# Detection of a Non-animal Source of Glycosaminoglycans from Edible Mushrooms in Northern Thailand

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## ABSTRACT

Several kinds of cultivated or local edible mushrooms exist in northern Thailand. While their nutritional features have been extensively analyzed, the presence of phytochemicals and glycosaminoglycans has yet to be investigated. Thus, in this study, we examined the presence of phytochemicals and glycosaminoglycans from 10 cultivated or local mushrooms: milk white russula, hygroscopic earthstar, log white fungi, log black fungi, shitake mushroom, rosy russula, sajor-caju mushroom, Jew's ear, bolete, and straw mushroom. Phytochemical analysis revealed the presence of carbohydrates, amino acids, and flavonoids in the extracts from most of the mushrooms. We detected glycosaminoglycans in all of the extracts using dimethylmethylene blue (DMMB) dye-binding assay and UV-Vis spectrophotometry. Hygroscopic earthstar contained the most glycosaminoglycans and sajor-caju mushroom the least. However, we were unable to distinguish the types of glycosaminoglycans, which should be considered for future study. In summary, the phytochemicals found here are crucial non-animal dietary sources of carbohydrates, amino acids, and antioxidants. In addition, the glycosaminoglycans detected in the mushroom extracts in this study potentially offer a non-animal source of glycosaminoglycans that may have clinical applications, apart from their nutritive value.

**Keywords:** Mushrooms, Phytochemicals, Glycosaminoglycans, Dye-binding assay, Spectrophotometry

## INTRODUCTION

Mushrooms are classified in the kingdom of fungi (Whittaker, 1969). Some edible species have been recognized for their health benefits (Flegg and Maw, 1997). Mushrooms have high nutritional value (Mattila et al., 2001; Barros et al., 2008; Reis et al., 2012; Carneiro et al., 2013); they are low-fat, high in protein, and contain vitamins, minerals, and trace element constituents with nutraceutical composition. Of the nutraceuticals, homopolysaccharides are the best known molecules; they play an essential role as an immunomodulator (Manzi and Pizzoferrato, 2000; Mayell, 2001; Vetvicka and Yvin, 2004), as well as exhibit anticancer, antibacterial, antioxidant, and anti-inflammatory properties (Yu et al., 2009; Zhang et al., 2011; Chang and Wasser, 2012; Finimundy et al., 2013). Therefore, mushrooms are not only a component of a balanced diet, but also provide key medicinal uses in the treatment of certain diseases.

Besides homopolysaccharides, most carbohydrates that have been found in mushrooms are monosaccharides, disaccharides, or sugar derivatives, such as, glucose, fructose, sucrose, inositol, mannitol, and sorbitol (Sanmee et al., 2003; Reis et al., 2012; Carneiro et al., 2013). However, glycosaminoglycans (GAGs), another type of polysaccharide, have yet to be investigated in mushrooms. GAGs are unbranched heteropolysaccharides containing a repeating disaccharide unit. They are composed of sugar acid and amino sugar; usually, one sugar is an uronic acid and the other is either N-acetylglucosamine (GlcNAc) or N-acetylgalactosamine (GalNAc). Various kinds of GAGs, including chondroitin sulfate, dermatan sulfate, heparin, heparan sulfate, and keratan sulfate (Kjellén and Lindahl, 1991; Varki et al., 2009) are bound to sulfate. Thus, the negative charge in various positions along the GAG chains is due to the sugar acid residues and/or sulfate groups (Prydz and Dalen, 2000). GAGs have been known to play many roles in both physiological and pathological conditions or diseases, including signaling activity during chondrocyte differentiation (Choocheep et al., 2010), angiogenesis, anti-coagulation, axonal growth, wound healing, pulmonary and vascular diseases, and tumor progression (Gandhi and Mancera, 2008). Additionally, chondroitin sulfate and hyaluronic acid have been reported to suppress cartilage degradation for the treatment of osteoarthritis (Monfort et al., 2005). Heparin also has important roles as an anti-coagulant for the treatment of thrombosis, thrombophlebitis and embolism (Gandhi and Mancera, 2008). Other GAGs, such as heparan sulfate, can act as receptors for proteases and protease inhibitors, or cooperate with integrins and other cell adhesion receptors to facilitate cell activities (Sarrazin et al., 2011), and keratan sulfate plays an essential role in visual functions (Pomin, 2015).

