsW1 came into void volume and there might be aggregates.
The results showed that LsW contained fractions with the molecular weight range from 2985,4 kDa to 2.4 kDa which may indicate that LsW contains a mixture of several polysaccharides. In general, linear polysaccharides exhibit shorter retention times than branched polysaccharides, probably because of their larger hydrodynamic volume. The crude water extract LsW contains three molecular weight populations. Therefore, it was interesting to find whether the different fractions contain the same or possible different types of polysaccharides. In order to find out it was necessary to perform a preparative fractionation and structural analysis on the obtained fractions. LsW was further purified by Sephacryl 500, HR Sephacryl 500 and Superdex 200 column.

10.5 Preparative SEC chromatography

Preparative SEC chromatography is suitable for neutral and anionic polysaccharides and separates these polymers based on their molecular weight and hydrodynamic volume. It was necessary to repeat the elution several times to collect enough fractions for further analysis. Three different gel filtration columns were used to obtain the best separated fractions: Sephacryl 500, HR Sephacryl 500 and Superdex 200 columns or eventual to find the most optimal column. The elution profiles for LsW showed almost the same pattern in each repetition with the same column. According to the obtained chromatograms, three different fractions were collected and combined.

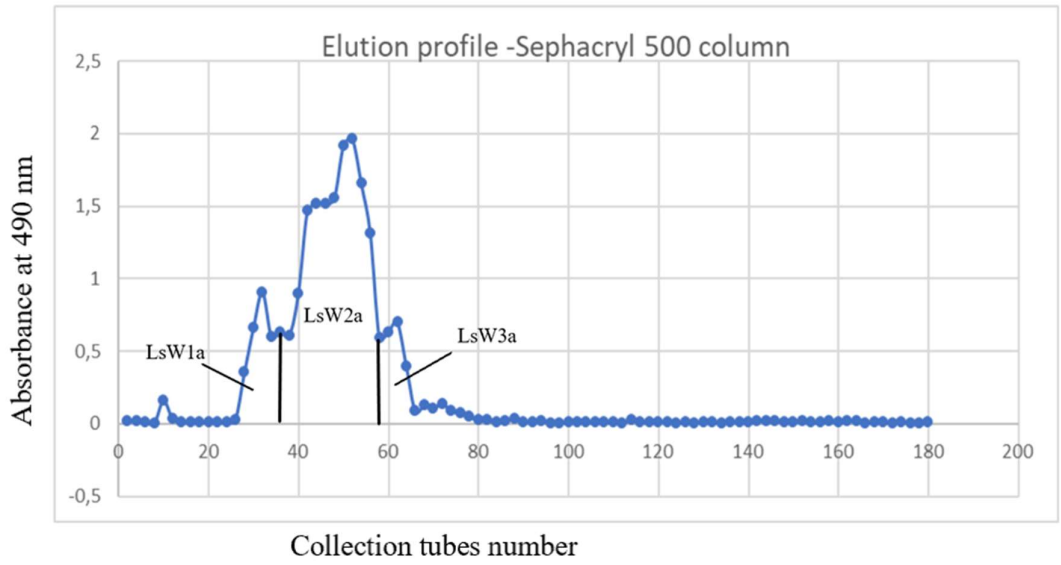


Figure 47. Elution profile after fractionation on **a Sephacryl S 500 gel filtration column**. Fractions: 26-36 (fraction 1), 38-58 (fraction 2) and 60-80 (fraction 3) were pooled and designated LsW1a, LsW2a and LsW3a, respectively. The elution profile was obtained using the phenol-sulphuric acid test.

The sample LsW was purified by Sephacryl S 500 column. Fractions gave elution profile after fractionation (see Figure 47). The phenol-sulphuric acid test was used to give carbohydrate profile of the pure collection tubes. The curves were obtained by plotting the fractions number tubes and absorbance, measured on 490 nm. The combined fractions, named LsW1a, LsW2a and LsW3a, were further subjected to GC and GC-MS analysis.

The crude extract LsW was then purified by Hi prep Sephacryl S 500 HR. The three different fractions, named LsW1b, LsW2b and LsW3b were collected and combined based on the elution profile (see Figure 48).

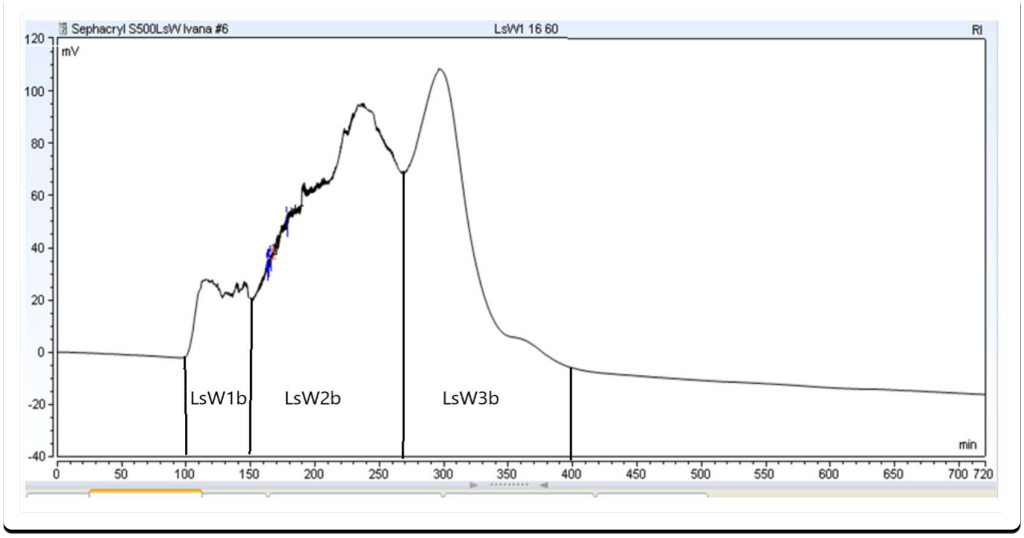


Figure 48. Elution profile of LsW after gel filtration on **Sephacryl S-500 HR**. The chromatogram shows the three different fractions which were combined and collected: fraction 1 (LsW1b): 100-150, fraction 2 (LsW2b): 151-270 and fraction 3 (LsW3b): 271-400. The elution was monitored by refractive detector (RI detector).

The LsW extract was purified by Superdex 200 HR column. There were obtained three different fractions: LsW1c, LsW2c and LsW3c. They were collected and combined according to the chromatogram (see Figure 49).

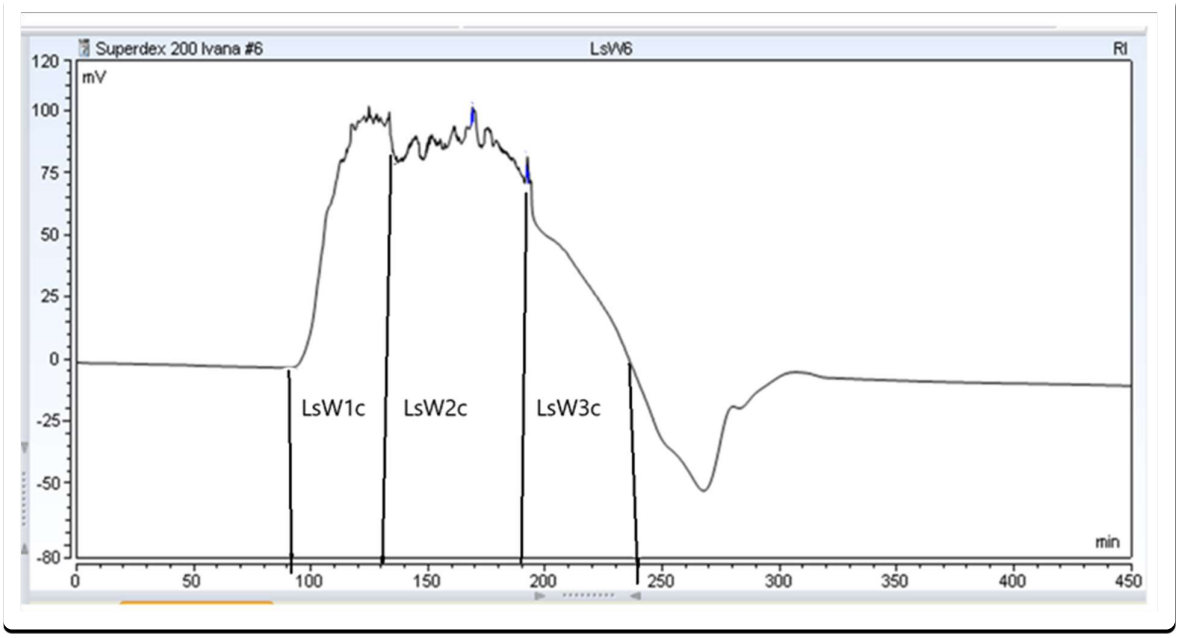


Figure 49. Elution profile of LsW after gel filtration on **Superdex 200 HR column**. There were obtained three

different fractions: LsW1c: 90-130; LsW2c: 131-190; LsW3c: 191-240. The elution profile was obtained by monitoring with refractive detector (RI detector).

Table 13: The yields of the three fractions obtained after purification with three different gel filtration columns

	Fraction 1 (mg)	**Yield (%)**	**Fraction 2 (mg)**	**Yield (%)**	**Fraction 3 (mg)**	**Yield (%)**
Sephacryl S-500 (51.2 mg applied)	5.3	10.4	23.5	45.9	3.3	6.5
Sephacryl S-500 HR (24.2 mg applied)	1.5	6.2	8.2	33.9	0.2	0.8
Superdex 200 HR (61.0 mg applied)	7.9	13.0	14.7	24.1	4.6	7,5

The total yields from different columns, Sephacryl S 500, Sephacryl S 500 HR and Superdex 200 HR were 63 %, 41% and 45 %, respectively. Sephacryl S 500 was packed at our lab previously, gave the highest yields of the fractions, except fraction 1. The highest yield of fraction 1 gave fractionation on third column, Superdex 200 HR. The second column showed the poorest yield of fraction 1 and 3. The best yields were obtained after fractionation on the first column (see Table 13).

10.6 The structural analysis of obtained SEC-fractions

The nine polysaccharide fractions: LsW1a, LsW2a, LsW3a, LsW1b, LsW2b, LsW3b, LsW1c, LsW2c and LsW3c were isolated from the crude water-soluble extract of *L. scrobiculatus and submitted to further analysis.*

The monosaccharide compositions of the obtained fractions were determined by methanolysis and GC. The ring size and glycosidic linkage position of monosaccharide units was established by methylation and GC-MS analysis.

10.6.1 SEC preparative chromatography with Sephacryl S 500 filtration column- quantitative and qualitative determination of the structure

Sephacryl S-500 gel filtration column was used for purification of the water extract and three fractions were obtained: **LsW1a, LsW2a** and **LsW3a**. According to the methanolysis data, the total amounts of the carbohydrates in the fractions LsW1a, LsW2a and LsW3a were 26.9%, 71.5% and 54.8%, respectively.

Table 14: The monosaccharide composition of polysaccharide fractions **LsW1a**, **LsW2a** and **LsW3a** after gel filtration on the Sephacryl 500 filtration column and GC analysis. The values are given in percentage (%) relative to total carbohydrate content.

