Chapter 3

Extraction and Fractionation of Polysaccharides from a Selected Mushroom Species, *Ganoderma lucidum*: A Critical Review

Yew-Keong Choong, Kavithambigai Ellan, Xiang-Dong Chen and Shaiful Azuar Mohamad

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.78047

Abstract

Fractionation plays a big role in most of the sample processing especially in mushroom polysaccharides extraction. This intermediate step will determine further purification process which will lead to the type of polysaccharides that will be obtained. Four types of *Ganoderma lucidum* cultured medium used in the research papers were randomly chosen. They are spores, mycelia, fruiting body and fermentation broth. For water soluble polysaccharides, hot water extraction is typically applied. The following ethanol precipitation could be appropriate used to sediment the component with OH-group including polysaccharide. The next step of fractionation consist of anion exchange chromatography or gel filtration enhance the purity of polysaccharides. Using these extraction and fractionation techniques, high quality polysaccharides could be successfully obtained from the mushroom that are useful for further studies. This review examined the various extraction and fractionation techniques used in the study of polysaccharides from *G. lucidum*.

Keywords: extraction, fractionation, polysaccharide, *Ganoderma lucidum*, anion exchange chromatography

1. Mushroom polysaccharides

Mushroom polysaccharides have been studied intensively since the therapeutic effects of hot water extract from several fungi were evaluated on Swiss albino mice [1]. The fungi, *Pleurotus ostreatus, Pholiota nameko, Pleurotus spodoleucus, Tricholoma matsutake, Flammulina velutipes* and *Lentinula edodes* have been shown to potentially inhibit the growth of transplanted Sarcome 180

tumor cells [2]. These significant results have prompted many scientists to purify and isolate the therapeutic compounds. One of the effective constituents was found to be β-D-glucan. It is one of the main constituents of medicinal mushroom, the main chain consisting of β-(1→3) linkages with some β-(1→6) branches as well as chitin, mannons, galactans, and xylans. **Figure 1** shows the molecular structure of β-D-glucan.

The long chain of main β-glucan molecules is capable of having branching side-chains with other monosaccharides in the position of C_1 and C_3 or C_1 and C_6 through condensation. Likewise, other types of molecules such as proteins are found in polysaccharide-K (**Figure 2**) [3].

Basically D-glucose units are the common forms of β-glucans and mostly they are binding with β-1,3 links. The β-glucans of mushroom, yeast or other fungi contains 1–6 side branches (**Figure 3**), while cereal β-glucans contain β-1,4 and β-1,3 backbone bonds.

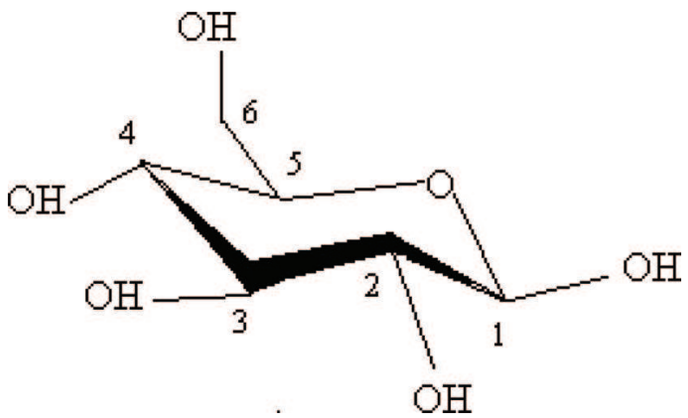

Figure 1. Six-sided D-glucose rings connected linearly at varying carbon positions. When the ═OH group at C_1 direct 45° toward C_3 at another molecule glucose to have glycosidic bond β(1→3) and the continuing linkage forming the larger polymer.

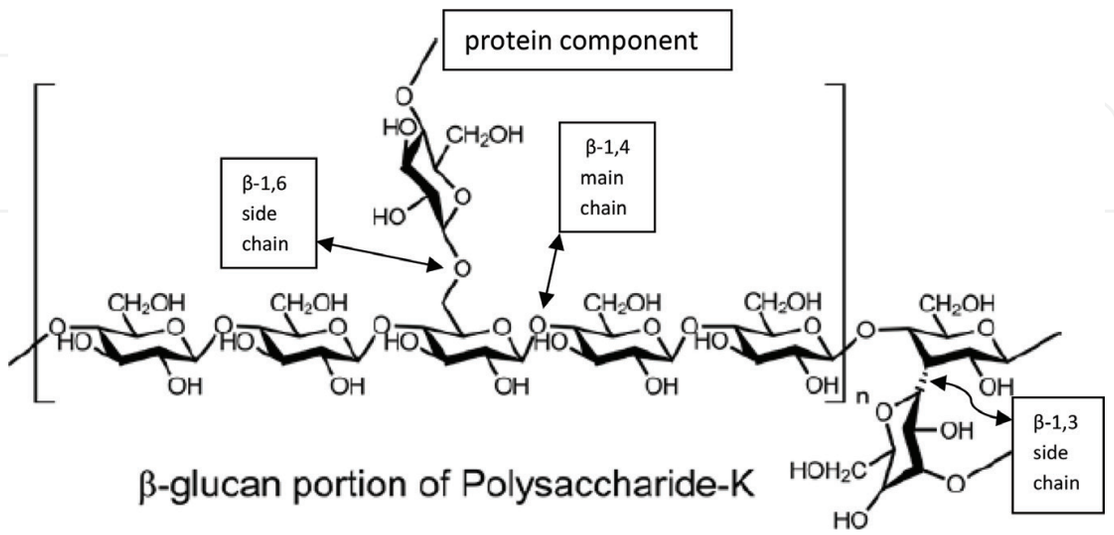

Figure 2. Side-chains of β-glucan with protein in polysaccharide-K. Polysaccharide-K (Krestin, PSK) is a protein-bound polysaccharide purified from the fruiting body of *Coriolus versicolor*. PSK predominantly consists of a beta-glucan β-1,4 main chain with β-1,3 and β-1,6 side chains. The molecular weight of PSK is 100,000 Da, its approximately 25% tightly bound protein is reported at the β-1,6 side chain.

http://dx.doi.org/10.5772/intechopen.78047

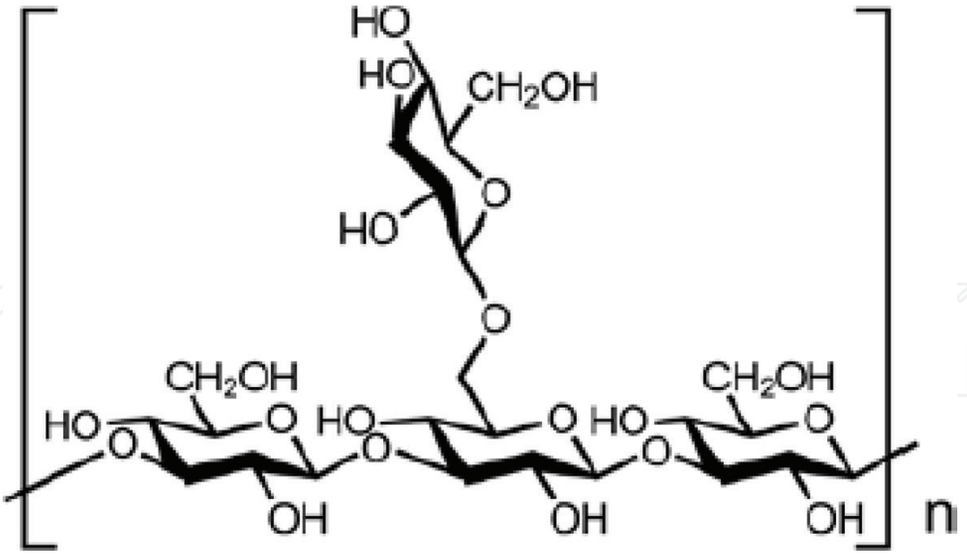

Figure 3. Structure of backbone β-1,3-glucan with β-1,6 branching. n is the alphabet numbers.

In short, there are many possible branches of different types of monosaccharides with glycosidic linkages. This scenario is closely associated with molecular weight of the polysaccharide.

Presently the effects of mushroom polysaccharides have been studied intensively which consist of antioxidant [4], anti-inflammation [5], anti-hyperglycemia [6], anti-tumor [7] and immunostimulation [8]. Nevertheless the process of discovery of new polysaccharides is still on-going. Moreover, research revealed that the chemical characteristics of the constituents of the mushroom polysaccharides, such as molecular weight, molecular structure, solubility in water, the location, length and frequency of the side-chains have a correlative relationship with the function of polysaccharides [9]. The issue of types of isolated polysaccharides have received considerable critical attention on the step of the process of extraction especially fractionation. Fractionation has a pivotal role in the intermediate stage between extraction and isolation. Polysaccharides fractionation is essential for a wide range of technologies for separation process including certain quantity of a crude polysaccharides. The crude will be continuously separated into smaller quantities with diverse compositions.