Mushrooms are a traditional ingredient in Thai cuisine. In addition, several kinds of cultivated or local edible mushrooms exist in northern Thailand, such as *Astraeus hygrometricus, Lentinus polychrous, Phaeogyroporus portentosus*, and *Russula* species (Sanmee et al., 2003; Valverde et al., 2015). Although the nutritive value of these mushrooms has been investigated, the presence of phytochemicals and GAGs has not been examined. Hence, the aim of this study was to examine the presence of phytochemicals and GAGs from 10 cultivated or local edible mushrooms.

## **MATERIALS AND METHODS**

#### **Collection and preparation of mushroom extracts**

The mushrooms were bought from markets in Wiang Pa Pao district, Chiang Rai province, or Mueang district, Chiang Mai province, both in northern Thailand. Ten fresh fruiting bodies of cultivated or local edible mushrooms – milk white russula, hygroscopic earthstar, log white fungi, log black fungi, shitake mushroom, rosy russula, sajor-caju mushroom, Jew's ear, bolete, and straw mushroom – were weighed, cut into small pieces, and ground by grinding machine. Then, 100 grams of each mushroom sample was extracted as previously described (Nakano et al., 2000), with some modifications. Briefly, the mushrooms were soaked in a water bath of deionized water of pH 4.5 at 37°C for 18 hours. Then, the extracts were boiled for 10 minutes, filtered through a muslin cloth and Whatman filter papers, and centrifuged. Next, the supernatant of the extracts was collected, and the same extracts were pooled together. These crude mushroom extracts were then freeze-dried to yield a powder, and stored at -20°C until use.

# Yield of mushroom extracts powder

The yield of each mushroom extract was calculated as follows: yield (%) = (weight of fresh mushrooms/weight of mushrooms powder) x 100.

# **Phytochemical screening**

Qualitative phytochemical analysis (Tiwari et al., 2011; Yadav et al., 2011; Vennila et al., 2012) was used to estimate the presence of carbohydrates, proteins, phenols, tannins, and flavonoids. About 200 mg of each mushroom extract powder was dissolved in 20 ml of deionized water and subjected to the phytochemical tests.

# **Detection of carbohydrates.**

- Molisch's test. Two ml of each mushroom extract sample was added by two drops of 10% (w/v)  $\alpha$ -naphthol in 95% ethanol and mixed well. Then, 1 ml of concentrated H<sub>2</sub>SO<sub>4</sub> was slowly added along the sides of the test tubes. The formation of a purple ring at the junction of the mixture indicated the presence of carbohydrates.
- Fehling's test. Two ml of each mushroom extract sample was added by 1 ml of both Fehling's solution A and B. Then, the mixtures were boiled for two minutes. The formation of a red precipitate in the mixtures indicated the presence of sugars.

# Detection of proteins and amino acids.

• **Biuret's test.** Two ml of each mushroom extract sample was added by 0.2 ml of both 1 M NaOH and biuret reagent. The formation of a blue-violet color in the mixtures indicated the presence of proteins.

• Ninhydrin test. One ml of each mushroom extract sample was added by 0.2 ml of 0.25% (w/v) ninhydrin in acetone. Then, the mixtures were boiled for two minutes. The formation of a purple color in the mixtures indicated the presence of amino acids.

**Detection of tannins.** Two point five ml of each mushroom extract sample was added by two drops of 1% (w/v) gelatin. The formation of a white precipitate in the mixtures indicated the presence of tannins.

**Detection of flavonoids.** Two point five ml of each mushroom extract sample was added by two drops of 10% (w/v) lead acetate. The formation of a yellow precipitate in the mixtures indicated the presence of flavonoids.

**Detection of phenolic compounds.** Two point five ml of each mushroom extract sample was added by two drops of 5 % (w/v) ferric chloride (FeCl<sub>3</sub>). The formation of a blue-green color in the mixtures indicated the presence of phenols.

#### Measurement of glycosaminoglycan levels

The levels of sulfated GAGs in mushroom extracts were determined by 1,9-dimethylmethylene blue (DMMB) dye-binding assay (Farndale et al., 1982) using chondroitin sulfate (C4384, Sigma-Aldrich, USA) as standard. Briefly, 50  $\mu$ l of either the extracts or standards were added into 96-well plates. Next, 200  $\mu$ l of DMMB solution was added and mixed. Then, the absorbance was read at 630 nm. The levels of GAGs were estimated by the calibration curve of chondroitin sulfate standards against their known concentrations.

## Scanning UV-Vis spectrophotometric analysis

The mushroom extracts were examined by spectrum mode on a UV-Vis spectrometer (UV2401, Shimadsu, Japan). The chondroitin sulfate standard and the extracts at 1 mg/ml in deionized water were scanned and analyzed from 190 to 500 nm.