Sample	LsW1a	LsW2a	LsW3a
6-deoxy-hexose	3.9	9.4	8.8
Xylose	1.7	2.3	1.6
Mannose	4.8	9.6	3.3
Galactose	14.3	25.6	28.4
Glucose	69.8	34.2	36.2
Glucuronic acid	-	3.3	1.0
Galacturonic acid	5.3	7.9	18.2

All fractions contained the same type monosaccharides but not in the same amount. LsW1a fraction contained higher amount of glucose (69.8%) than LsW2a (34.2%) and LsW3a (36.2%) (Table 14). Opposite to this, the amount of galactose was highest in fraction LsW3a. The amounts of 6-deoxy-hexose were almost the same in fractions 1 and 2. The mannose content was 9.6 % in LsW2a and 4.8 % in LsW1a. The xylose was found in small amount. The highest amount of galacturonic acid was in fraction 3, LsW3a.

Structural analysis was performed on these fractions by methylation and GC-MS.

Table 15: Semi quantitative linkage distribution of fractions **LsW1a, LsW2a** and **LsW3a,** obtained after purification on the Sephacryl 500 filtration column and determined by methylation and GC-MS. The values are given in percentage (%) relative to total carbohydrate content.

Sample:	LsW1a	LsW2a	LsW3a
T-6-deoxy hexose (*p*)	3.7	8.6	7.2
T-Xyl*p*	1.0	0.9	0.6
(1 → 3)- Xyl*p*	0.6	-	-
(1 → 2)- Xyl*p*	-	1.2	0.7
(1 → 3)- Man*p*	4.5	7.1	2.0
(1→ 3,6)-Manp	-	1.7	0.7
T-Glc*p*	21.6	10.6	11.1
(1 → 3)-Glc*p*	31.3	12.6	7.6

(1 → 4)- Glc*p*	0.5	1.1	2.7
(1 → 6)-Glc*p*	0.4	2.8	6.4
(1→ 3,6)-Glcp	12.1	4.3	1.7
T-Gal*p*	1.1	0.5	0.5
(1 → 6)- Gal*p*	7.4	14.0	14.5
(1 → 2,6)- Gal*p*	5.0	8.9	8.1

The results from GC-MS analyse indicate that fraction LsW1a has higher amount of (1 → 3)-linked Glc*p*, T-Glc*p* and (1 → 3,6)-linked Glc*p* than fractions LsW2a and LsW3a. The amount of T-Glc*p* is high, probably because of the possible overlaping of T-Man*p* and T-Glc*p* peaks, which was found in GC-MS chromatogram. But it was difficult to separate them. LsW2a and LsW3a contained higher amount of (1 → 6)-Gal*p* and (1 → 2,6)-Gal*p* than LsW1a (see Table 15). (1 → 4)-linked Glc*p* has not been observed before in the water extract, but it was found here in small amount. Methylation analysis revealed that all monosaccharides were in the pyranose form (*p*).

10.6.2 SEC preparative chromatography with a Sephacryl S 500 HR column-quantitative and qualitative determination of the structure

After filtration of the crude extract LsW through a Sephacryl S 500 HR column, LsW1b and LsW2b fractions were obtained. Fraction 3 was obtained as a trace amounts and therefore could not be analysed further. The possible reason could be because the column had a small capacity. The total amounts of the carbohydrates in the fractions LsW1b and LsW2b were 20.4% and 62.3%, respectively. Fraction LsW1b consisted mainly of glucose (82.3%) and galactose (7.4%). The other monosaccharides mannose, xylose and 6-deoxy hexose were in small amounts. Content of glucose was higher in LsW1b than in LsW2b, while galactose was in higher amount in fraction 2 than in fraction 1. The amount of 6-deoxy-hexose, mannose and glucuronic acid were higher in LsW2b than in other two fractions (see Table 16).

Table 16: The monosaccharide composition of polysaccharide fractions **LsW1b**, and **LsW2b** after gel filtration on the Sephacryl 500 HR column and determined by methanolysis. The values are given in percentage (%) relative to total carbohydrate content.

Sample	LsW1b	LsW2b
6-deoxy-hexose	2.0	8.3
Xylose	1.2	3.3
Mannose	3.3	7.6
Galactose	7.4	35.5
Glucose	82.3	36.1
Galacturonic acid	3.3	7.7

The polysaccharide fractions: LsW1b and LsW2b were methylated by method 6.4.2 and the partially methylated alditol acetates were analysed by GC-MS in order to determine the structure of polysaccharides.

Table 17: Semi quantitative linkage distribution of fractions **LsW1b** and **LsW2b,** obtained after purification on the Sephacryl S 500 HR column and determined by methylation and GC-MS. The values are given in percentage (%) relative to total carbohydrate content.

Sample:	**LsW1b**	**LsW2b**
T-6-deoxy hexose (*p*)	1.9	7.7
T-Xyl*p*	0.2	1.1
(1 → 3)- Xyl*p*	0.1	-
(1 → 2)- Xyl*p*	0.9	1.9
(1 → 3)-Man*p*	3.2	5.3
(1→3,6)- Manp	-	1.7
T-Glc*p*	49.8	10.5
(1 → 3) -Glc*p*	19.5	14.0
(1 → 4)-Glc*p*	1.9	1.4
(1 → 6)-Glc*p*	2.1	2.7
(1 → 3,6) Glcp	6.3	4.6
T-Gal*p*	3.8	0.3
(1 → 6)- Gal*p*	2.3	18.7
(1 → 2,6)-Gal*p*	1.1	13.8

The results after methylation and GC-MS analysis indicates that fraction LsW1b showed a higher amount of (1 → 3)-linked Glc*p* (19.5%) and (1 → 3,6)-linked Glc*p* (6.3%) compared to LsW2b, 14.0 % and 4.6%, respectively (Table 17). The reason of appearance of high percentage of T-Glc*p* might be incompletely methylation. Methylation analysis revealed that all monosaccharides were in the pyranose form (*p*).

The amounts of (1 → 6)-linked Gal*p* (18.7%) and (1 → 2,6)-linked Gal*p* (13.8%) were higher in LsW2b than in fraction LsW1b, 2.3% and 1.1%, respectively. This may indicate that glucans are present in higher amounts in fraction 1 than in fraction 2 and galactans in fraction 2 than in fraction1. (1 → 4)-linked Glc*p* was not found before in the crude water extract, but it was found here in small amounts in both fractions: LsW1b (1.9%) and LsW2b (1.4 %).

10.6.3 SEC preparative chromatography with a Superdex 200 HR column quantitative and qualitative determination of the structure

The water extract was fractionated by gel filtration with Superdex 200 HR column and separated into three fractions: LsW1c, LsW2c and LsW3c. The total amounts of the carbohydrates in the fractions LsW1c, LsW2c and LsW3c were respectively 71.9%, 82.2% and 52%.

LsW1c contained high amounts of glucose (55.8%) and galactose (20.9%) and small amounts of galacturonic acid (10.1%), mannose (6.5%), 6-deoxy-hexose (4.4%) and xylose (2.2%). Fraction LsW2c contained almost the same amount of glucose (37.6%) and galactose (33%). It contained more 6-deoxy-hexose (9.7%) than LsW1c, but less than fraction 3- LsW3c (11.6%). The difference between fraction 2 and 3 in content of galacturonic acid was not significant. The monosaccharides mannose and xylose were lower in fraction 3 than in fraction 1 and 2 (see Table 18).

Table 18: The monosaccharide composition of polysaccharide fractions **LsW1c**, **LsW2c** and **LsW3c** after gel filtration on the Superdex 200 HR column and determined by methanolysis. The values are given in percentage (%) relative to total carbohydrate content.

Sample:	**LsW1c**	**LsW2c**	**LsW3c**
6-deoxy-hexose	4.4	9.7	11.6
Xylose	2.2	2.5	1.7
Mannose	6.5	6.1	4.0
Galactose	20.9	33.0	32.8
Glucose	55.8	37.6	35.9
Galacturonic acid	10.1	11.2	13.9

The polysaccharide fractions were further subjected to methylation and then analysed by GC-MS (see Table 19).

Table 19: Semi quantitative linkage distribution of fractions **LsW1c, LsW2c** and **LsW3c**, obtained after purification on the Superdex 200 HR column and determined by methylation and GC-MS. The values are given in percentage (%) relative to total carbohydrate content.

Sample:	**LsW1c**	**LsW2c**	**LsW3c**
T-6-deoxy hexose	4.0	8.6	10.0
T-Xyl*p*	0.6	0.9	0.6
(1 → 3)-Xyl*p*	0.2	-	-
(1 → 2)-Xyl*p*	1.2	1.3	0.9
(1 → 3)-Man*p*	5.8	5.4	2.4
(1→3,6)-Manp	-	-	1.0
T-Glc*p*	13.6	14.0	9.5

(1 → 3) -Glc*p*	24.1	12.7	10.0
(1 → 4)- Glc*p*	1.3	-	2.7
(1 → 6)- Glc*p*	1.0	3.4	4.4
(1 → 3,6)-Glcp	10.3	3.2	4.3
T-Gal*p*	0.3	-	0.4
(1 → 6)-Gal*p*	10.4	17.4	11.7
(1 → 2,6)-Gal*p*	8.1	11.9	16.1

The GC-MS results indicates that (1 → 3)-linked Glc*p* is in higher amount in LsW1c (24.1%) than in fraction LsW2c (12.7%) and fraction LsW3c (10.0%). Also, (1 → 3,6)-linked Glc*p* is higher in fraction LsW1c (10.3%) than in fractions LsW2c (3.2%) and LsW3c (4.3%). Methylation analysis revealed that all monosaccharides were in the pyranose form (*p*).

(1 → 4)-linked Glc*p* has not been observed before in the crude water extract LsW, but it was found here in small amounts, only in two fractions LsW1c and LsW3c.

10.6.4 Enzymatic hydrolysis with endo-(1 → 3)-β-glucanase

In fractions LsW2b and LsW2c were found more galactose than glucose by methanolysis and also (1 → 6)-Gal*p* and (1 → 2,6)-Gal*p* by methylation. Therefore, it was proposed that galactan was present in these fractions. Since, the galactan was not isolated by SEC preparative, it was tried to isolate with another method. Therefore, the two fractions LsW2b and LsW2c were subjected to enzyme degradation. The purpose of this method was to remove a (1 → 3)- Glc*p* residues and obtain the purer fractions with galactose. The enzyme endo-(1 → 3)-β-glucanase catalyses hydrolysis of (1→3)-β-Glc*p* linkages. After the hydrolysis with enzyme and methanolysis of fraction LsW2b, it was found that the amount of glucose was decreased (from 36.1% to 24.4%) and amount of galactose (from 35.5% to 43.4%) and galacturonic acid (from 7.7 to 11.6%) were increased (see Table 20).

Table 20: The monosaccharide composition of polysaccharide fraction before **LsW2b, LsW2c** and after enzymatic treatment, **LsW2bE** and **LsW2cE** determined by methanolysis. The values are given in percentage (%) relative to total carbohydrate content.

Sample:	**LsW2b**	**LsW2bE**	**LsW2c**	**LsW2cE**
6-deoxy hexose	8.3	9.4	9.7	8.0
Xylose	3.3	3.3	2.5	2.8
Mannose	7.6	7.9	6.1	7.6
Galactose	35.5	43.4	33.0	41.4
Glucose	36.1	24.4	37.6	29.4
Galacturonic acid	7.7	11.6	11.2	10.7

After enzymatic hydrolysis and methanolysis of fraction LsW2c, the amount of the glucose was decreased (from 37.6% to 29.4%) whereas the amount of galactose was increased (from 33% to 41.4%) (see Table 20).

Structural analysis was performed by methylation and GC-MS. Linkage determination showed (Table 21) that there was a relative increase of (1 → 6)-linked Gal*p* in LsW2bE fraction (from 18.7 to 22.1 %) compared to LsW2b, before treatment with the glucanase. Also, both T-Gal*p* and (1 → 2,6)-linked Gal*p* increased in fraction LsW2bE (from 13.8 to 16 %). The amount of glucose was decreased in hydrolysed fraction LsW2bE. The amount of (1 → 3)-linked Glc*p* was decreased from 14.0 to 6.2 %; and (1→ 3,6)-linked Glc*p* from 4.6 to 2.6 %, compared to fraction LsW2b. The amount of galacturonic acid was increased (from 7.7 to 11.6%) after enzymatic degradation which can indicate that galacturonic acid is a part of the main chain or branching of galactans.