2. Polysaccharides of selected species, *Ganoderma lucidum*

Ganoderma lucidum is one of the popular medicinal mushrooms. It belongs to the basidiomycetous and assigned to the family *Polyporaceae*. It has been used in Chinese traditional medicine for more than 4000 years [10]. This fungus with long medicinal history has been studied intensively. Typically such studies included its genetic profiles, chemical composition, multiple physiological testing, toxicology and clinical trials. The bioactive compounds especially the polysaccharides of this mushroom generate much attention due to its host dependent action [11] and involving immune stimulation system [12].

Polysaccharides of *G. lucidum* have been studied and are found typically to contain the pure polysaccharides, but some are also binding with proteins or peptides. It was previously reported that the molecular weight of these polysaccharides varied widely, e.g., a polysaccharide peptide

with a molecular weight of 584,900 Da was isolated from a hot water extract of wood-cultured *G. lucidum* by Cao *et al.* [13]. It was found to contain more than 40% β-D-glucan. Another report by You *et al.* showed a type of polysaccharide peptide extracted from the same species with an average molecular weight of 513,000 Da. It contains 16 kinds of amino acids [14]. Most of the polysaccharides extracted and bonded with protein had high molecular weight and played a specific function related to immune stimulation system. It was reported by Giavasis [15] and Ho *et al.* [16] that bioactive polysaccharides which are higher in molecular weight have been shown to exhibit significant antitumor properties, immunomodulatory activity, antioxidant activity, and neuroprotection. A series of fractionation has been conducted with the purpose of studying this little known fungus using advanced technology. A pure *G. lucidum* polysaccharide labeled as GLP-1-1 was isolated from a culture broth with molecular weight of 22,014 Da [17]. Its monosaccharide content contained glucose, mannose, and galactose with molar percentages of 92.33, 7.55, and 0.22%, respectively. A water-soluble polysaccharide [18] extracted from the spores of *G. lucidum*, subsequently went through water extraction and sequential alcohol precipitating method and purified by anion-exchange and gel filtration chromatography. It was eluted as a single and symmetrical sharp peak corresponding to an average molecular weight of 8000 Da as determined by high performance gel permeation chromatography. Sugar compositional analysis showed that it is only composed of D-glucose. Wang *et al.* [19] used a native *G. lucidum* polysaccharide in a linear, water-insoluble β-D-(1→3)-glucan to prepare sulfated polysaccharides. The molecular weight of *G. lucidum* polysaccharide was 133,000 Da. In addition, another water-soluble neutral polysaccharide was isolated from the fruiting bodies of *G. lucidum* by DEAE Sepharose Fast Flow and Sephacryl S-500 High Resolution Chromatography [20]. GC analysis showed that this polysaccharide was mainly composed of glucose and galactose in the molar ratio of 34:1 and its average molecular weight was approximately 2,500,000 Da. Contradictorily, there are two low-molecular weight of glucan purified from a crude *G. lucidum* polysaccharide preparation [21]. Their physiochemical properties marked a glucan with 5200 Da and another glucan with molecular weight 15,400 Da. The later was composed of glucose, galactose and mannose in a ratio of 29:1.8:1.0. This wide range of molecular weight of *G. lucidum* polysaccharides apparently clearly indicated that they are still abundant polysaccharides of *G. lucidum* yet to be discovered.

The molecular structure of *G. lucidum* polysaccharides which is associated closely with glycosidase linkage is another factor vitally influencing the bioactivities. The types of monosaccharide used to be the backbone play an important role as skeleton to support the other types monosaccharide as branches. Actually, it is a complex polymer upon which the different types of monosaccharides were involved plus the different position of carbon binding. For example, two peptidoglycans named Ganoderan B and C isolated from the fruiting body of *G. lucidum*, were shown to exhibit hypoglycemic efficacy with molecular weight 7400 and 5800 Da [22]. Chemical and physicochemical studies demonstrated that the backbone of ganoderan B contain D-glucopyranosyl β-1→3 and side chains with β-1→6 linkages. The backbone of Ganoderan C contains glucopyranosyl β-l→3 and side chains with β-1→6 linkages and a D-galactopyranosyl β-1→6 linkage.

3. Review of available *G. lucidum* polysaccharides extraction and fractionation methodology

Mushroom polysaccharides are present as structural components of fungal cell wall which is composed of two major types of polysaccharides. They are divided into a rigid fibrillar of

cellulose and a matrix-like glycoprotein, α-glucan or β-glucan [23]. Selection of mushroom polysaccharides extraction method normally relies on the cell wall structure. A reliable procedure for successful extraction of polysaccharides from either cultivar mycelia or fruiting body has been developed [24]. The extraction method commonly involves 80% ethanol for elimination of low molecular substances from mushroom material, followed by 3–5 successive repeating extractions with water (100°C, 2–4 h). The alternative choice is 5% sodium hydroxide (80°C, 6 h) or 2% ammonium oxalate (100°C, 6 h). In fact, the hot water extraction yields water-soluble polysaccharides, while the extraction with alkali solution is the best method to produce water-insoluble polysaccharides. Although various types of extraction method can be applied depending on the structure and water-solubility of polysaccharides, however, it is important to rupture the hard cell wall from the outer layer to the inner layer with weak-to-strong extraction conditions (pH and temperature). A combination of techniques should be chosen for further extraction of polysaccharides, normally ethanol precipitation will be the first choice as it excludes the impurities from the extracted polysaccharides. Other techniques included fractionation precipitation [20], acidic precipitation with acetic acid [21], ion-exchange chromatography [22], gel filtration [23] and affinity chromatography [24].

Comparing to the ancient era, definitely today's new technology is enhancing the yields of the extraction. A lot of new polysaccharides of *G. lucidum* have been discovered and reported to possess certain level of therapeutic efficacy [25, 26]. However, the method of polysaccharides extractions of this mushroom including the fractionation performed by the researchers are not standardized. Numerous methods resulted in the extraction of incorrect polysaccharides especially when using the hot water extraction method. The objective of this review paper is to discuss several polysaccharides extraction methods and verify these methods which include fractionation on the selected mushroom, *G. lucidum*.

3.1. Extraction and fractionation of a novel water-soluble β-D-glucan from the spores of *G. lucidum* using ethanol precipitation

Analysis was based on the conceptual framework proposed in five papers authored by Bao *et al.* from 2000 to 2002 [27] (please refer to **Figure 4**). The subject model was the spore of *G. lucidum* which was sporoderm-broken. With reference to the paper by Bao *et al.* [28], a novel water-soluble β-D-glucan was discovered after 10 years of study. Most of the steps of the extraction and fractionation were similar except that several procedures were modified in order to obtain better results. This illustrated that older methods could still work well and these methods could give rise to more effective methods that could lead to new discoveries.

Normally 95% ethanol is added at the beginning of extraction to remove the lipids especially after the sporoderm of the spores were broken. The exposure to the environment actually escalated the oxidation of the content of spores. Two types of spores breaking mechanisms are applicable nowadays. The traditional way, i.e., via mechanical vibration grinding resulted in the bioactive ingredients and unsaturated oil being directly exposed to the air, while the Supersonic Airflow Pulverizer would only break the outside hard chitinous layer and keep the inner covering layer complete [29]. The methodology in this study did not mention the types of spores breaking mechanism and storage period of sporoderm-broken spores before starting the extraction.

The defatted step, conducted twice, 5 days duration in each, would remove maximum lipid contents. After centrifugation, the residues, with most of the ethanol content being removed, have to be air-dried to ensure complete ethanol free. The hot water extraction is particularly useful to