#### RESULTS

#### Appearance of the 10 edible mushrooms

The appearance of the 10 fresh edible mushrooms, including 5 cultivated mushrooms; log white fungi, shitake mushroom, sajor-caju mushroom, Jew's ear, straw mushroom; and local mushrooms; milk white russula, hygroscopic earthstar, log black fungi, rosy russula, and bolete are shown in Figure 1.





Note: A, Milk white russula; B, Hygroscopic earthstar; C, Log white fungi; D, Log black fungi; E, Shitake mushroom; F, Rosy russula; G, Sajor-caju mushroom; H, Jew's ear; I, Bolete; J, Straw mushroom.

## Yields of the mushroom extract powders

The amount and yield of the mushroom extract powders per 1 kg fresh mushrooms are shown in Table 1. Log black fungi and log white fungi had notably high yields, followed by shitake mushroom, hygroscopic earthstar, milk white russula, sajor-caju mushroom, rosy russula, straw mushroom, Jew's ear, and bolete.

Mushrooms	g powder/kg wet weight	Yield (%)		
Milk white russula	37	3.70		
Hygroscopic earthstar	42	4.20		
Log white fungi	58	5.80		
Log black fungi	59	5.90		
Shitake mushroom	49	4.90		
Rosy russula	28	2.80		
Sajor-caju mushroom	29	2.90		
Jew's ear	13	1.30		
Bolete	10	1.03		
Straw mushroom	23	2.30		

Table 1. The yields of 10 mushroom extracts calculated from 1 kg of fresh mushrooms.

## Phytochemicals screening of the mushroom extracts

The phytochemical analysis of the mushroom extracts are shown in Table 2. Screening for carbohydrates by Molisch's test detected carbohydrates in 9 of the 10 mushroom extracts, all but rosy russula. Fehling's test detected reducing sugars in only three of the extracts: shitake mushroom, sajor-caju mushroom, and bolete. This suggested that most of the mushrooms studied here contained carbohydrates, some of which were reducing sugars.

Analysis of amino acids by ninhydrin test detected amino acids in all of the mushroom extracts, except milk white russula, bolete, and Jew's ear. This contrasted with the biuret test, which detected no proteins in any of the mushroom extracts. Analysis of tannins by gelatin test found no tannins in any of the mushroom extracts. In contrast, analysis of flavonoids by lead acetate test detected flavonoids in eight of the mushroom extracts, excluding rosy russula and Jew's ear. The ferric chloride test detected phenolic compounds in hygroscopic earthstar and shitake mushroom; thus, these two mushrooms contained both flavonoids and phenolic compounds. The results suggested that they could be a potential source of antioxidant compounds (Balasundram et al., 2006; Armassa et al., 2009; Ferreira et al., 2009; Thetsrimuang et al., 2011a, 2011b).

Thus, the 10 cultivated or local edible mushrooms studied here are potential sources of carbohydrates, amino acids, and flavonoids.

	Test						
Mushroom	Molish	Fehling	Biuret	Ninhydrin	Gelatin	Lead acetate	Ferric chloride
Milk white russula	+	-	-	-	-	+	-
Hygroscopic earthstar	+	-	-	+	-	+	+
Log white fungi	+	-	-	+	-	+	-
Log black fungi	+	-	-	+	-	+	-
Shitake mushroom	+	+	-	+	-	+	+
Rosy russula	-	-	-	+	-	-	-
Sajor-caju mushroom	+	+	-	+	-	+	-
Jew's ear	+	-	-	-	-	-	-
Bolete	+	+	-	-	-	+	-
Straw mushroom	+	-	-	+	-	+	-

 Table 2. The qualitative phytochemical screening of 10 mushroom extracts.

Note: (+) Positive; (-) Negative.

# Glycosaminoglycan screening of the mushroom extracts

Glycosaminoglycans (GAGs) were present to some degree in all of the mushroom extracts (Figure 2). Hygroscopic earthstar contained the most, followed by log white fungi, bolete, log black fungi, shitake mushroom, rosy russula, straw mushroom, Jew's ear, milk white russula, and sajor-caju mushroom, respectively. Interestingly, the three most popular local edible mushrooms contained the most GAGs – hygroscopic earthstar (19  $\mu$ g/ml), log white fungi (12  $\mu$ g/ml), and bolete (11  $\mu$ g/ml).



Figure 2. Dimethylmethylene blue (DMMB) dye-binding assay of glycosaminoglycans (GAGs) from 10 mushroom extracts. Note: The levels of GAGs in the 10 mushroom extracts are presented as mean ± S.D.