Table 21: Semi quantitative linkage distribution of **LsW2b, LsW2c** and after hydrolysis **LsW2bE**, **LsW2cE** determined by reduction, methylation and GC-MS. The values are given in percentage (%) relative to total carbohydrate content.

Sample:	**LsW2b**	**LsW2bE**	**LsW2c**	**LsW2cE**
T-6-deoxy hexose	7.7	8.3	8.6	7.1
T-Xyl*p*	1.1	1.2	0.9	1.1
(1 → 2)-Xyl*p*	1.9	1.7	1.3	1.4
(1 → 3)-Man*p*	5.3	7.9	5.4	3.5
(1→3,6)- Manp	1.7	-	-	3.3
T-Glc*p*	10.5	9.1	14.0	10.0
(1 → 3)-Glc*p*	14.0	6.2	12.7	7.5
(1 → 4)- Glc*p*	1.4	-	-	-
(1 → 6)- Glc*p*	2.7	3.8	3.4	5.1
(1 → 3,6)-Glc*p*	4.6	2.6	3.2	3.6
T-Gal*p*	0.3	0.4	-	0.4
(1 → 6)- Gal*p*	18.7	22.1	17.4	20.8
(1 → 2,6)-Gal*p*	13.8	16.0	11.9	15.9

In LsW2cE, (1 → 3)-linked Glc*p* was decreased (from 12.7 to 7.5 %) and T-Glc*p* (from 14.0 to 10.0 %), compared to LsW2c fraction. Furthermore, it was observed an increase in amount of (1 → 6)-linked Gal*p* (from 17.4 to 20.8%), (1 → 2,6)-linked Gal*p* (from 11.9 to 15.9 %), compared to LsW2c (see Table 21). There were obtained more purified fractions, with more galactose and less glucose.

10.7 Alkali extract (LsA)

10.7.1 Monosaccharide composition of polysaccharides in LsA

GC analysis of alkali extract LsA was performed after methanolysis and TMS derivatization. According to methylation analysis, LsA was contained mainly glucose (79%). Other monosaccharides appeared in small amounts: mannose (9.1%), xylose (6.7%), galactose (2.2%) and 6-deoxy-hexose (2.0%) (see Table 22). The total amount of the carbohydrates in the alkali extract LsA was 68.3%.

Table 22: Monosaccharide composition of fraction LsA. The values are given in percentage (%) relative to total carbohydrate content.

Sample	LsA
6-deoxy-hexose	2.0
Xylose	6.7
Mannose	9.1
Galactose	2.2
Glucose	79.0
Glucuronic acid	1
Galacturonic acid	1

After methanolysis, the fractions were subjected to methylation and GC-MS analysis.

Table 23: Semi quantitative linkage distribution of fraction LsA by methylation and GC-MS. The values are given in percentage (%) relative to total carbohydrate content.

Sample:	LsA
T-6-deoxy hexose	2.0
T-Xyl*p*	2.9
(1 → 2)-Xyl*p*	3.6
(1 → 3)-Man*p*	6.9
(1→3,6)- Manp	1.9
T-Glc*p*	28.1
(1 → 3) -Glc*p*	18.4
(1 → 4)- Glc*p*	13.2
(1 → 6)- Glc*p*	10.6
(1 → 3,6)- Glcp	7.0
(1 → 6)- Gal*p*	1.5
(1 → 2,6)-Gal*p*	0.7

The GC-MS results (see Table 23) showed the occurrence of (1 → 3)-linked -D-Glc*p* (18.4%), (1 → 6)-linked -D-Glc*p* (10.6%) (1 → 3,6)-linked -D-Glc*p* (7.0%) and T- Glc*p* (28.1%) which are common monosaccharides found in mushrooms. The amount of (1 → 4) -linked -D-Glc*p* is 13.2 %. The presence of (1 → 4)-α D-glucans was supported by iodine-potassium iodide assay and methylation.

10.7.2 Estimation of the molecular weight distribution of LsA

HPLC chromatogram (see Figure 50) shows three very broad peaks of alkali extract with low intensity because of low solubility of LsA in water and possible forming of aggregates. At the same chromatogram are represented pullulan standard peaks after analysis on the TSK-column.
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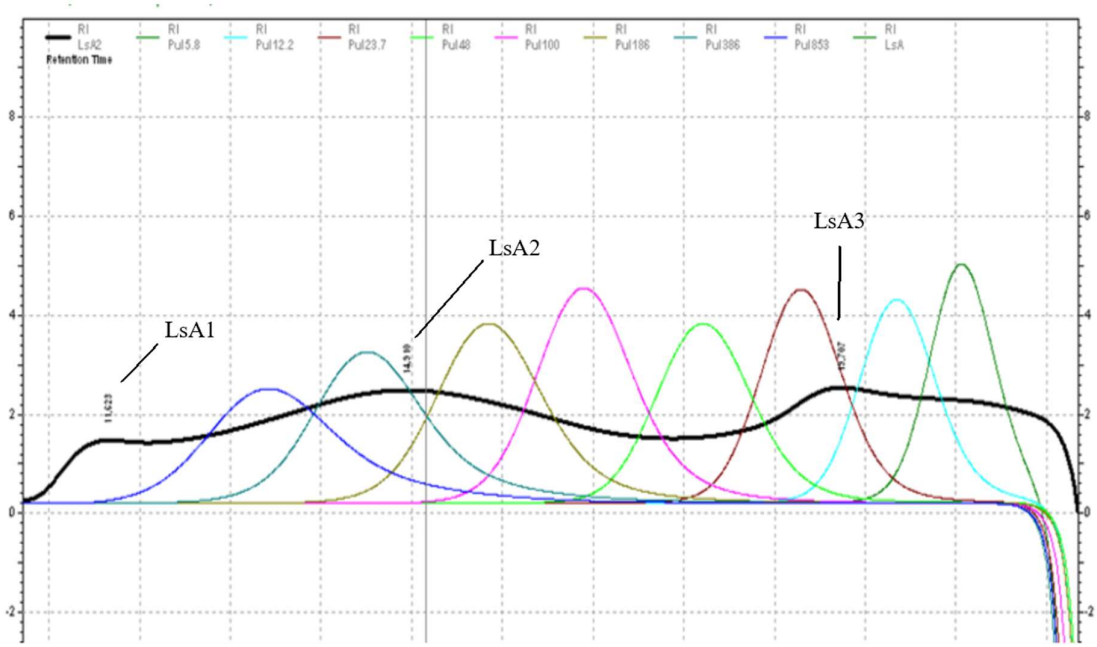

Figure 50. HPLC chromatogram of alkali extract LsA and pullulan standards after analysis on the TSK-Gel® G5000PW$_{XL}$ column: fraction LsA1, Rt= 11.623; LsA2, Rt=14.910; LsA3, Rt=19.707

Fractionation of LsA revealed three molecular weight populations. LsA1 had a peak molecular mass, Mp= 2772.0 kDa. LsA2 was smaller and had a wider peak with molar mass, Mp=352.7 kDa and LsA3 had a peak molecular mass, Mp= 17.4 kDa (see Table 24).

The polysaccharide fraction corresponding to the peak 1, consisted of the large macromolecules, which were eluted in the exclusion volume. The molecular weight of LsA1 (top 1) was outside of the standard curve and therefore was calculated with uncertainty.

Table 24: Peak molecular weight (Mp) of the fractions LsA1, LsA2 and LsA3 compared to pullulan standards

LsA sample	Mp (kDa)
Peak 1	2772.0
Peak 2	352.7
Peak 3	17.4

10.7.3 The presence of (1→4)-α-glucans

The water extract LsW and the alkali extract LsA were tested for presence of (1→4)-α-D-glucans (Method 5.2.3). The fractions were visually compared to the positive control (starch) and the negative control (water). LsA fraction had a positive reaction by the addition of iodine-potassium iodide reagent and gave a dark blue-coloured complex (see Figure 51, well A2). The amount of (1→4)-α-D-Glc*p* was 13.2 %, according to methylation analysis and GC-MS. Amylose and amylopectin in starch have different structures. The amylose forms a left-handed helical structure with six glucose units per turn and the number of turns in the amylose helix is necessary to provide the appearance of blue colour. The reagent form polyiodides which enter the (1→4)-α-D-Glc*p* helix forming a blue- black coloured complex. Therefore, the reaction indicated that (1→4)-D-Glc*p* residues had α- anomeric configuration (Tomasik & Schilling, 1998).

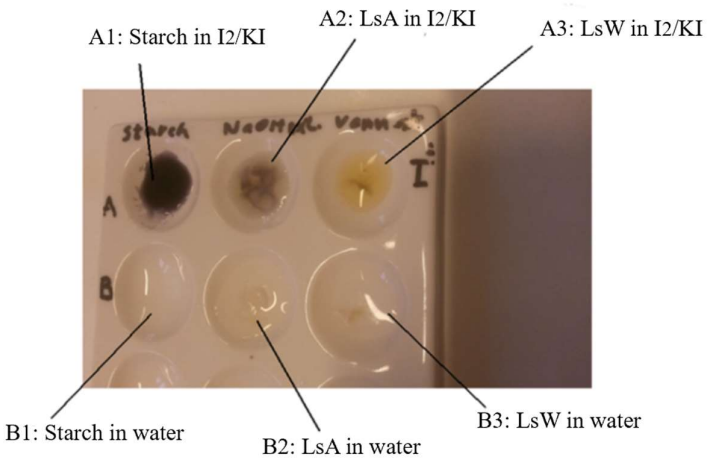

Figure 51. Row A: Iodine-potassium iodide (I_2/KI) was added in: A1: Starch – positive control A2: LsA A3: LsW; Row B1: Water was added in all well of row B: B1: water-negative control, B2: LsA B3: LsW

The result of the test confirmed the presence of (1→4)-α-D-glucans in both LsA and LsW. LsA contained a significant amount of (1→4)-α-D-glucans. LsW extract showed a little change of the colour and possible presence of (1→4)-α-D-glucans (see Figure 51, well A3).

10.8 Discussion about fractionation of LsW on three different columns

Polysaccharides are quite heterogenous group, which differ in primary structure, degree of polymerization (mono-, oligosaccharides), macromolecular characteristics (linear and branched structure) and linkage (α and β glycosidic linkage). So, the analysis of polysaccharides can be difficult to achieve because of the high molecular weight and polydispersity of the polymer chains.

The yield of the extracts depends mainly on the selected methods of extraction and variables such as temperature of extraction and chosen solvents. The water extract, LsW yielded 2.8 % while the alkali extract yielded 18 %. The LsW was further fractionated on three different columns Sephacryl S500, Sephacryl S500 HR and Superdex 200 HR columns.

The purification of polysaccharides with three different columns was carried out with different yields. The total yield was 63%, 41 % and 45%, respectively, after use of Sephacryl S 500, Sephacryl S 500 HR and Superdex 200 HR.

There were chosen both columns Sephacryl S 500 HR and Superdex 200 HR for purification because of their good product characteristics and high-resolution features. They are commercial products, with well-packed column. Sephacryl S 500 HR was chosen, because of its fast, high recovery separations and Superdex S 500 HR because of high resolution, a short run times and high recovery (see Table 25). Considering the properties of the columns, they should provide high yields. But, no one of the columns has showed ideal results. Only, the first column has showed the acceptable results of purification.