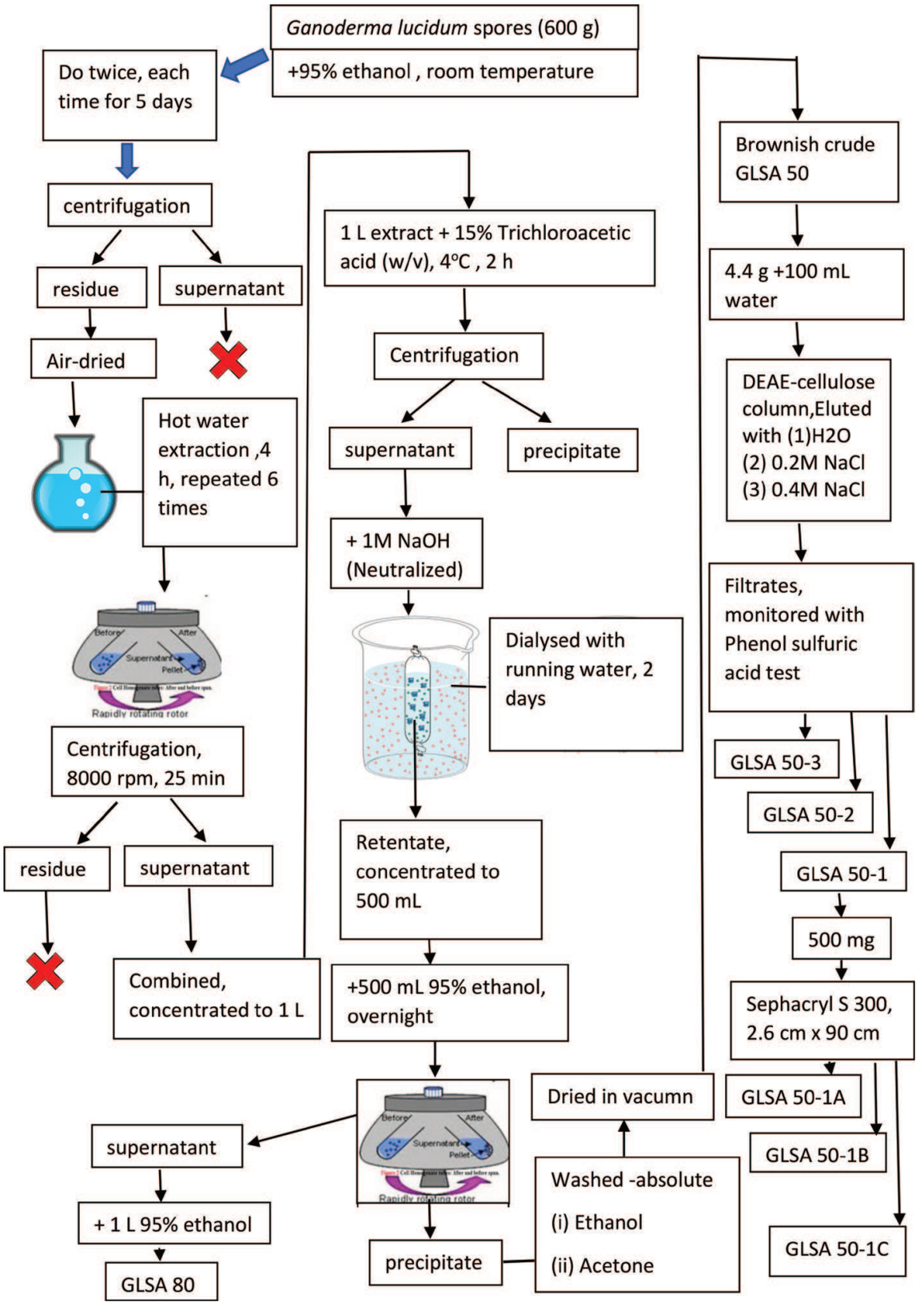

Figure 4. Flowchart of extraction, fractionation and isolation methodology of polysaccharides from *Ganoderma lucidum* spores.

extract water-soluble polysaccharides in the mushroom. Six replicates of extraction with boiling water with interval of 4 h each would allow the changing of new batch of hot water. This suggested that the bigger spin probe provided at the center of the container with appropriate speed would reduce the bubbles and increase the volume of spores with water. The extraction of substances with OH group under power of polarity of water and temperature could ensure the effective extraction of reishi spores polysaccharides. Therefore the accumulated 60 L of hot water filtrate needs to be centrifuged until 1 L of concentrated filtrate is obtained for further processing.

For this study, 15% of trichloroacetic acid (TCA) was used to procure protein precipitation reaction [30]. In fact, most of the protein components have been denatured during boiling. Continuously added TCA eliminated components that interrupted the polysaccharide purification process. The next step was dialysis of the supernatant with 1 M of sodium hydroxide aimed to neutralize the TCA. The dialysis was completed after 2 days under running water. However, Bao *et al.* [28] reported using 3 days of running water followed by 1 day of distilled water to remove components with molecular weight of 3000 Da. Later the concentrated retentate was stirred slowly and 95% ethanol was added at low temperature to promote polysaccharides precipitation. After centrifugation the precipitate was washed with two solvents before being dried in vacuum. Compared to previous study [28], the precipitate obtained was dissolved in water at room temperature, 0.5 L of ethanol was added and another round of centrifugation was conducted. The resulting brownish crude product was the key component used for further fractionation. The packed column with DEAE-cellulose (diethylaminoethyl) was used in anion-exchange chromatography. It achieves separation by using positively charged ion exchange matrix with an affinity for molecules having net negative surface charges [31]. The elution by using water and various affinity salts would be adequate to dissolve the polysaccharide components which are retained in the matrix. The fractions collected in tube were colorless, and the best determination method was the phenol-sulfuric acid method [32], since the yellowish end product could be used for the calibration graph based on absorbance at 490 nm versus number of fractions. The peak determined from the graph could be used to determine combination of fractions for further purification. Thus sephacyl-S-200HR [33] is used as size exclusion ordinal isolating each different polysaccharides based on their molecular weight. In this case, the compound labeled as GLSA50-1B was chosen for the characterization as a novel β-D-glucan.

The key aspect of this experiment was isolation of the pure polysaccharides and not total carbohydrates. Hence the definition of polysaccharide needs to be fully understood. There was confusion on the nature of polysaccharides when the experiment was terminated at the time when only accumulated filtrates were obtained after a few rounds of hot water extractions. The claim that polysaccharide could not be substantiated, when steps included protein-precipitation, dialysis, ion-exchange chromatography and size exclusion, were ignored. The center concept of polysaccharide is that it consists of the skeleton of the sugar backbone structure, the glycosidase linkage of branches, and specific rotation to indicate the configuration. The result of this study provided further support of new and significant new polysaccharides from mushroom spores.

3.2. Extraction and fractionation of *G. lucidum* polysaccharide from the mycelia of *G. lucidum* using agitation and filtration

The topic has mentioned "polysaccharides" which implied many types of polysaccharides. and this was frequently attributed to hot water extraction of mushroom resources [34] (please refer to **Figure 5**). The methodology described seems short and insufficient to determine the major characteristics of polysaccharides. To date, studies investigating *G. lucidum* polysaccharides have produced equivocal results that contributed to misunderstanding of polysaccharides. Firstly, when the extraction steps were carried out using hot water extraction and terminated after precipitation with 95% ethanol, most of the claimed polysaccharides (labeled as GLP) comprised total carbohydrates. In addition, majority of the polar compounds with plenty of –OH groups could possibly have been evacuated from the matrix. The experiment was unable to encompass the entire full scope of detailed molecular level of specific polysaccharides. Secondly the composition of GLP included alduronic acid (19.27%) and protein (5.39%). This scenario directly indicated the GLP content was not purely polysaccharides. The objectives of this study focused on efficacy of GLP treatment in plasma insulin concentration and gut microbiota composition in mice. There is notable paucity of empirical research focusing especially on polysaccharide.

The extraction started with pulverized *G. lucidum* mycelia with water. The process was repeated twice and the mixture was agitated for 4 h at 70–80°C. Comparing the common hot water extraction which provided 100°C of boiling water, it can be seen that the reduced temperature used was to avoid the formation of bubbles. The agitation was accomplished by continued shaking for 4 h. These mechanical activities are adjustable to ensure the extraction would be maximum. However, there is no mention on the speed of shaking in the experiment and the condition of the mycelia.

A variety of perspectives were expressed at the end of study. The striking result to emerge from the data was the positive and significance of GLP treatment. However, this finding cannot be extrapolated to determine the effectiveness of polysaccharides due to the impurity of GLP.

3.3. Extraction and fractionation of *G. lucidum* polysaccharides F31 from the fruiting body of *G. lucidum* using absolute ethanol at 4°C

Comparing with the common hot water extraction, this study applied 80°C instead of 100°C water [35] (please refer to **Figure 6**). Recent trends using temperature lower than 100°C have led to the preservation of protein content and elimination of bubble forming. One of the greatest challenges is the extraction of the polysaccharides with hot water, concurrently preventing the denaturation of the protein content. Typically, when the temperature is above 41°C, the protein will probably be melted but most of the peptides maintain their primary structures. Questions have been raised about the degree of denaturation of protein at temperature below 80°C compared to 100°C. Eventually the secondary and tertiary structures of protein will be destroyed under higher temperature. Heat could be a contributing factor for the production of end products during the process of water extraction. In this case, protein analysis had been