Figure 3. UV-Vis spectrophotometry pattern of glycosaminoglycans from 10 mushroom extracts. (A), baseline; (B), standard chondroitin sulfate; (C), Jew's ear; (D), rosy russula; (E), shitake mushroom; (F), sajor-caju mushroom; (G), hygroscopic earthstar; (H), milk white russula; (I), bolete; (J), log black fungi; (K), straw mushroom; (L), log white fungi.

## Scanning UV-Vis spectrophotometric analysis

Scanning UV-Vis spectra of the mushroom extracts are shown in Figure 3. The absorption pattern in the mushroom extracts resembled the chondroitin sulfate standard, with two peaks around 190-300 nm in most extracts. Since GAGs polymers are composed of either iduronic acid or glucuronic acid and N-acetyl chromophores (Kjellén and Lindahl, 1991; Varki et al., 2009), the highest peaks around 190-210 nm, arising from the carboxylate chromophore of iduronate and glucuronate present in the GAGs chains, were responsible for the majority of the absorption pattern (Lima et al., 2011).

## DISCUSSION

Several studies have reported that different extraction techniques yielded varying degrees of chemical and biological components (Sasidharan et al., 2010). The yield in this study was rather low, although we used the extraction method specific to GAGs or GAG-peptides (Nakano et al., 2000). This is probably due to the naturally low occurrence of GAGs in these mushrooms, or their geographic origin and phytochemical content (Ncube et al., 2008).

According to Sanmee et al. (2003), rosy russula contained carbohydrates; however, we found none using phytochemical analysis. This inconsistency may be due to limitations of the extraction or detection methods used. Most mushrooms are high in protein, providing a good dietary source (Sanmee et al., 2003; Valverde et al., 2015; Li et al., 2017). Nevertheless, we only detected amino acids in some of the mushroom extracts. A non-optimal pH can affect

protein structure, leading to protein dysfunction or liberating free amino acids. For example, an acid environment can denature proteins through non-enzymatic hydrolysis (Marcus, 1985; Lauer et al., 2016). This may explain our results, as we extracted the mushrooms with acidic water (Nakano et al., 2000); this condition may have degraded the proteins, resulting in release of free amino acids. Furthermore, the absence of either amino acids or proteins in the mushroom extracts could be due to the sensitivity of the qualitative methods used in this study. Given we did find free amino acids in some of the extracts, and the limitations discussed above, it remains possible that the mushrooms of northern Thailand could still be a good source of non-animal proteins, especially for vegetarians.

Besides their nutritional value, mushrooms contain biologically active compounds with beneficial properties (Valverde et al., 2015; Filipa et al., 2017). For instance, numerous mushroom extracts have been shown to contain antioxidants, including phenolic compounds, vitamin C, vitamin E, and carotenoid (Ferreira et al., 2009). Phenolic compounds are aromatic hydroxylated molecules that can be found in either simple or complex phenolic molecules. The phenolics are composed of many compounds, such as phenolic acids, flavonoids, tannins, and lignans (Cote et al., 2010; D'Archivio et al., 2010), that generate antioxidant activity. This activity is related to their capacity to act as reducing agents, scavenge free radicals, quench singlet oxygen, or chelate metal ions (Balasundram et al., 2006; Ferreira et al., 2009). Furthermore, several studies have shown that the presence of phenolic compounds in mushroom extracts correlated with antioxidant activity, which depended on the type of mushroom, stage of development, age of the fresh mushroom, storage conditions (Guillamón et al., 2010), or preparation procedures. For example, the methanolic extract and crude polysaccharides of log black fungi (L. polychrous) found in northern and northeastern Thailand exhibited antioxidant activity and an inhibitory effect on breast cancer cells (Armassa et al., 2009; Thetsrimuang et al., 2011a; 2011b). Aqueous and butanol extracts of sajor-caju mushroom (*P. sajor-caju*) have shown high antioxidant activity linked to their total phenolic content (Kanagasabapathy et al., 2011). These results indicate that the extraction method used with different mushroom species affect the levels of active compounds and their range of bioactive properties. Based on our current results, we found no tannins in any of the mushroom extracts, but we detected flavonoids in eight of the mushroom extracts (all but rosy russula and Jew's ear), and detected phenolic compounds only in hygroscopic earthstar and shitake mushroom. These findings are thought to be the consequence of using acidic water (Nakano et al., 2000) for preparing mushroom extracts, as we did in this study; this method may not be suitable for extracting tannins or other phenolic compounds of some mushroom species (Tiwari et al., 2011). We did find flavonoids in most of the mushroom extracts; this finding supported the presence of catechin in shitake mushroom, log white fungi, and log black fungi (Attarat and Phermthai, 2015; Chowdhury et al., 2015). Some studies have demonstrated that bioactive compounds in mushrooms play an essential role in the human immune system by acting as immunomodulating molecules. The main groups of immunomodulating compounds are terpenes, terpenoids, lectins, fungal immunomodulatory proteins (FIPs) and polysaccharides (Enshasy and Kaul, 2013). Of these, the best known immunomodulating molecules are lentinan and schizophyllan; both are are  $\beta$ -1, 3-D-glucans with  $\beta$ -1, 6 branches and have been found in shitake mushroom and spilt gill mushroom, respectively (Reis et al., 2012). They have been used to treat gastric cancer (lentinan) and head and neck cancer (schizophyllan), in combination with chemotherapy (Ina et al., 2013; Filipa et al., 2017). In addition, other polysaccharides rich in  $\beta$ -1, 3- D-glucans,  $\beta$ -1, 4-D-glucans,  $\beta$ -1, 6-D-glucans,  $\alpha$ -1, 4-D-glucans,  $\alpha$ -1, 6-D-glucans, and glucomannan have been isolated from *A. blazei*; these have been shown to have immunomodulatory and antineoplastic properties (Biedron et al., 2012).