Table 25: Characteristics of the two high resolution columns

Sephacryl S-500 HR	Lower medium pressure system Macromolecule separation Product line covering wide fractionation range	**Superdex 200 HR**	Upper-medium pressure system High recovery High stability High selectivity

The fractionation range defines the range of molecular weights that have access to the pores to the matrix.

The reasons of the obtained lower yields than expected could be, for example, macromolecules of the sample could aggregate on the column. If the sample precipitates in a gel filtration column, column will be clogged, and the sample may be lost. Also, polysaccharides are so viscous relative to the eluent (NaCl) that it could cause a high loss of resolution. Resolution depends on the ratio of sample volume to column volume.

The capacity of the column means the quantity of the sample (its volume and concentration) which can be applied. Sample volumes of the total column volumes for Sephacryl S 500 HR and Superdex 200 were very low, 1-2% and both columns Sephacryl S-500 HR and Superdex 200 HR had the small capacity. The highest yield was obtained from fraction 2, from 24% (Superdex 200 HR) to 46 % (Sephacryl S 500).

The Sephacryl S 500 column had the highest capacity of the columns and largest sample volume that has to do with the largest capacity. Large sample volume requires large bead volume and large column. The porosity of Sephacryl S 500 column is controlled by dextran components and gave five types with different fractionation range. So, the Sephacryl S 500 column which was packed in our lab, Department of Pharmaceutical Chemistry, showed the best features, good separation and the highest yields of the obtained fractions except fraction 1. Total yield was 63 % and fractionation was good. The disadvantage of the column was probably that the column clogged after first elution. The reasons of clogging might be that the sample was too viscous, aggregates of macromolecules precipitated on the column or poorly packed column. Therefore, it was necessary to wash it with NaOH after use.

The obtained elution profiles of three columns showed three different fractions. But the profiles have not exhibited the clear differentiation between the peaks. This can imply that there were possible aggregation and inhomogeneity of the purified sample. High sample viscosity causes instability of the separation and an irregular flow pattern ("GE Healthcare," 2014). The fractions 2 and 3 (LsW2a and LsW3a), obtained on a Sephacryl S500 showed a clearer line of separation between the two fractions than what was obtained on Sephacryl S 500 HR and Superdex 200 HR. The elution profile of fractionation on column Superdex 200 HR showed the poorest differentiation between fractions LsW1c, LsW2c and LsW3c or the poorest resolution and lowest intensity of RI signal than the two others of the columns tested. There were not registered three different peaks (Figure 64). The signal intensity depended on concentration of the sample and the capacity of the column. Also, the elution profile after fractionation on Sephacryl S 500 column exhibited the stronger peak differentiation which was closer to the baseline, compared to the other two columns. The appearance of the elution profile on Sephacryl S 500 HR column was similar to the elution profile on Sephacryl S 500, but not exactly.

The yield of fraction 1 was almost the same after purification on Superdex 200 column (13%) than Sephacryl S 500 (10.4%). But, during the elution, the column Superdex 200 HR was probably clogged and gave significant lower yields in fraction 2 and 3. On the other side, the second column gave the poor yield in fraction 1 and 3 because of the small capacity and very viscous sample which was flown through the column with small capacity.

Comparing the obtained fractions after preparative SEC, the best fractionation was accomplished with Sephacryl 500 column. The fractions LsW2a and 3a contained higher amount of galactose and less glucose than fraction 1, LsW1a. SEC preparative on second column, Sephacryl 500 HR, gave only 2 fractions. LsW2b had also higher amount of galactose and less glucose than fraction LsW1b. The obtained fractions LsW2c and LsW3c showed no big difference in content of monosaccharides like fraction 1. For example, the amount of (1 → 6)-linked Gal*p* and (1 → 2,6)-linked Gal*p* showed insignificant difference between three fractions which indicated that separation on Superdex 200 column was not as well as with the first two columns.

10.9 Discussion of the structure of polysaccharides, present in the water extract, LsW and its fractions

Analytical SEC on a TSK-column revealed that the molecular weight of the first peak (Mp) was 2985.4 kDa, the second 19 kDa and the third was 2.4 kDa relative to the pullulan standards. The first molecular weight population was outside of the pullulans standard curve and therefore the calculated value is quite uncertain. The results also showed that LsW contained fractions with the molecular weight range from 2985,4 kDa to 2.4 kDa which may indicate that LsW contains a mixture of several polysaccharides. In general, linear polysaccharides exhibit shorter retention times than branched polysaccharides, probably because of their larger hydrodynamic volume. The figure also shows that LsW1 came into void volume and there might be aggregates.

Monosaccharide composition analysis of LsW indicated that LsW mainly composed of glucose and galactose with the same amount of unknown 6 deoxy-hexose and galacturonic acid, following mannose and a small amount of xylose. According to the literature, a (1 → 3)- linked-β-D-Glc*p* backbone substituted with single β-D-Glc*p* at position 6, is one of the most important common constituents of fungal cell wall (see Figure 52) (Dalonso et al., 2015) (Andrea Caroline Ruthes, Smiderle, & Iacomini, 2015). Besides β-glucan, (1 → 6)-linked-D-glucan and heteroglycans are probably presented (Wasser, 2002).

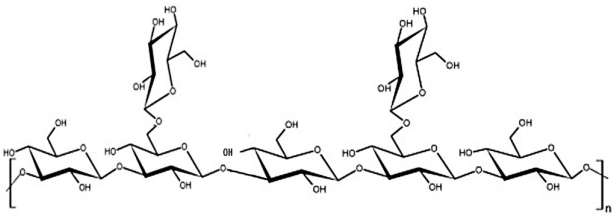

Figure 52: A possible β-glucan structure: (1 → 3)-linked β-D-Glc*p* in the main chain and substitutions at position 6, which was found in many mushrooms and probably in LsW

Methylation analysis revealed that LsW contained (1 → 3), (1 → 3,6), (1 → 6)- linked and terminal-D-Glc*p* in the ratio 15:5:2:12. According to the obtained results, (1 → 3)-linked -Glc*p* is a dominant sugar monomer in LsW. This indicates that the main chain might be composed of (1 → 3)-linked -Glc*p* and (1 → 3,6)-linked Glc*p* with branching in position O-6. The

repeating unit of the glucan contain probably side chain of T-Glc*p* or a (1 → 6)-linked -D-Glc*p*. However, it appears that branching units are presented on every fourth unit along the main (1 → 3)-linked -D Glc*p* chain (see Figure 53). Methylation analysis also revealed that all monosaccharides were in the pyranose form (*p*).

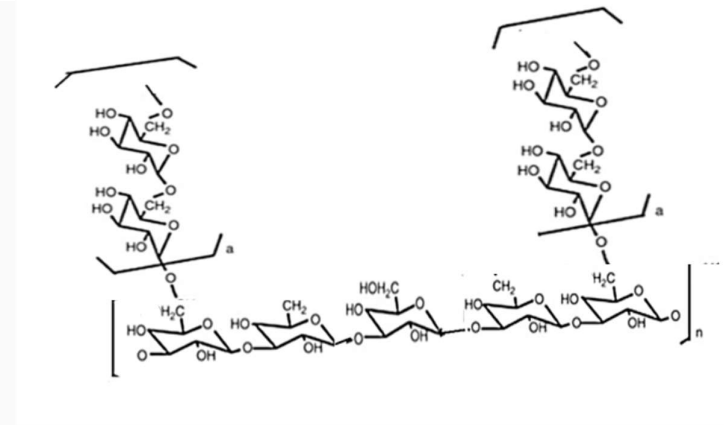

Figure 53: Possible structure of β- glucan in LsW: repeated (1 → 6)- linked glucose units as a side chain are presented on every fourth unit along the main (1 → 3)-linked glucose chain.

Also, the obtained pure fractions from LsW showed that the first eluted fractions: LsW1a, and LsW1b after fractionation on the two different columns had higher amount of glucose than galactose suggesting that they might contain more glucans than galactans in these fractions.

The previous studies have also reported that the same β-glucan, with a main chain of (1 → 3)-linked-Glcp residues, substituted at O-6, was isolated from mushroom *Schizophyllum commune* (Klaus et al., 2011; Sietsma & Wessels, 1977), *Grifola frondosa* (Fang et al., 2012) and *Flammulina velutipes* (Fhernanda R. Smiderle et al., 2006).

Also, a (1 → 3)-Glc*p* backbone might have branching with the terminal-D-Glc*p* at position 6 and it is necessary to investigate in the future. T-Glc*p* residue appeared in high amount in LsW and some fractions of LsW which indicates that for example the methylation was incompletely at branching points or the possible overlap of T-Man*p* and T-Glc*p* peaks, which was found in GC-MS chromatogram. But it was difficult to divide the two peaks.

Furthermore, it was isolated soluble (1 → 3)- β-D-glucan with branching in position O-6, from *Lactarius rufus,* with a higher degree of branching, substituted by some (1 → 3)-linked β-D-Glc*p* side chains (A. Ruthes et al., 2013). This finding can indicates that β-glucan in *L.scrobiculatus* may have the similar structure.

The results obtained from the methylation analyses: (1 → 6)-linked -D-Gal*p* and (1 → 2.6)-linked -D-Gal*p* and T-Gal*p* in the ratio 16:12:0.3 indicated that the polysaccharide might consist the main chain composed of (1 → 6)-linked -D-galactan with branching in position O-2. According to the obtained results by methanolysis, from second, LsW2a, LsW2b, LsW2c and third (LsW3 and LsW3c) fractions, galactans might be more present in these fractions. It might

be mainly (1 → 6)-linked D-galactan with branching at position O-2. A (1 → 6)-linked α-D-galactomannan from *Cordyceps sinensis* was identified (J. Wang et al., 2018).

After methylation analysis was found the presence of terminal 6-deoxy-hexose (9.6%) in *L.scrobiculatus,* which might be a part of side chain, attached to O-2 of (1 → 6)-D-galactan (see Figure 54). The ratio 6-deoxy-hexose and galactose was 1:3 by methanolysis. Therefore, it might be a side chain with branching at every third unit along the main chain composed of (1 → 6)-linked -D-Gal*p* and (1 → 2.6)-linked -D-Gal*p*. T-Gal*p* is present in trace amount (0.3%) and therefore it could not be in side chain. Another monosaccharide might be present. So, 6-deoxy hexose which is terminal bonded and has a non-reducing end, might be a part of sidechains. The amounts of 6-deoxy hexose corresponded well to the amounts of branching residues.

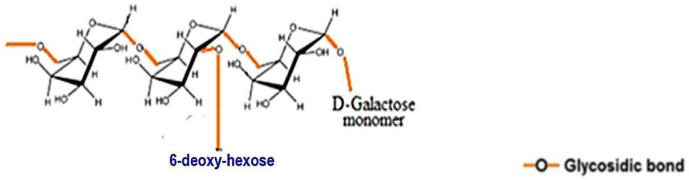

Figure 54: Possible structure of polysaccharide (1 → 6)-D-galactan. It was probably composed of (1 → 6)-D-Glc*p* as a main chain with terminal 6-deoxy-hexose, attached to O-2

Also, it was confirmed the presence of unidentified 6-deoxy hexose in fraction 2 and 3 (LsW2a, LsW2b, LsW2c) and (LsW3a and LsW3c) in a certain amount, indicating that it might be residue, attached to (1 → 6)-linked-D-galactan at position O-2. In order to determine the branching points and conformation of (1 → 6)-linked D-galactose, it is necessary to perform further NMR analysis.