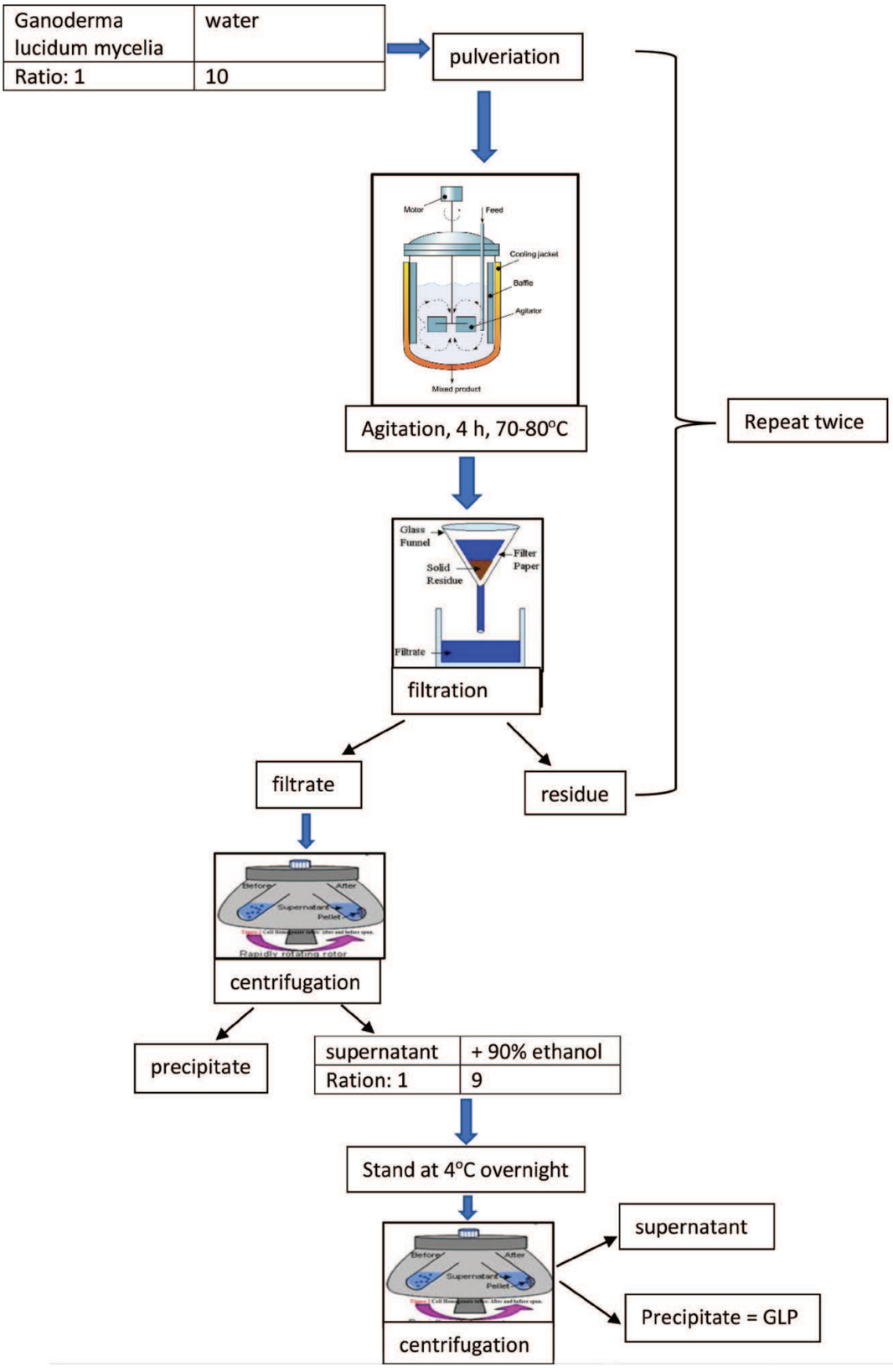

Figure 5. Flowchart of extraction, fractionation methodology of polysaccharides from *Ganoderma lucidum* mycelia.

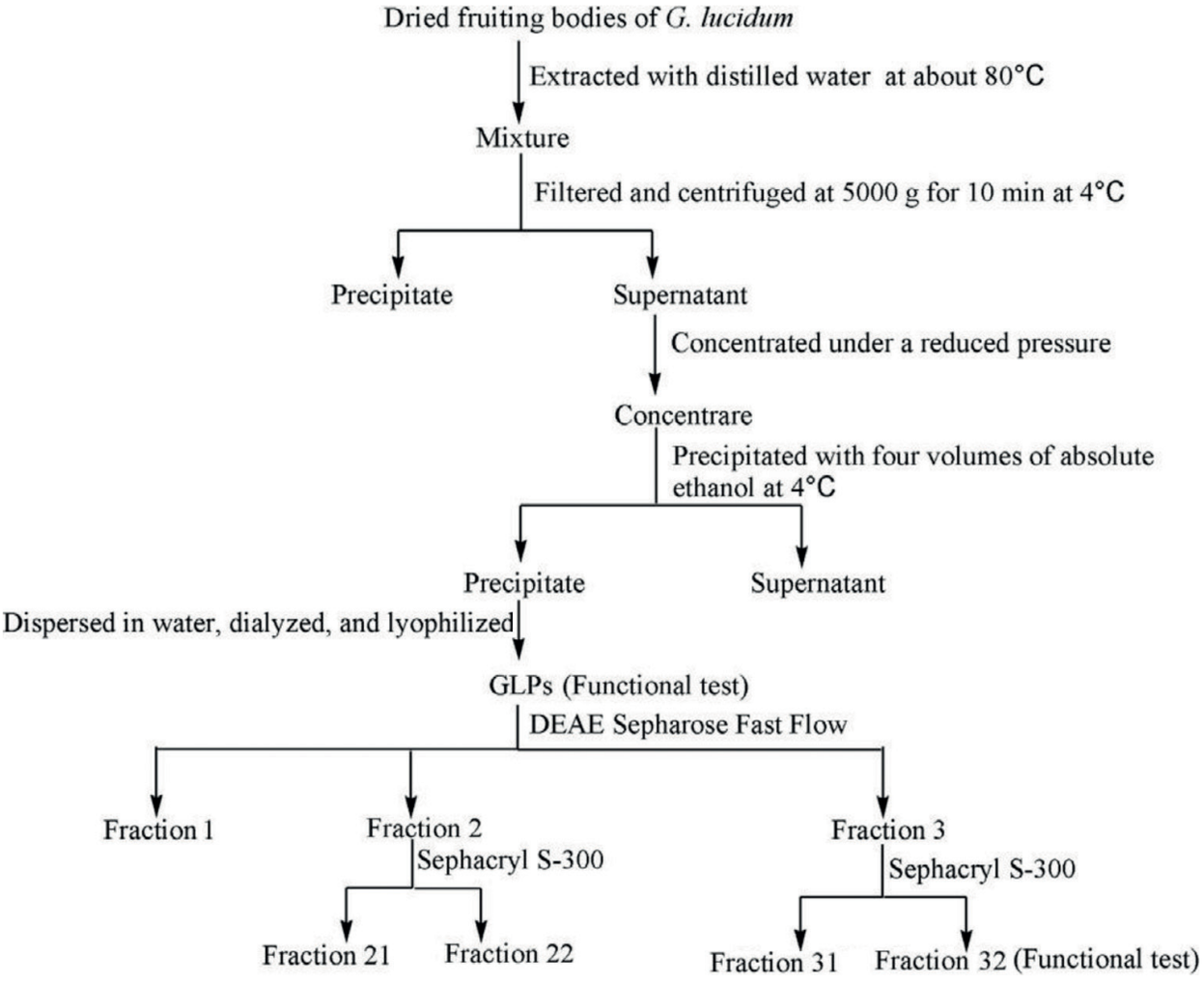

Figure 6. Flowchart of extraction, fractionation and isolation methodology of polysaccharides from *Ganoderma lucidum* fruiting body. The gel DEAE Sepharose Fast Flow and Sephacryl S-300 were the main tools to obtain the pure polysaccharides.

carried on during the process of extraction. Despite the fact that the main objective of the study was to accentuate the polysaccharides extraction, certain conditions were required to determine of protein or peptide content.

After the precipitation of GLPs by DEAE-sepharose Fast Flow, fractionation was conducted using affinity chromatography. The fractionation has been applied intensively with the purpose of obtaining single pure compound. The subsequent usage of Sephacryl-S-300 resin [36] as high resolution size exclusion chromatography allows rapid and reproducible purification of polysaccharides, proteins and other macromolecules. In this study, this resin purified the polysaccharides with fractionation range of globular polysaccharides size 1×10^4 to 1.5×10^6 Da.

The results showed that P31 has been successfully fractionated and characterized with subsequent determination of molecular weight. The different techniques of analytical results indicated that P31 is a pure compound. Thus, this study showed an example of obtaining pure polysaccharides for bioassay guided test.

3.4. Extraction and fractionation of high molecular weight bioactive-β-glucan from the fruiting body of *G. lucidum* using centrifugation and high performance anion exchange chromatography

Previous studies have reported that the fractionation for polysaccharides from mushroom sample normally started with hot water extraction [37] (please refer to **Figure 7**). This initial step, albeit old, ensures the polysaccharides are the main compounds obtained at the end of the extraction. The function of polysaccharide is largely based upon empirical studies that demonstrated a strong and consistent association with immune system of host.

Similar procedure of hot water extraction was used on dried dark brownish powder form of fruiting body and freeze-dried white powder (labeled GLP20 in this paper), compared to procedure for mycelia and spores. The minor differences of the temperature, duration of hot water extraction, speed and time of centrifugation and volume of ethanol could be due to the larger sample of volume being used.

The fractionation of GLP20 started when 2 M trifluoroacetic acid (TFA) was added at 110°C for 3 h. This colorless strong acid has a higher acid ionization constant compared to acetic acid and trichloroacetic acid, due to the fluorine atom exerting the inductive effect that strategically stabilizes the CO^{2-} charge of the carboxylic anion more than the other corresponding acids [38, 39]. Therefore the glycosidic linkage of GLP20 was broken down and hydrolyzed to monosaccharides composition. One possible implication of this is the separation of the sugar units of the polysaccharide as its large molecular structure, and later the determination of the monosaccharides which can be done with analytical column. However, another issue has to be considered. GLP20 was the product of the precipitation by ethanol. In this study, open column for further purification of GLP20 was not used, despite the fact that references were cited in the section of results on the purification by columns such as DEAE cellulose chromatography and Sephadex series size exclusion chromatography. The authors commented that this process was tedious and the yield of purified polysaccharides was low. The present studies yielded 0.37% (w/w) GLP20 on the basis of the dry weight of fruiting bodies. The mean 5.55 g of GLP20 was obtained by using 1500 g fruiting body of *G. lucidum*. If each batch of analysis needed 2 mg of GLP20, a total of 2775 times of analysis can be conducted.

The present study was over-reliance on self-initiated methodology. The advantages of using a series of chromatography for isolation at the final stage of fractionation are for the purpose of maximizing the purification. The use of 20% ethanol precipitation was supposed to precipitate all substrates with ―OH group. Even at the higher % of ethanol applied, there are increasing precipitation of substrates including polysaccharides.

The degree of purification of GLP20 was uncertainly proven. Thus the content of monosaccharides reported later did not appear to support the assumption of single polysaccharide obtained from this extraction, since the process of hydrolysis was not specific to determine the bonding of each sugar component. Generally, hydrolysis or saccharification is a step in the degradation of a polysaccharide substance. The cleavage of H―O bond reaction of cation and anion or both with water molecule takes place due to pH conditions [40]. It is possible that these reports were influenced by lack of information on glycosidic linkages. The final analysis step included total protein test.

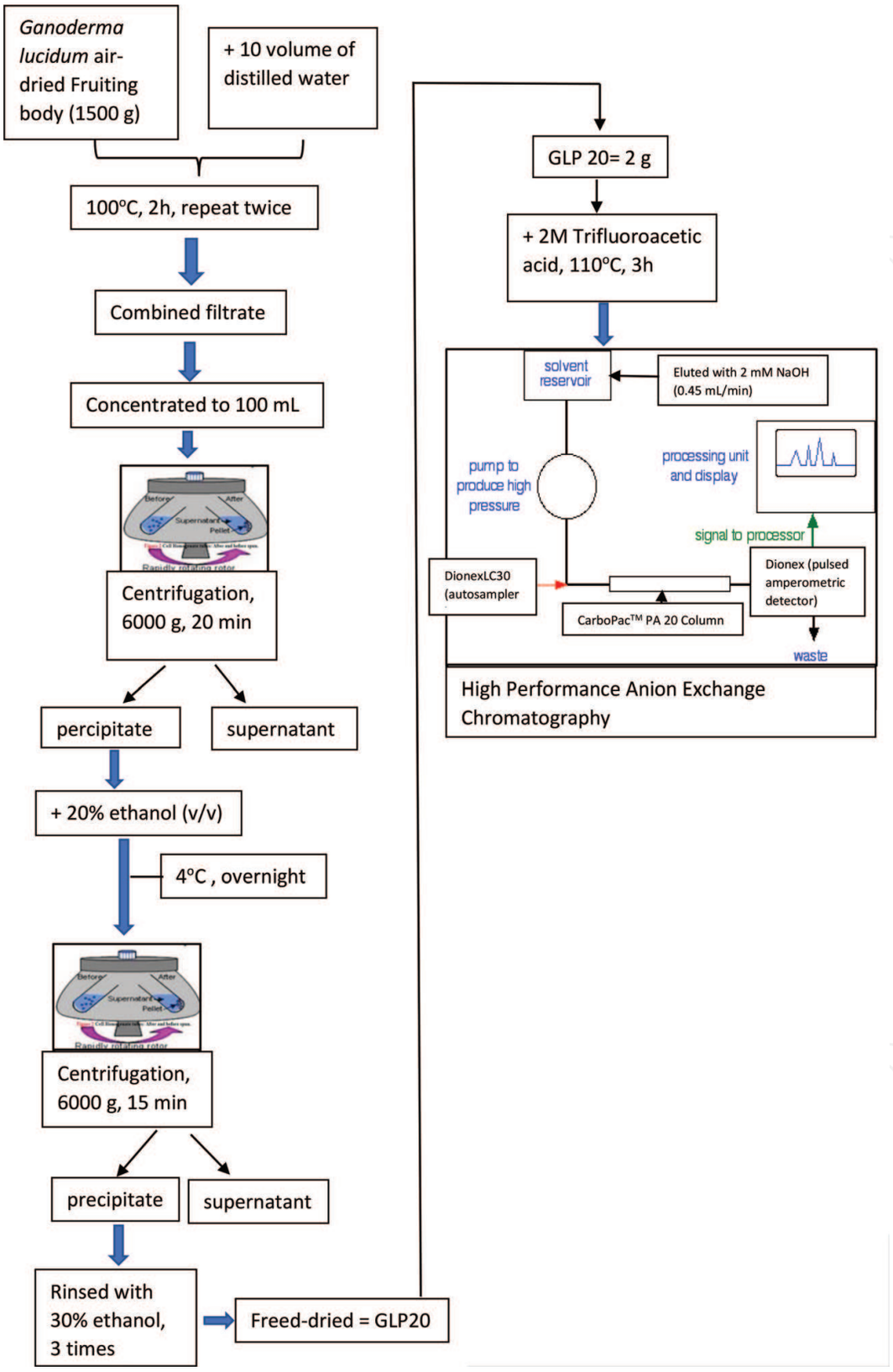

Figure 7. Flowchart of extraction, fractionation and isolation methodology of polysaccharides from *Ganoderma lucidum* fruiting body.

3.5. Extraction and fractionation of mushroom polysaccharides from the mycelia and fruiting body (dried of *G. lucidum* and *Poria cocos* using soxhlet extractor and anthrone sulfate)

Two types of mushroom have been used [41] (please refer to **Figure 8**). They were *G. lucidum* and *Poria cocos*. The methodology used was similarly applied on both mushrooms. Sample mushrooms were in the form of mycelia and fruiting body, respectively. In other words, the method aimed to produce total polysaccharide as mentioned in the article and there was no specific technique to produce targeted polysaccharide from either mycelia or fruiting body. There was no indication whether the samples were in fresh or dried condition, and it was assumed that 2 g of dried powder sample plus 90 mL of distilled water processed in soxhlet extractor. The final concentrated extract after distillation for few hours was diluted with distilled water. This step is important because the final reading of absorbance at 625 nm is limited at 3. When 10% volume of diluted soxhlet extract was added with ethanol, it was possible that the micro-molecules that existed during the soxhlet heating were eliminated. Hence the next step of chilling at 4°C provided ample time for the suspension of the unwanted debris. The speed and duration of centrifugation were commonly applied to most of precipitate. After separation, the precipitate still needs to be diluted for determination of carbohydrate by the anthrone method. The principle of anthrone method is that dehydrated carbohydrates in various form included monosaccharides, disaccharides, starch, gums, glycosides, dextrins and definite polysaccharides during the process will condense with anthrone to form a green color complex which can be measured colorimetrically at 625 nm.

This soxhlet extraction method is one of the practical ways to exclude types of carbohydrate from the mushroom. Therefore, the polysaccharides mentioned in the chapter would refer to various forms of carbohydrates which included polysaccharides as one of them. The result and conclusion has positively quoted the significance of said production of polysaccharides potentially acting as prebiotics by manipulating gut microbiota composition. It was decided that the best method to adopt for this investigation was to provide a platform for beneficial bacteria associated with polysaccharides treatment, making these polysaccharides candidate prebiotics. However, there was a lack of result specifically on types of polysaccharides from both basidiomycetes.

3.6. Extraction and fractionation of three kinds of *G. lucidum* polysaccharides from the fermentation broth, mycelia, fruiting body using open column with resin DEAE-Sephadex A-25 and Sepharose 6B

Three different life stages of *G. lucidum* were involved in this study [42] (please refer to **Figure 9**). Since it takes around 3 months for cultivation of *G. lucidum* fruiting body, the mycelia-based and broth-based products have assumed extraordinary importance because of the requirement for heightened quality-control and year-round production [43]. The processes and the different types of growth parameters including temperature and pH concerned in submerged mycelial medium, are simple to be standardized under controlled circumstances. In addition, the purification and other succeeding processing of active components such as polysaccharides excreted into the culture medium normally involve relatively simple procedures. The cultivation