The similar structure, fucogalactan, was found in a white button mushroom *Agaricus bisporus Lactarius rufus* and *Albatrellus ovinus* (Andrea C. Ruthes, Rattmann, Carbonero, Gorin, & Iacomini, 2012; Andrea C. Ruthes et al., 2013; Samuelsen et al., 2019). The polysaccharide consisted of a (1 → 6)-linked-α-D-Glc*p* main chain, partially substituted at O-2 by α-L-Fucose. The similar polysaccharide, fucomannogalactan, was isolated from *L.edodes* (Andrea C. Ruthes et al., 2013). It was composed of (1 → 6)-linked-α-D-Glc*p* main chain, partially substituted at O-2 by β-D-mannopyranose or α-D- fucopyranose (E. R. Carbonero et al., 2008). A fucomannogalactan, (1 → 6)-linked-α-D-Glc*p* main chain, substituted at O-2 by α-L-Fuc*p* and a small amount of β-D-Man*p*, was identified in mushroom *Amanita muscaria* (Andrea Caroline Ruthes et al., 2013).

Another study showed that was found very complex polysaccharide in *Lactarius lividatus* (Tako et al., 2013). The polysaccharide was composed of a (1 → 6)-linked- and (1 →2,6)- linked-α-galactan substituted at C-6 with terminal 6-deoxy-D-altrose and α-D-galactose (see Figure 55).

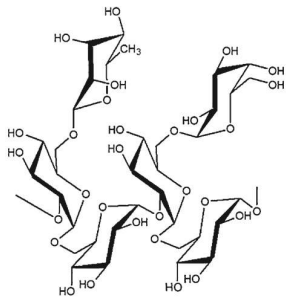

Figure 55: The structure of the polysaccharide (with 6-deoxy-D-altrose), isolated from *L.lividatus*

Mannose is often found in the mushrooms cell wall. Xylose and mannose are present in the small amounts and their positions were not identified. There were carried out any methods that could show this. They can be a part of the side chain, as xylomannans (Andrea C. Ruthes et al., 2016) or galactomannans (F. R. Smiderle et al., 2008).

All fractions contained (1 → 4)-linked-α-D-glucan in small amount which was determined by iodine-potassium iodide assay og methylation. An amylose like linear and water soluble (1→4)-α-D-glucan was isolated from basidiocarps of *Agaricus blazei* (Gonzaga, Ricardo, Heatley, & Soares, 2005). The result of the test confirmed the presence of (1→4)-α-D-glucans. LsW extract showed a little change of the colour and possible presence of (1→4)-α-D-glucans. This (1 → 4)-linked-α-D-glycan was identified in fractions: LsW1a, LsW2a, LsW3a, LsW1b, LsW2b, LsW1c and LsW3c according to methylation and GC-MS analysis in range from 0.5 to 2.7%.

Also, galacturonic acid is present in all fractions. Galacturonic acid was in higher amount in fraction 2 and 3 after purification on Sephacryl 500 column (LsW2a and LsW3a, 7.9% and 18.2 %) which contained more galactose, than fraction 1 (LsW1a, 5.3%). The second column fractions also gave more galacturonic acid in fraction 2, LsW2b than in fraction 1, LsW1b.The amount of galacturonic acid is almost the same after purification on the third column, which indicates that separation of fractions was not satisfied. Therefore, it is possible that galacturonic acid is located inside and /or branched in galactans.

The different fractions obtained by purification on three different columns showed that glucose was present mainly in fraction 1, while in fraction 2 and 3 galactose was dominant monosaccharide. This might indicate that fraction 1 contained one type glucan and fraction 2 and 3 contained galactan. In addition, it was more present unknown terminal 6-deoxy hexose in fractions 2 and 3 than in fraction 1 which indicates that this monosaccharide might be bonded to galactan.

The results after enzymatic degradation showed that the amount of glucose was decreased compared to the amount of glucose in LsW2b and LsW2c fractions, before treatment with enzyme. (1 → 3)-β-glycosidic bonds were broken and amount of (1 → 3)-linked Glc*p* was decreased. Amount of galactose in both fractions was increased compared to fractions before

enzymatic degradation. Enzymatic degradation with the same enzyme endo-(1 → 3)-β-glucanase could be repeated and eventual treatment with new enzymes which has ability to catalyse glucosidic bonds can be performed in order to obtain more purified fractions of polysaccharides.

10.10 Discussion of the structure of LsA

The results from SEC-HPLC showed that LsA contains of the structures with the molecular weight range from 2772 kDa to 17.4 kDa which indicates that LsA might consists of a mixture of polysaccharides.

The results showed that glucose was the predominant monosaccharide in the alkali extract with molar percentage 79 % and this indicates that glucans might be present in LsA. Mannose (9.1%) and xylose (6.7%) were present in small amount. Galactose (2.2%) and 6-deoxy-hexose (2%) were present in low levels. A small amount of both galacturonic and glucuronic acid were also present in the extract (1%).

Linkage determination revealed that all monomers were in the pyranose form (*p*). According to obtained GC-MS results, the ratio between (1 → 3)-linked Glc*p*, (1 → 6)-linked Glc*p* and (1 → 3,6)-linked Glc*p* was 18:11:7. The ratio of (1 → 3)-linked Glc*p* and (1 → 3,6)-linked Glc*p*, 18:7, indicates that they might be a main chain of one polysaccharide: (1 → 3)-linked Glc*p* with a branching in position O-6. It appears that branching units are presented on every fifth unit along the main (1 → 3)-linked -D Glc*p* chain. LsA might contain another types of glucan: a (1 → 6)-linked-D-glucan as a main chain with branching in position 3. The branching units might be presented on every third unit along the main (1 → 6)-linked -D Glc*p* chain. In side chain might be (1 → 6)-linked -D Glc*p* residue as in Lentinan (Sasaki & Takasuka, 1976) or terminal linked-D Glc*p* residue as i *Ganoderma lucidum* (Y. W. Chang & Lu, 2004).

Alkali extract of mushrooms often contains long glucan chains with less branching and (1 → 3)-linked β-D- glucan is believed to be involved in maintaining of wall mechanical strength and rigid structure, Thus, it is insoluble in water and can form aggregates (Zekovi, Kwiatkowski, Vrvi, Jakovljevi, & Moran, 2005).

Some of these glucans that contained a (1 → 3) Glc*p* as a main chain with branching in position 6, as lentinan from *L.edodes,* branched to every third residue (K.-P. Wang et al., 2014) ((Y. Zhang, Li, Wang, Zhang, & Cheung, 2011). The similar polysaccharide but more branched, to every second residue was found in *Grifola frondosa* (Fang et al., 2012). Also, similar polysaccharide was found in *Shizophyllum commune* and *Sclerotium glucanicum* (Synytsya & Novák, 2013) (Giavasis, 2014).

Another type of glucan, (1 → 6)-β-D-linked Glc*p* as main chain with a single β-D Glc*p* -residue at the O-3 position might also be presented in LsA extract. It was found in medicinal mushroom *Agaricus blazei* (Gonzaga, Menezes, de Souza, Ricardo, & Soares, 2013). The similar β-glucan was identified from the fruiting body of *Amillariella mellea.* The main chain of this mushroom consists of (1 → 6)-linked β-D-Glcp residues, substituted at O-3 by β-D-Glc*p* (Yan

et al., 2018). The exact structure of β-glucan in LsA can be identified using more purified fractions and NMR analysis.

The GC-MS and iodine-potassium iodide assay has indicated the presence of high amounts of (1 → 4)-α-D-glucan. It might be amylose like linear (1 → 4)-α-D-glucan (see Figure 56). A linear, water soluble (1→4)-α-D-glucan was isolated from basidiocarps of *Agaricus blazei* (Gonzaga et al., 2005).

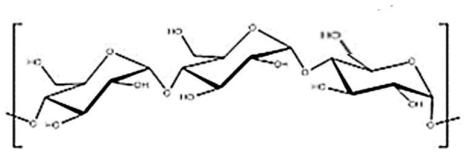

Figure 56: Proposed structure of α-glucan in LsA

The presence of (1 → 4)-α-D-glucan, but more branched has been demonstrated in mushroom *Agaricus bisporus*. This polysaccharide was identified as a main chain of (1→4)-α-D-Gal*p*, substituted at position O-6 by α-D-Glcp single units (F. Smiderle et al., 2010). Also, this type of (1 → 4)-α-D-glucan with a single α-D-Glcp at position O-6 is found in mushroom *Flammulina velutipes* (X. Pang et al., 2007). Siden (1 → 4)-α-D-Glc*p* residues were not registered by methylation, it might be not present a branched (1 → 4)-α-D-glucan. A linear (1 → 4)-α-D-glucan might be present in LsA.

So, it is possible that some of these glucans exist in alkali extract *L.scrobiculatus*. But it is necessary to obtain the more purified fractions and then to analyse the carbohydrates content. In order to find the sequence, i.e. which monomers are linked to one another in the polymer, it is needed to perform several types of NMR experiments and also to determine the anomeric configuration.

11 The other extracts, ^{1}H NMR, results and discussion

11.1 LsK

The white crystals were appeared as a precipitate in the Soxhlet extraction with ethanol, named LsK. In order to find out what was in LsK, ^{1}H NMR was performed.

^{1}H NMR of LsK had the identical characteristics ^{1}H spectrum (the same pattern) (see Figure 57) as mannitol standard ^{1}H NMR spectrum, found in literature, from the Human Metabolome Database("Human Metabolome Database," 2005). Mannitol is a naturally occurring alcohol found in fruit and vegetables and used as an osmotic diuretic.

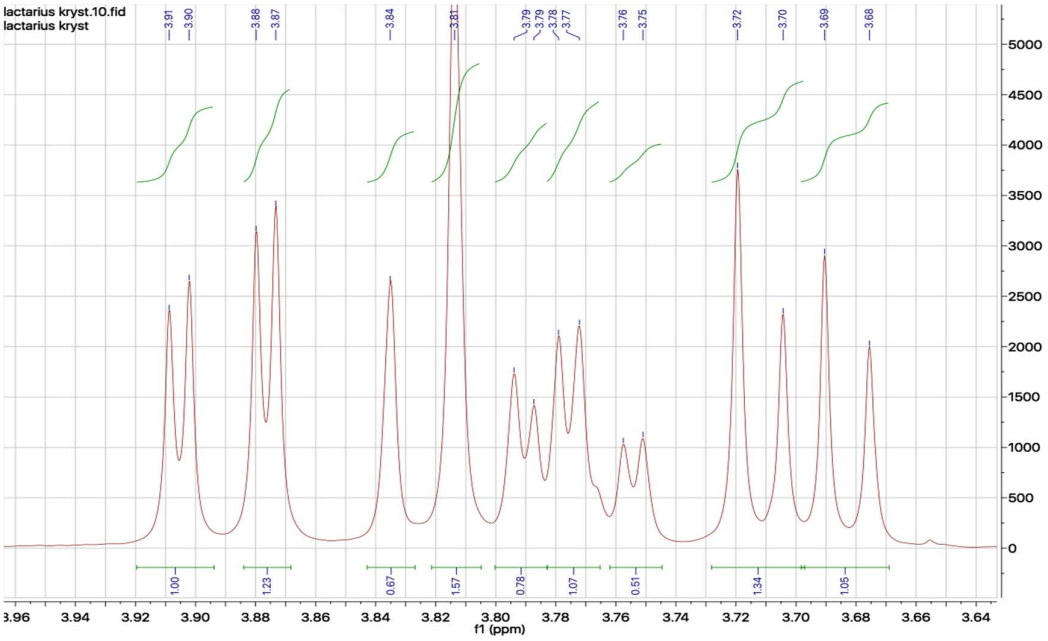

Figure 57 Obtained ^{1}H NMR spectrum (proton spectrum) for the fraction LsK, 400 MHz

^{1} H spectrum of mannitol ($C_6H_{14}O_6$) contained strongly coupled multiplets, covering a chemical shift range from 3.67 to 3.92 ppm. ^{1}H NMR was a rapid technique to determinate mannitol in the ethanol extract precipitate (LsK).