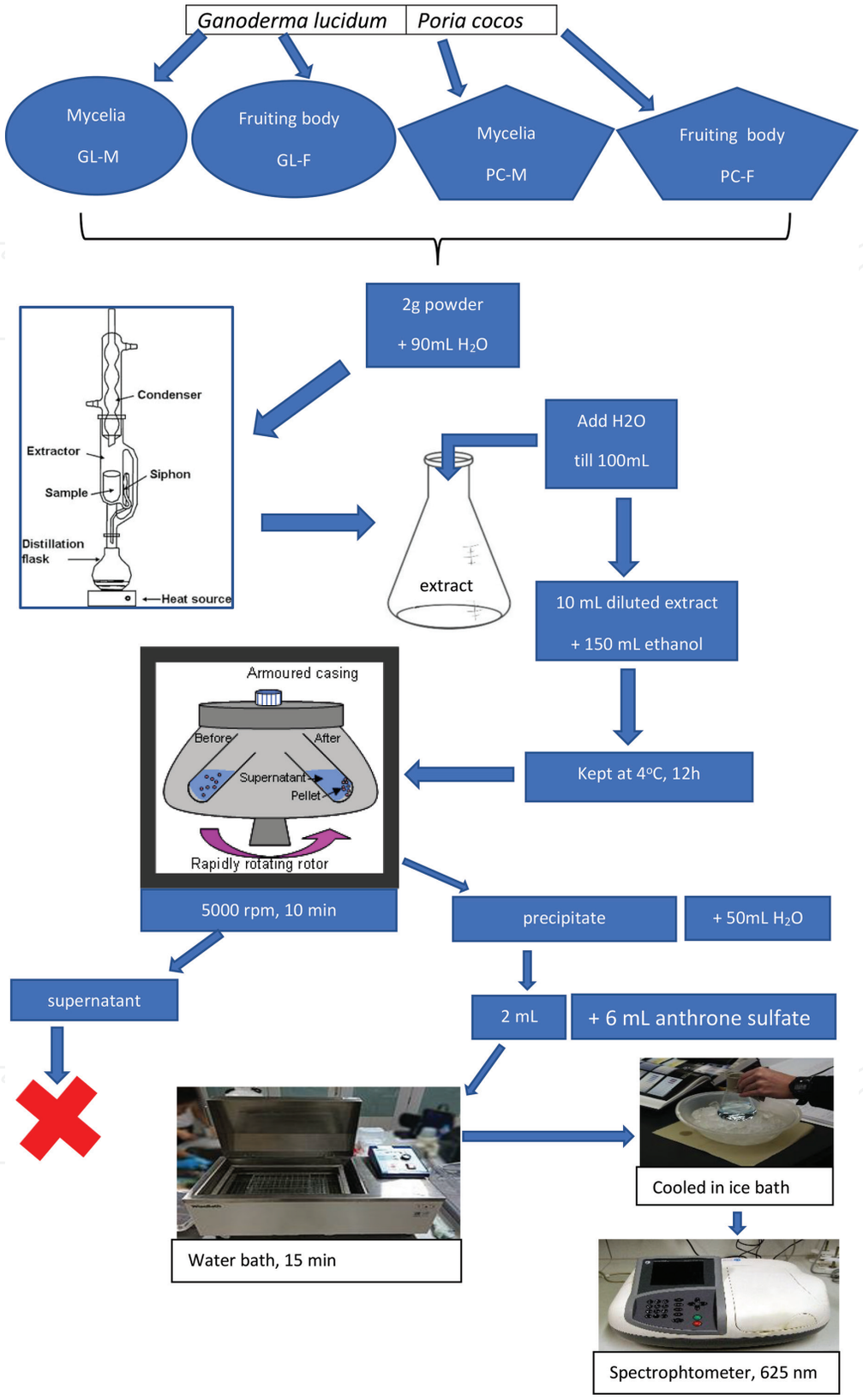

Figure 8. Flowchart of extraction, fractionation methodology of polysaccharides from *Ganoderma lucidum* and *Poria cocos*, fruiting body and mycelia.

http://dx.doi.org/10.5772/intechopen.78047

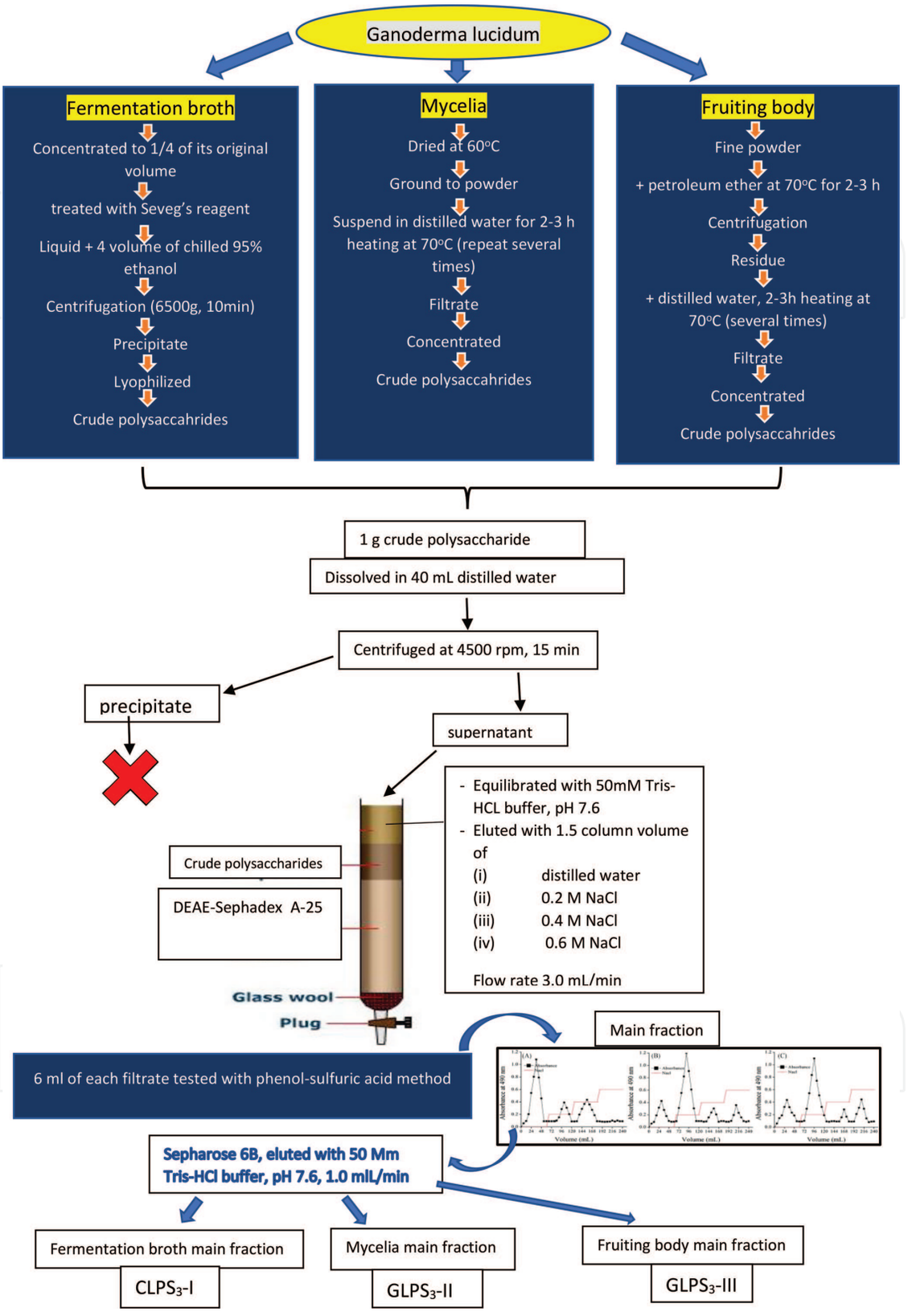

Figure 9. Flowchart of extraction, fractionation and isolation methodologies of polysaccharides from *Ganoderma lucidum* fermentation broth, mycelia and fruiting body.

environment and the ingredient of medium have also been reported to strongly impact the growth of mycelia and the production of exopolysaccharides [44]. A report by Yang and Liau [45] noted that production of *G. lucidum* polysaccharides by fermenter-grown mycelia cultivar was optimum at pH of 4–4.5 and temperature 30–35°C. Acceleration of mycelial growth and bioactive components could be produced by addition of supplements such as fatty acids.

Three types of dissimilar pre-treat ways were applied on the different life stages of mushroom before the hot water extraction. An investigation of the non-volatile composition of *G. lucidum* [46] reported that the mushroom contains 59% crude fiber, 26–28% carbohydrate, 7–8% crude protein, 3–5% crude fat and 1.8% ash. Nonetheless, a wide variety of bioactive molecules are also found in *G. lucidum* which include terpenoids, phenols, nucleotides and their derivatives, steroids, glycoproteins and polysaccharides. *G. lucidum* proteins contain most of the essential amino acids and are especially affluent in leucine and lysine. The fermentation broth contained media, mycelia and spores. After concentration, the volume of liquid was reduced. Addition of Sevag's reagent for the purpose of deproteinization could eliminate the protein ingredient in the mixture. Xia Li *et al.* [47] proposed an explanatory theory of the advantage of using Sevag's reagent. With regards to deproteinization rate and polysaccharide residual rate, the process of optimization, was based on the following parameters: deproteinization frequency (example: 4 times), time of oscillating (example: 11 min), the volume ratio of sample to Sevag's reagent (2:1), and the volume ratio of chloroform to n-butanol (5:1), The resulting deproteinization rate was 64.2%, the polysaccharide loss rate was 34.3%, and the purity of polysaccharide was raised by about 2.8 times. Compared to other stage of cultivation, mycelia of *G. lucidum* were treated simply and without any step of deproteination and removal of fatty substances. One of the reasons for these could be that at the initial stage of mycelial mat forming, little protein was available. Excluding the step of deproteination allowed the hot water extraction at 70°C to be conducted directly and shorten the time for processing polysaccharides. Similarly, deproteination for fruiting body powder is also not required. Instead, petroleum ether is used to remove fatty and fat-soluble substances. The high proportion of polyunsaturated fatty acids and low total fat content relative to the total fatty acids of *G. lucidum* are considered significant contributors to the heath value of *G. lucidum*. [48]. The residues accumulated after the defatted step would allow the hot water extraction to proceed.

In this case, the main concern at the end of experiment was isolation of polysaccharides. As in other studies, from the accumulated filtrates of several replicates of hot water extraction, the precipitate treated by addition of ethanol and freeze-dried would contain crude polysaccharides, indicating that several types of polysaccharides are found in the crude. The subsequent step in this study was the application of DEAE-Sephadex A-25 column as anion exchange chromatography intended for the further separation of each type of crude polysaccharides. According to the data file of ion exchange [49], Sephadex ion exchangers are derived from either Sephadex G-50 or Sephadex G-25. The G-50 matrix is less highly cross-linked than the G-25. Ion-exchangers based on Sephadex-50 have less rigidity and thus swell more than those based on G-25, which are more rigid. These properties mean that A-50 and C-50 types are better suited to larger biomolecules, such as polysaccharides or proteins in the molecular weight range of 30,000–100,000 MW, whereas A-25 and C-25 type ion exchangers are a better choice for small molecules up to about 30,000 MW.

In this stage of fractionation, Sephadex A-25 was used instead of A-50 and followed by another stage of fractionation with Sepharose 6B. Hence, most of the molecules more than 30,000 MW could pass through. In order to completely isolate the polysaccharides, the main fraction of each stage of *G. lucidum* was successfully eluted with 50 mM Tris-HCl buffer.

The excellent results presented in this study illustrated the effective methodology for purification of polysaccharides from *G. lucidum* using three different life stages. The dissimilar way of pre-treating the sample in the beginning has potentially separated all the polysaccharides. The results clearly showed each main isolated polysaccharide contained different total sugar content, monosaccharides composition, molecular weight and the bioactivity. This study had successfully isolated the main polysaccharides from fermentation broth, mycelia and fruiting body of *G. lucidum*.

4. Fractionation and its application in the extraction of polysaccharides from the mushroom species, *G. lucidum*

The discovery of new polysaccharides from *G. lucidum* has long been a research of great interest in a wide range of field. Bao *et al.* [50] reported six different functionalized derivatives of the (1→3)–α-D-glucan which were isolated from the spores of *G. lucidum*. The process included aminopropylated, sulfated, carboxymethylated, carboxymethylated and sulfated, and benzylamidated-carboxymethylated with varying degrees of substitution synthesized. These modified derivatives have shown potent stimulating effects on the lymphocyte proliferation and antibody production. On the other hand, the induction of carboxymethyl group with low degree of substitution was the best choice for the improvement of the immunostimulating activity. A one-step to prepared selenium nanoparticles (SeNPs) decorated by the water–soluble derivative of *G. lucidum* polysaccharides (SPS) was tested on their anti-inflammatory activity against murine RAW264.7 macrophage cells induced by lipopolysaccharides [51]. The results suggested that seNPs-SPS complexes possessed anti-inflammatory potential modulating pro/anti-inflammation cytokine secretion profiles. The isolated polysaccharide (molecular weight 1×10^6 Da) isolated from the sporocarps of *G. lucidum* was used to determined radioprotective property *in vivo* and *in vitro* by survival studies [52]. The findings indicated *Ganoderma* polysaccharides were significantly protective against radiation-induced damages. Another study showed positive effect of polysaccharides extracted from *G. lucidum* on blood glucose, serum level, lipid peroxidation, nonenzymaic and enzymic antioxidants in the plasma and liver of streptozotocin-induced diabetic rats [53]. The neutral crude polysaccharides extracted from *G. lucidum* fruiting body claimed by Chen *et al.* [54] exhibited the higher DPPH-, O- and OH- free radical scavenging activities. It could significantly enhance the antioxidant enzyme activities (SOD, CAT and GPx) and reduce levels of IL-1β, IL-6 and TNF-α in rats with cervical cancer. Two fractions purified from the fruiting body of *G. lucidum* were tested on activation of macrophage cell (RAW 264.7) and antitumor activities on the human breast cancer cell (MDA-MB-231) [55]. The results indicated that both fractions increased the proliferation and pinocytic activity of macrophage significantly and played an inhibiting effect on the cancer cell.

5. Conclusion

The method of *G. lucidum* polysaccharides extraction and fractionation varied greatly according to the objectives of each study. This targeted end products and the yields of polysaccharides extraction normally started with hot water extraction. The temperature applied could be lower than boiling point of water in order to preserve the proteins. Such temperature can soften some fibers and the water molecules in the liquid phase are easily absorbed by the mushroom cells. Therefore, most of the initial steps of polysaccharide extraction need to ensure the physical form of resources are pulverized to obtain the maximum effects on the samples, since the polysaccharides contain many ―OH groups which can be easily extracted by the water. Although most of the methodology of the *G. lucidum* polysaccharides extraction and fractionation are different they all followed a similar principle, i.e., using ethanol to precipitate the main polysaccharides. However, at this stage, the extract is still considered crude. Some reported studies would terminate at this stage and use the crude for therapy. In fact, the extraction should continue to the stage of purification in order to separate the different types of polysaccharides in crude. This paper has reviewed six different types of fractionations from different media of *G. lucidum.* All these studies reported positive results in accordance to the study objective(s).

Acknowledgements

The authors thank the Director General of Health, Malaysia for the permission to publish and the Director of Institute for Medical Research (IMR), Kuala Lumpur for the support. This work was financially supported by the Ministry of Health Research Grant Scheme (Grant ID: NMRR-11-953-10736). The authors also thank Prof. Vikineswary (University of Malaya), Dr. Wan Abd Al Qadr Imad Wan mothar (UM) and Dr. Rahayu Ahmad (MIP) for critically reviewing this chapter.

Author details

Yew-Keong Choong[1]*, Kavithambigai Ellan[2], Xiang-Dong Chen[3] and Shaiful Azuar Mohamad[4]

*Address all correspondence to: yewkeong11@yahoo.co.uk

1 Phytochemistry Unit, Herbal Medicine Research Center, Institute for Medical Research, Jalan Pahang, Kuala Lumpur, Malaysia

2 Virology Unit, Infectious Disease Research Center, Institute for Medical Research, Jalan Pahang, Kuala Lumpur, Malaysia

3 Institute for Medical Research, Jalan Pahang, Kuala Lumpur, Malaysia

4 Malaysia Nuclear Agency, Kajang, Selangor, Malaysia

References

[1] Ikekawa T, Uehara N, Maeda Y, Nakanishi M, Fukuoka F. Antitumor activity of aqueous extracts of edible mushrooms. Cancer Research. 1969;**29**:734-735

[2] Rahar S, Swami G, Nagpal N, Nagpal MA, Singh GS. Preparation, characterization, and biological properties of β-glucans. Journal of Advanced Pharmaceutical Technology & Research. 2011;**2**:94-103

[3] Kobayashi H, Matsunaga K, Oguchi Y. Antimetastatic effects of PSK (Krestin), a protein-bound polysaccharide obtained from basidiomycetes: An overview. Cancer Epidemiology, Biomarkers & Prevention. 1995;**4**(3):275-281

[4] Zeng D, Zhua S. Purification, characterization, antioxidant and anticancer activities of novel polysaccharides extracted from Bachu mushroom. International Journal of Biological Macromolecules. 2018;**107**:1086-1092

[5] Liu M, Li S, Wang X, Zhu Y, Zhang J, Liu H, Jia L. Characterization, anti-oxidation and anti-inflammation of polysaccharides by *Hypsizygus marmoreus* against LPS-induced toxicity on lung. International Journal of Biological Macromolecules. 2018;**111**:121-128

[6] Wang J, Wang C, Li S, Li W, Yuan G, Pan Y, Chen H. Antidiabetic effects of *Inonotus obliquus* polysaccharides in streptozotocininduced type 2 diabetic mice and potential mechanism via PI3K-Akt signal pathway. Biomedicine & Pharmacotherapy. 2017;**95**:669-1677

[7] Yang L, Zhang J, Meng Z. Purification, characterization and anti-tumor activities of polysaccharides extracted from wild *Russula griseocarnosa*. International Journal of Biological Macromolecules. 2018;**109**:1054-1060

[8] Hea X, Wang X, Fang J, Chang Y, Ning N, Guo H, Huang L, Huang X, Zhao Z. Structures, biological activities, and industrial applications of the polysaccharides from *Hericium erinaceus* (Lion's mane) mushroom: A review. International Journal of Biological Macromolecules. 2017;**97**:228-237

[9] Daba AS, Ezeronye OU. Anti-cancer effect of polysaccharides isolated from higher basidiomycetes mushrooms. African Journal of Biotechnology. 2003;**2**(12):672-678

[10] Zhang J, Meng G, Zhai G, Yang Y, Zhao H, Jia L. Extraction, characterization and antioxidant activity of polysaccharides of spent mushroom compost of *Ganoderma lucidum*. International Journal of Biological Macromolecules. 2016;**82**:432-439

[11] Nagaia K, Uenob Y, Tanakab S, Hayashib R, Shinagawaa K, Chayama K. Polysaccharides derived from *Ganoderma lucidum* fungus mycelia ameliorate indomethacin-induced small intestinal injury via induction of GM-CSF from macrophages. Cellular Immunology. 2017;**320**:20-28

[12] Liu Y, Tang Q, Zhang J, Xia Y, Yang Y, Di W, Cui SW. Triple helix conformation of β-D-glucan from *Ganoderma lucidum* and effect of molecular weight on its immunological activity. International Journal of Biological Macromolecules. Available online 13 March 2018