Figure ^{1} H NMR spectra for mannitol was taken from the Human Metabolome Database (SEE Figure 58). The chemical structure of mannitol: (2R, 3R, 4R, 5R)-hexane-1.2.3.4.5.6-hexol.

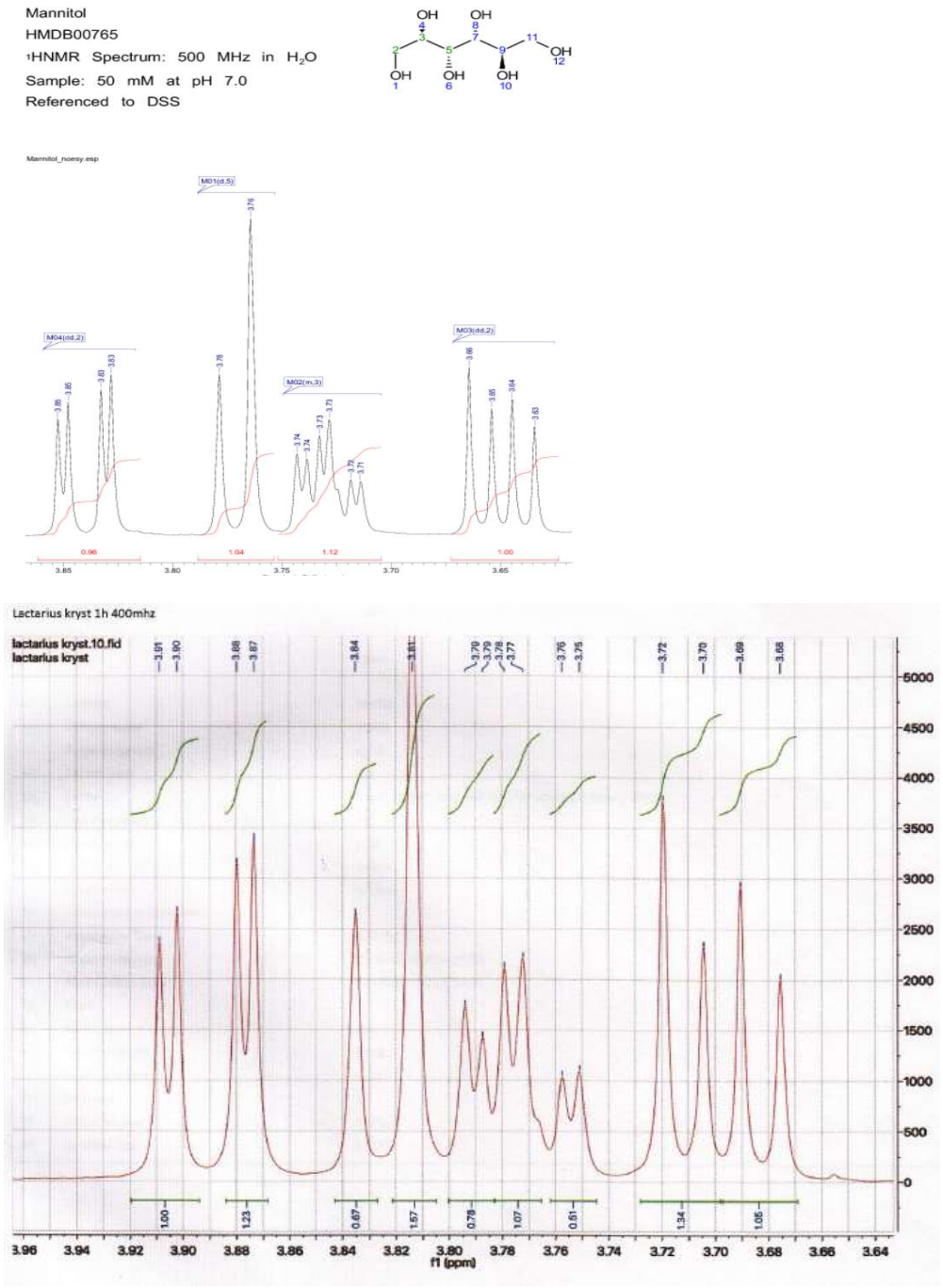

Figure 58 Characteristic ^{1}H NMR spectra for mannitol, was taken from ("Human Metabolome Database," 2005)

^{1}H NMR was a rapid technique to determinate mannitol in the ethanol extract precipitate (LsK).

In this way, the presence of mannitol was determined in ethanol extract precipitate. In this way, the presence of mannitol was determined in ethanol extract precipitate. The presence of mannitol was confirmed in other mushrooms for example in *Amanita Muscaria* 10-11 mg per 1 g of dry mass (Maciejczyk & Kafarski, 2013), in *Pleurotus ostreatus* 1.8 % of dry weight (Hammond, 1980) and in *Agaricus brasiliensis* 21.8 g in 100 g dry weight (Cho, Jang, Park, & Park, 2008). Because of the presence of mannitol, mushroom are useful as diabetic foods (Cho et al., 2008).

11.2 LsEt

After Soxhlet extraction and concentrating on rotary evaporator, a dark brown extract, LsEt was obtained. To get insight in the chemical composition in general, ^{1}H NMR analysis was performed. Indications of chemical components present in NMR spectrum is presented as chemical shifts (δ, ppm) relative to internal standards. Internal standard is normally set to 0 ppm.

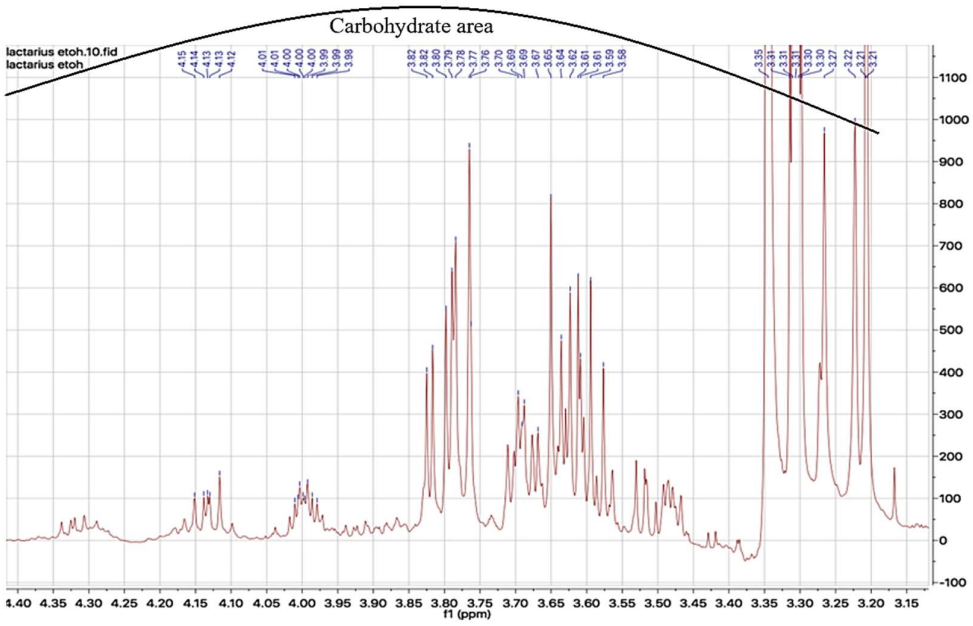

Figure 59. 1 H NMR spectrum of the fraction LsEt, 1h, 400 MHz, Carbohydrate area 3.2-4.5 ppm, mannitol (3.67-3.92)

The strong signals were observed in regions from 3.2 to 4.4 ppm (see Figure 59), indicating that the extract contained monosaccharides, oligosaccharides and mannitol which was identified in ethanol extract precipitate. The signal of residual methanol in D_2O in the sample occurred in the spectrum at 3.3 and water at 4.8 ppm.
The general representative peaks for carbohydrates are described in literature (Cui, 2005) as follows: H2, H3, H4, H5 and H6 appear at 3.2-4.5 ppm (see Figure 59). In the region of 5 to 6

ppm appears the α-anomeric proton while β-anomeric proton appears mainly in the 4 to 5 ppm region (see Figure 60). There are many examples from literature, for example ^{1}H NMR spectrum of *L.camphoratus* showed the signal peak of the remaining proton of monosaccharides from 3.4 to 4.17 ppm (F. Wang et al., 2013).

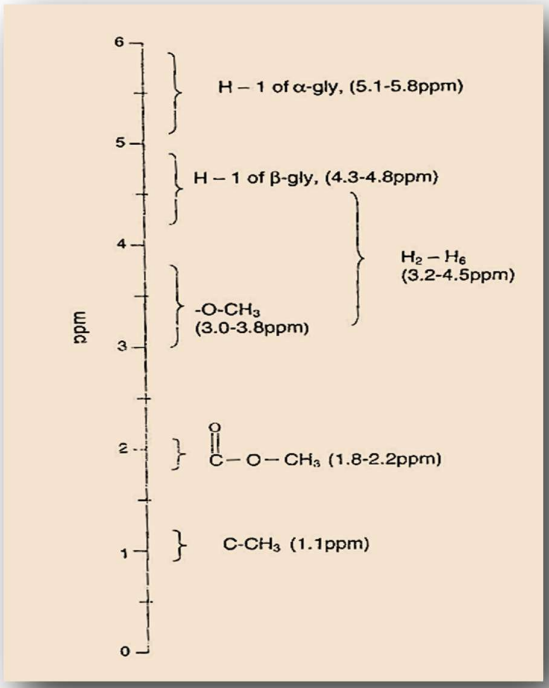

Figure 60 Chemical shifts of carbohydrates in 1H NMR spectra (Cui, 2005)

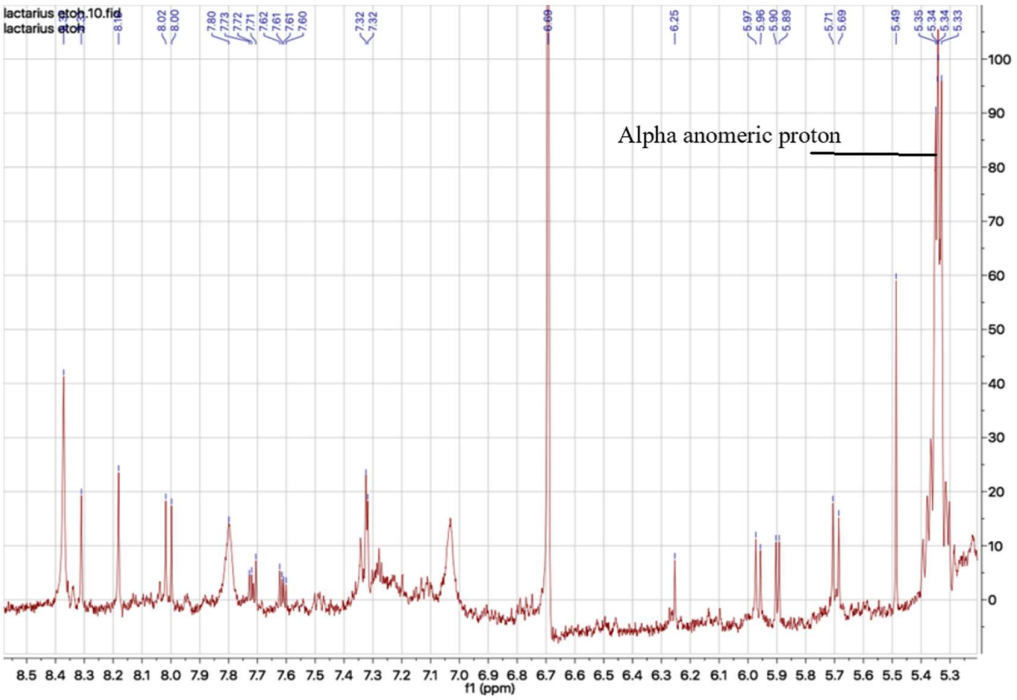

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Figure 61: α-anomeric proton appears in the region 5-6 ppm, here at 5.34 ppm

The ^{1}H spectrum at 400 MHz of the ethanol extract LsEt is presented in Figure 61 and 62.

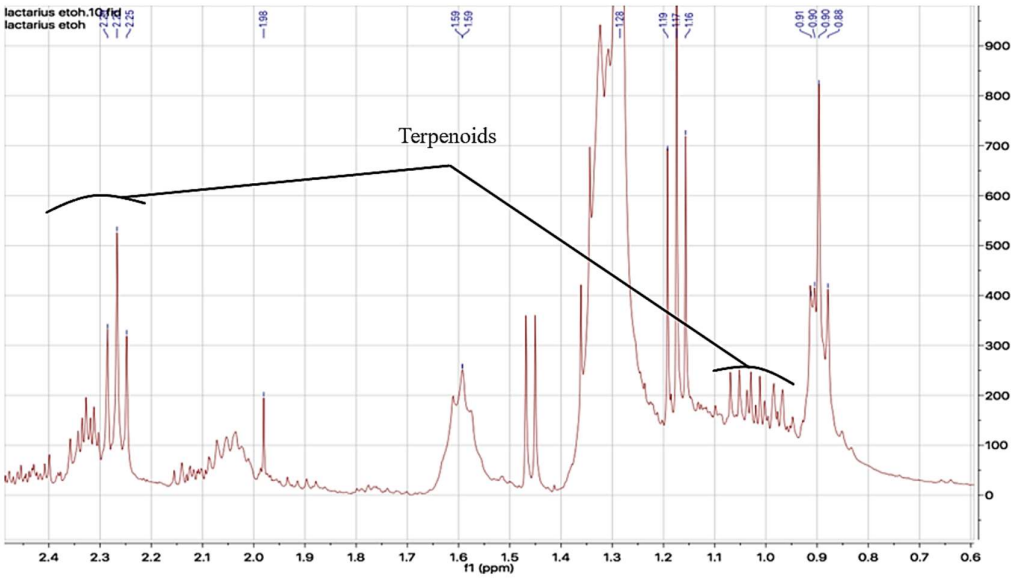

Figure 62 ^{1} H NMR spectra of the fraction LsEt, 1h, 400 MHz

Chemical signals from possible terpenoids in LsEt appears in the range 0.95-1.1 ppm and 2.25-2.4 ppm (see Figure 62). This was found in the literature (De Bernardi et al., 1993). Sesquiterpenes from *L.scrobiculatus,* were identified from ^{1}H NMR spectrum and appeared in the range 0.69-1.2 ppm and 2.1-2.7 ppm. For example, position of proton H 3 was at position 2.15, 2.63; H12 at position 0.69, 1.12 and H 10 at position 2.37, 2.53. Chrysorrhelactone and chrysorrhedial (see Figure 18 (2)) were identified by NMR (De Bernardi et al., 1993).

11.3 LsDCM

The dichloromethane extract LsDCM was subjected to ^{1}H-NMR analysis in order to reveal the types of chemical components present. Figure 63 shows the ^{1}H NMR spectrum of LsDCM. According to the data found in literature, LsDCM probably contained terpenoids, phenolic compounds, lipids and other compounds (De Bernardi et al., 1993; Yokokawa & Mitsuhashi, 1981) (L.D.Field, 2015).

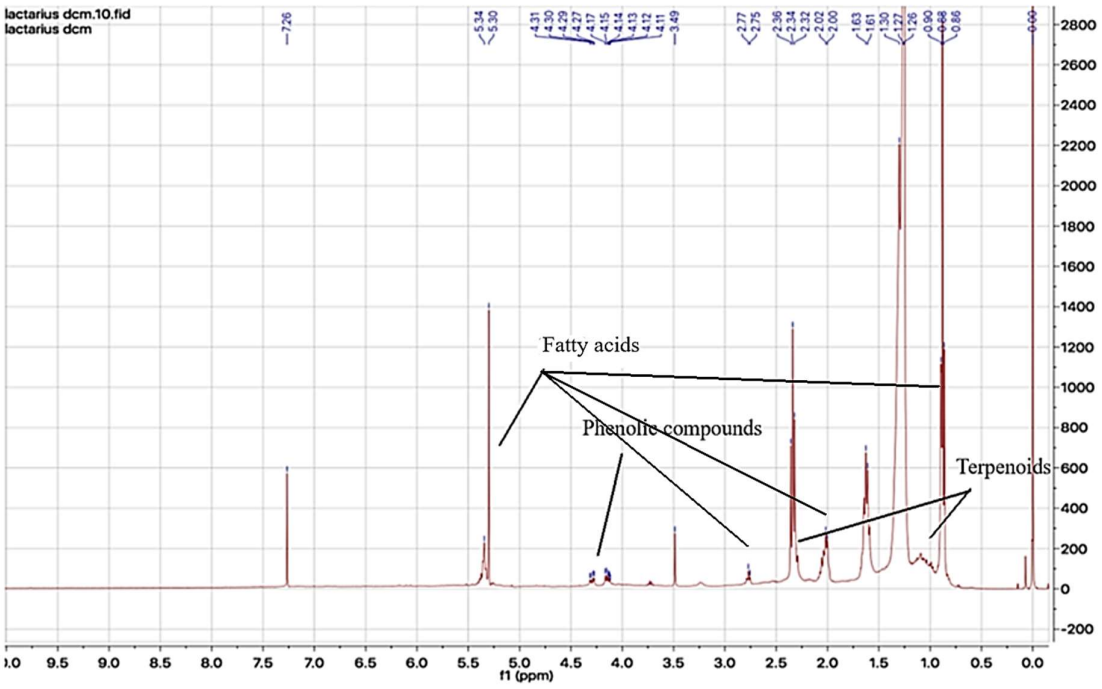

Figure 63 ^{1} H NMR spectra of the fraction LsEt, 1h, 400 MHz

Chemical signals for terpenoids were found in almost same region as in LsEt, in range 0.9-1.2 ppm and 2.0-2.4 ppm.

The chemical signals for quantitating unsaturated fatty acids were found in the literature: terminal CH_3 group protons (0.8-0.9 ppm), protons attached to the allylic carbons (2.0-2.1ppm), protons attached to the bis-allylic carbons (2.7-2.8 ppm) and olefinic protons (5.3-5.4 ppm)

(Knothe & Kenar, 2004). The same pattern of fatty acid signals was found in LsDCM (see Figure 63).

The phenolic compounds chemical shift was found in LsDCM and also in literature in the range 4-6 ppm (L.D.Field, 2015).

12 BIOLOGISK ACTIVITY ASSAY-results and discussion

12.1 Measurement of the inhibition of *MgtA*

E. coli is an anaerobic, gram-negative bacterium. Infection by *E. coli* can cause a wide range of clinical manifestations such as diarrhea, colitis, uremic syndrome and death. It is necessary to find out compound, which can inhibit bacteria's P-ATPase. Therefore, the ability of mushroom extracts to inhibit *MgtA* activity of *E. coli* was examined.

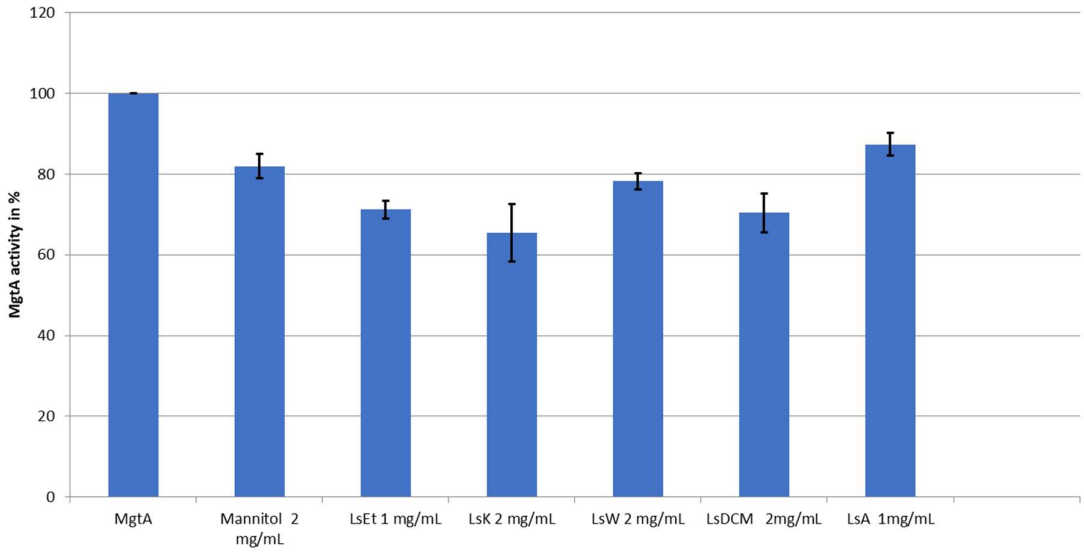

Figure 64. Inhibition of MgtA activity: MgtA- protein; mannitol- pure mannitol; the mushroom extract: LsEt-ethanol extract; LsK-ethanol extract precipitate; LsW- the water extract; LsDCM- dichloromethanol extract and LsA- alkali extract

Mushroom extracts: LsA, LsW, LsEt, LsK and LsDCM were dissolved in water or in 50 % DMSO and water to the concentration of 1 or 2 mg/ml. Some of them could not be completely dissolved in the water, so there were used in those cases DMSO and water as a solvent. This test was performed to determine which extracts may affect *MgtA* activity and which of them can be used further for concentration-dependent effect.

As shown in Figure 64, it was a decrease in *MgtA* activity with *L. scrobiculatus* extracts, compared to control. This inhibition of protein activity was not specific inhibition, only

general inhibition. LsK, LsDCM and LsEt gave about 30 % inhibition, in general but not specific inhibition. LsW and mannitol reduced *MgtA* activity by about 20 %.

LsK-crystals obtained in Soxhlet extraction, contained the clear mannitol showed about 34% inhibition of activity *MgtA*. Also, mannitol standard gave about 18 % inhibition. So, this indicate that LsK contains the other compounds which were inhibited activity of *MgtA*. In addition, LsEt-ethanol extract showed about 29 % inhibition. So, mannitol contributes a certain inhibition of activity in addition to other components which exist in ethanol extract, LsEt and ethanol extract precipitate, LsK.

The results showed that alkali extract LsA had a little ability to affect *MgtA* activity (<20%).

12.2 Concentration-dependent inhibition of *MgtA in vitro*

Dichlorometane extract LsDCM and the water extract (LsW) were tested on concentration-dependent inhibition.

LsDCM showed a small concentration dependent activity against *MgtA* (see Figure 65). Taking into consideration three points (0.25 mg/ml, 0.5 mg/ml and 1.5 mg/ml of the extract LsDCM), it is observed a small concentration dependent inhibition of *MgtA*.