[13] Cao LZ, Lin ZB. Regulatory effect of *Ganoderma lucidum* polysaccharides on cytotoxic T-lymphocytes induced by dendritic cells in vitro. Acta Pharmacologica Sinica. 2003;**24**:321-326

[14] You YH, Lin ZB. Protective effects of *Ganoderma lucidum* polysaccharides peptide on injury of macrophages induced by reactive oxygen species. Acta Pharmacologica Sinica. 2002;**23**:787-791

[15] Giavasis I. Bioactive fungal polysaccharides as potential functional ingredients in food and nutraceuticals. Current Opinion in Biotechnology. 2014;**26**:162-173

[16] Ho YS, Yu MS, Yang XF, So KF, Yuen WH, Chang RC. Neuroprotective effects of polysaccharides from wolfberry, the fruits of *Lycium barbarum*, against homocysteine-induced toxicity in rat cortical neurons. Journal of Alzheimer's Disease. 2010;**19**(3):813-827

[17] Ai-Lati A, Liu S, Ji Z, Zhang H, Mao J. Structure and bioactivities of a polysaccharide isolated from Ganoderma lucidum in submerged fermentation. BIOENGINEERED. 2017;**8**(5):565-571

[18] Guo L, Xie J, Ruan Y, Zhou L, Zhu H, Yun X, Jiang Y, Lü L, Chen K, Min Z, Wen Y, Gu J. Characterization and immunostimulatory activity of a polysaccharide from the spores of *Ganoderma lucidum*. International Immunopharmacology. 2009;**9**:1175-1182

[19] Wang J, Zhang Y, Yuan Y, Yue T. Immunomodulatory of selenium nano-particles decorated by sulphated *Ganoderma lucidum* polysaccharides. Food and Chemical Toxicology. 2014;**68**:183-189

[20] Huang S-Q, Li J-W, Li Y-Q, Wang Z. Purification and structural characterization of a new water-soluble neutral polysaccharide GLP-F1-1 from *Ganoderma lucidum*. International Journal of Biological Macromolecules. 2011;**48**:165-169

[21] Liu W, Wang H, Pang X, Yao W, Gao X. Characterization and antioxidant activity of two low-molecular-weight polysaccharides purified from the fruiting bodies of *Ganoderma lucidum*. International Journal of Biological Macromolecules. 2010 May 1;**46**(4):451-457

[22] Tomoda M, Gonda R, Kasahara Y, Hikino H. Glycan structures of ganoderans b and c, hypoglycemic glycans of *Ganoderma lucidum* fruit bodies. Phytochemistry;**25**(12):2817-2820

[23] Ruiz-Herrera J. Fungal Cell Wall: Structure, Synthesis, and Assembly. Boca Raton, Ann Arbor, London: CRC Press; 1956

[24] Mizuno T. Development of antitumor polysaccahrides from mushroom fungi. Food and Food Ingredient Japanese Journal. 1996;**167**:69-87

[25] Pan D, Wang L, Chen C, Teng B, Wang C, Xu Z, Hu B, Zhou P. Structure characterization of a novel neutral polysaccharide isolated from *Ganoderma lucidum* fruiting bodies. Food Chemistry. 2012;**135**:1097-1103

[26] Jiang H, Sun P, He J, Shao P. Rapid purification of polysaccharides using novel radial flow ion-exchange by response surface methodology from *Ganoderma lucidum*. Food and Bioproducts Processing. 2012;**90**:1-8

[27] Dong Q, Wang Y, Shi L, Yao J, Li J, Ma F, Ding K. A novel water-soluble b-D-glucan isolated from the spores of *Ganoderma lucidum*. Carbohydrate Research. 2012;**353**(2012):100-105

[28] Bao XF, Liu CP, Fang JN, Li XY. Carbohydrate Research. 2001;**332**:67-74

[29] http://tcmadaptogen.com/msg.php?id=310

[30] Rajalingam D, Loftis C, Jiashou JX, Kumar TKS. Trichloroacetic acid-induced protein precipitation involves the reversible association of a stable partially structured intermediate. Protein Science. 2009;**18**(5):980-993

[31] http://www.bio-rad.com/en-my/applications-technologies/anion-exchange-chromatography

[32] DuBois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. Anlytical Chemistry. 1956;**28**:350-356

[33] https://www.gelifesciences.com/shop/chromatography/resins/size-exclusion/sephacryl-s-200-hr-p-05621?current=17058410

[34] Xu S, Dou Y, Ye B, Wu Q, Wang Y, Hu M, Ma F, Rong X, Guo J. *Ganoderma lucidum* polysaccharides improve insulin sensitivity by regulating inflammatory cytokines and gut microbiota composition in mice. Journal of Functional Foods. 2017;**38**(2017):545-552

[35] Xiao C, Wu Q, Zhang J, Xie Y, Cai W, Tan J. Antidiabetic activity of *Ganoderma lucidum* polysaccharides F31 downregulated hepatic glucose regulatory enzymes in diabetic mice. Journal of Ethnopharmacology. 2017;**196**(2017):47-57

[36] https://www.gelifesciences.com/shop/chromatography/resins/size-exclusion/sephacryl-s-300-hr-p-05620?current=17059901

[37] Liu Y, Zhang J, Tang Q, Yang Y, Guo Q, Wang Q, Wu D, Cui SW. Physicochemical characterization of a high molecular weight bioactive-β-glucan from the fruiting bodies of *Ganoderma lucidum*. Carbohydrate Polymers. 2014, January 2014;**101**(30):968-974

[38] https://en.wikipedia.org/wiki/Trifluoroacetic_acid

[39] https://www.quora.com/Which-one-is-more-acidic-trichloroacetic-acid-or-trifluoroacetic-acid

[40] https://en.wikipedia.org/wiki/Hydrolysis

[41] Khan I, Huang G, Li X, Leong W, Xia W, Wendy Hsiao WL. Mushroom polysaccharides from *Ganoderma lucidum* and *Poria cocos* reveal prebiotic functions. Journal of Functional Foods. 2018;**41**:191-201

[42] Li K, Yu M, Hu Y, Ren G, Zang T, Xu X, Qu J. Three kinds of *Ganoderma lucidum* polysaccharides attenuate DDC-induced chronic pancreatitis in mice. Chemico-Biological Interactions. 2016;**247**:30-38

[43] Sanodiya BS, Thakur GS, Baghel RK, Prasad GB, Bisen PS. Ganoderma lucidum: A potent pharmacological macrofungus. Current Pharmaceutical Biotechnology. 2009;**10**(8):717-742

[44] Boh B, Berovic M, Zhang J, Zhi-Bin L. *Ganoderma lucidum* and its pharmaceutically active compounds. Biotechnology Annual Review. 2007;**13**:265-301

[45] Yang FC, Liau CB. The influence of environmental conditions on polysaccharide formation by *Ganoderma lucidum* in submerged cultures. Process Biochemistry. 1998;**33**:547-553

[46] Mau JL, Lin HC, Chen CC. Non-volatile components of several medicinal mushrooms. Food Research International. 2001;**34**:521-526

[47] Li X, Zhao R, Zhou HL, Wu DH. Deproteinization of polysaccharide from the stigma maydis by sevag method. Advanced Materials Research. 2012;**340**:416-420

[48] Borchers AT, Stern JS, Hackman RM, Keen CL, Gershwin ME. Minireview: Mushrooms, tumors and immunity. Proceedings of the Society for Experimental Biology and Medicine. 1999;**221**:281-293

[49] http://www.chembio.uoguelph.ca/educmat/chm357/sephadex.pdf

[50] Bao XF, Duan J, Fang X, Fang J. Chemical modifications of the (1→3)-α-D-glucan from spores of *Ganoderma lucidum* and investigation of their physiochemical properties and immunological activity. Carbohydrate Research. 2001;**336**:127-140

[51] Wang J, Zhang Y, Yuan Y, Yue T. Immunomodulatory of selenium nano-particles decoreated by sulfated *Ganoderma lucidum* polysaccharides. Food and Chemical Toxicology. 2014;**68**:183-189

[52] Pillai TG, Nair CKK, Janardhanan KK. Polysaccharides isolated from *Ganoderma lucidum* occurring in southern part of India, protects radiation induced damages both in vitro and in vivo. Environmental Toxicology and Pharmacology. 2008;**26**:80-85

[53] Jia J, Zhang X, Hu Y-S, Wu Y, Wang Q-Z, Li N-N, Guo Q-C, Dong X-C. Evaluation of in vivo antioxidant activities of*Ganoderma lucidum* polysaccharides in STZ-diabetic rats. Food Chemistry. 2009;**115**(1):32-36

[54] Ping CX, Yan C, Bing LS, Guo CY, Yun LJ, Ping LL. Free radical scavenging of *Ganoderma lucidum* polysaccharides and its effect antioxidant enzymes and immunity activities in cervical carcinoma rats. Carbohydrate Polymers. 2009;**77**:389-393

[55] Zhao L, Dong Y, Chen G, Hu Q. Extraction, purification, characterization and antitumor activity of polysaccharides from *Ganoderma lucidum*. Carbohydrate Polymers. 2010;**80**:783-789