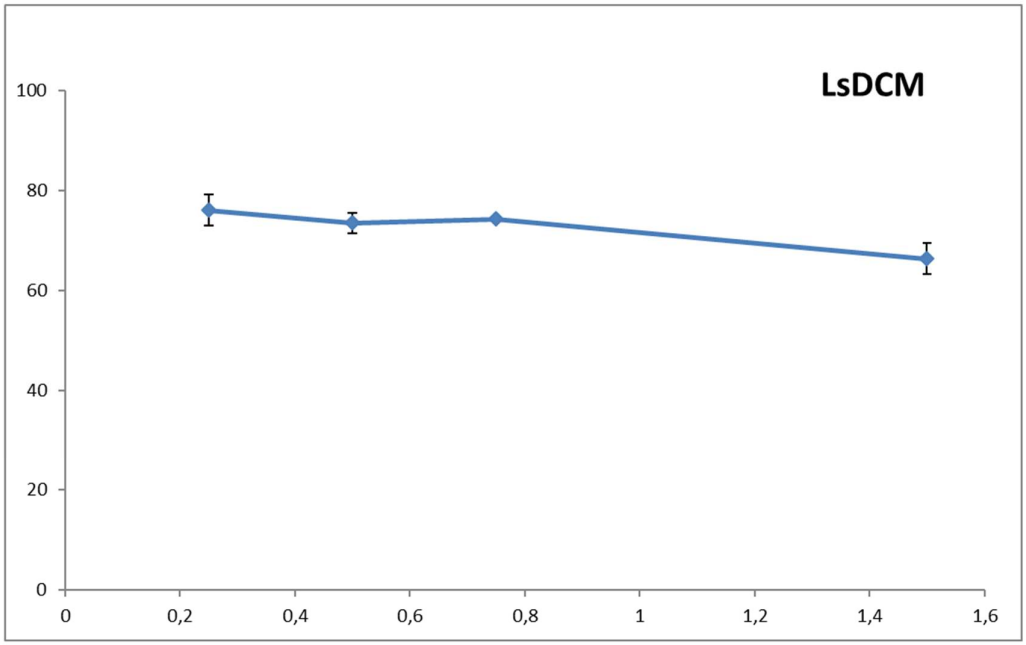

Figure 65 *In vitro* inhibition of the activity of *MgtA* stimulated with different concentration of LsDCM

The LsDCM extract of concentration 1.5 mg/ml showed highest inhibition of the protein compared to two other concentrations of the LsDCM extracts 0.5 mg/ml and 0.25 mg/ml which showed less inhibition.

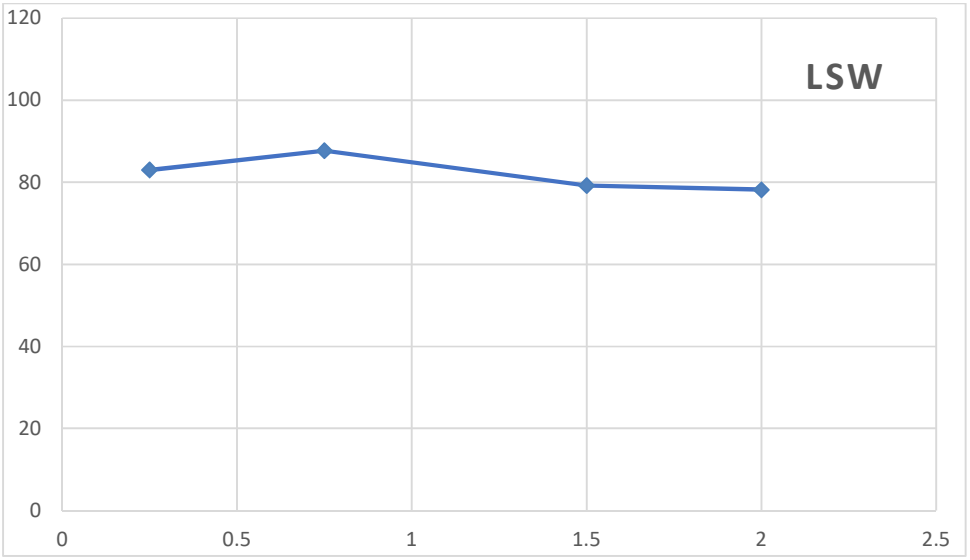

Figure 66 *MgtA* activity after stimulation with different concentration of the water extract LsW

The LsW extract was dissolved in water to different concentrations: 0.25 mg/ml, 0.5 mg/ml, 0.75 mg/ml, 1 mg/ml, 1.5 mg/ml and 2 mg/ml. This extract did not give dose dependent inhibition of the activity of *MgtA* and the results varied between different concentrations of the extract. The possible reason could be incomplete water solubility and viscous solution which caused incorrect added concentrations of the extract. If the concentration of 0.75 mg/ml was excepted, it can be observed a little concentration-dependent inhibition (see Figure 66).

12.3 Discussion of biological assay

The purpose of the biological assay was to evaluate the ability of *L.scrobiculatus* extracts to inhibit activity of membrane protein Mg^{2+}-ATP-ase of bacteria *E.coli.* The results of this screening test showed that some extracts had influence on the activity of *MgtA in vitro*. Inhibition of the Mg^{2+} ATP-ase of *E.coli* has not been investigated previously. But, in the literature, it was found that two compounds had ability to inhibit other type of P-ATP-ase. They have probably the same mechanism of action as Mg^{2+}ATP-ase.

For more than twenty years ago, the two- dimensional crystals of a renal $Na^{+}K^{+}$-ATP-ase were first obtained by incubating kidney-cell membrane by vanadate (Cantley et al., 1977).

The thapsigargin which was isolated from the Mediterranean plant *Thapsia garganica*, was used as inhibitor of the SR Ca^{2+} ATP-ase in mammalian cells. This guaianolide compound of

plant has become popular in investigating the mechanisms of intracellular Ca^{2+}signalling and it is most widely used SERCA (a class of transporters named sarco-endoplasmic reticulum Ca^{2+}-ATP-ases) inhibitor (Treiman, Caspersen, & Christensen, 1998).

The thapsigargin has ability to inhibit E2 form of the sarcoplasmic reticulum of Ca^{2+}ATP-ase while the vanadate inhibited E2-P form of the Na^{+}/K^{+}-ATP-ase (Werner, 2004).

Furthermore, it was reported that Bafilomycin, a macrolide antibiotic, inhibited the enzymatic activity of $Na^{+}K^{+}$ATP-ase. It was not effective against *E.coli* membrane but was highly specific for the class of vacuolar ATP-ase from fungus, plant and animals (Bowman, Siebers, & Altendorf, 1988).

The mushroom extracts of *L.scrobiculatus* affected the activity of bacterial membrane protein, MgtA. The pure mannitol showed 18 % inhibition. Other extracts LsK and LsEt which contain mannitol gave about 30% inhibition. LsDCM, LsEt mainly contain lipid soluble and low molecular weight compounds. This indicates that these bioactive components, present in mushrooms, such as phenolic compounds, terpenoids and others might inhibit MgtA proteins. In the literature are found many articles which confirm that secondary metabolites of mushroom could have antibacterial activity, for example *Laetiporus sulphurous*, *Ganoderma lucidum* and *Lentinus edodes* (Alves et al., 2012). The wild mushrooms from genus *Lactarius*, *Lactarius turpis* and *Lactarius citriolens* showed also antioxidant and antibacterial activity. They also contained mannitol, phenolic compounds, organic acids, fatty acids and terpenoids (Vieira et al., 2014). It is necessary to isolate these compounds and subject to *MgtA* biological activity test. LsDCM and LsW showed a small concentration dependent activity against *MgtA*, probably because of the small solubility of extracts in water or DMSO and water. They were viscous, colloid solutions and this incompletely solubility of extracts could affect the results.

This is a pioneer study, since, as far as we know, there are no reports on the inhibition of bacterial *MgtA* by mushroom extracts.

13 Conclusion

Different extracts of *Lactarius scrobiculatus* fruiting body were obtained by sequential extraction with different solvents. The highest yield of extraction was obtained from alkali extract LsA (18%) and the lowest from water extract LsW(2.8%). The yield of other extracts, LsDCM, LsEt and LsK was about 10%.

After purification of the water extracts on three different columns, the nine fractions were obtained. No one of the columns has showed ideal results. Sephacryl S 500 filtration column with large capacity, which was packed at our lab, showed best separation and highest yield of fractions compared to other two columns: Sephacryl S 500 HR and Superdex 200 HR column.

The results from SEC-HPLC showed that LsW contained three molecular weight populations in range from 2985,4 kDa to 2.4 kDa while LsA contained fractions with the molecular weight range from 2772 kDa to 17.4 kDa. This may indicate that both LsW and LsA contained a mixture of several polysaccharides.

Structural and linkage determination of LsW showed that the isolated polysaccharide fractions contained a certain amount of (1 → 3) -linked Glc*p,* (1 →6)-linked Glc*p* as well as (1 → 3,6) -linked Glc*p* residues, which are common structures for β-glucans, found in mushrooms. In addition, the water extract LsW contained more (1 →6)-linked Gal*p* residue while alkali extract LsA contained more (1 → 4)-α-D-Glc*p* residues.

The methylation and methanolysis analysis of water extract, LsW suggested the appearance of a (1 → 3)- linked-glucan, (1→6)-galactan and a small amount of (1→4)-α-glucan. Unidentified 6-deoxy-hexose might be attached to (1→6)-galactan. The similar structure fucogalactan was found in some mushrooms.

The results obtained by methanolysis and methylation of alkali extract, LsA showed the presence of glucans: (1 → 3)-linked -D-Glc*p,* (1→ 6)-linked -D -Glc*p* and (1→3,6)-linked -D-Glc*p* which suggested the presence of (1→ 3)- D -glucan and probably a (1→6)- D-glucan. Also, significant amount of a (1 → 4)-α-glucan was determined. The presence of high amount of (1 → 4)-α-D-glucan was showed by iodine-potassium iodide assay and methylation analysis.

The extracts: LsK, LsDCM and LsEt gave about 30 % inhibition of *MgtA* of *E.coli in vitro.* LsW and mannitol decreased *MgtA* activity by about 20 %. Mannitol contributes a certain inhibition of activity. But, probably other components which exist in ethanol extract also had ability to inhibit *MgtA*. Alkali extract LsA had a little ability to affect *MgtA* activity (<20%). LsDCM and LsW showed a weak nonspecific concentration dependent activity against *MgtA.* It requires to perform more tests to confirm this biological activity.

14 Suggestion to further studies

Mushrooms are used for medicinal purposes and the screening of molecules which can possess biological activities. LsW contained a (1 → 3)-linked β-D-glucan with branches in O-6, a (1 → 6) -linked D-galactan with branching in position 2 with a terminal unidentified 6-deoxy hexose in its fractions. To achieve better results, it may be useful to separate these glucans from each other by repeating the treatment with enzyme endo-(1 → 3)-β-D-glucanase, or treatment with new enzymes for example endo-(1 → 6)-glucanase, to remove (1 → 6)-Glc*p* residue. Then, qualitative and quantitative identification with the purer fractions would performed. Both extracts LsW and LsA contained galacturonic acid which might be also removed by ion-exchange chromatography and obtain more purified fractions. The mushroom extracts and the obtained fractions can be subjected to ^{13}C NMR, COSY, NOESY analyses in order to clarify more the structure of polysaccharides, for example unidentified 6-deoxy hexose and conformation of (1 → 6) -linked D-galactan. Furthermore, the monosaccharide composition is an important characteristic of polysaccharides and there is a clear relationship between structure and bioactivity. So, it is necessary to obtain pure fractions which can give better results of quantitative and qualitative determination of the structure.

Inhibition of *MgtA* activity in vitro was a screening test in order to investigate whether mushroom extracts may inhibit activity of the important membrane protein of G- bacteria *E.coli*. It can be interesting to isolate the secondary metabolites such as phenolic compounds, terpenoids from extracts and perform the *MgtA* activity assay. However, *Lactarius scrobiculatus* extract can be an interesting resource of compounds that may be a potential inhibitor of *MgtA*. New inhibitors of bacterial P-ATP-ase may have important implications for finding new antibiotics and thus improvement of people health.

15 References

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