Several studies mentioned that most bioactive polysaccharides isolated from mushrooms are branched or unbranched homopolysaccharides (Biedron et al., 2012; Ina et al., 2013; Filipa et al., 2017), which usually contain long chains of glucose polymer. In our study, most of the mushroom extracts were positive with Molisch's test – a general test for detecting carbohydrates, including monosaccharides, disaccharides, and polysaccharides – indicating that the extracts contained carbohydrates. Since, GAGs are carbohydrates (heteropolysaccharides), we then specifically tested for their presence using a DMMB dyebinding assay; all of our mushroom extracts also contained GAGs. This suggested that some of the carbohydrates that were detected by Molisch's test were probably GAGs. In contrast to most of our mushroom extracts, rosy russula extracts were negative for carbohydrates using both the Molish and Fehling tests. This result also contrasted with Sanmee et al. (2003), who found monosaccharides in rosy russula. These conflicting results could be due to differences in the preparation procedures, origin and stage of the mushrooms, or detection methods.

Most GAGs are attached to a core protein to form proteoglycans; they are found predominantly in vertebrates and invertebrates. For example, aggrecan, the major proteoglycan present in cartilage (Heinegård and Axelsson, 1977), has a core protein that is covalently linked to sulfated glycosaminoglycans (GAGs) side chains, mainly chondroitin sulfate and keratan sulfate (Meyer et al., 1958). Other sulfated GAGs, such as heparan sulfate and dermatan sulfate, are also found in cartilage and several tissues (Heinegård and Axelsson, 1977; Gandhi and Mancera, 2008). These GAGs have distinct biological functions; for instance, heparan sulfate plays an essential role as co-receptors for various receptor tyrosine kinases (Miserocchi et al., 2001), while chondroitin sulfate can prevent and maintain the structural-functional activity of cartilage during inflammation (Takagaki et al., 2002; Raman et al., 2005). In addition, chondroitin sulfate, as nutraceuticals or prescribed drugs in combination with glucosamine (GlcN) sulfate, has been increasingly employed to treat the symptoms of osteoarthritis and osteoarthrosis (Bishnoi et al., 2016). These pharmaceutical preparations of chondroitin sulfate are usually obtained from animal parts, such as shark or bovine trachea cartilage (Garnjanagoonchorn et al., 2007). Thus, detecting GAGs from the mushroom extracts in this study could potentially provide non-animal sources of chondroitin sulfate and other GAGs. However, these mushroom extracts might also contain some other sulfated polysaccharides as have also been found in algae and other mushroom types (Ina et al., 2013; Filipa et al., 2017).

Algae, another non-animal source, contains the most abundant GAG-resembling sulfated glycans (Vasconcelos et al., 2017). Brown algae contains sulfated fucans that exhibit biomedical functions in inflammation, coagulation, angiogenesis, and cell adhesion (Cumashi et al., 2007). Green and red algae also contain sulfated galactans that involve many pathophysiological systems. These sulfated glycans occur as structural components in the cell walls of algae (Chevolot et al., 2001), and their structures are maintained among phyla. Most sulfated glycans found in algae are composed of disaccharide repeating units with distinct sulfation patterns. Whereas GAG-resembling sulfated glycans of algae have been

studied extensively (Vasconcelos and Pomin, 2017), very few studies have researched sulfated glycans or GAGs of local edible mushrooms found in northern Thailand. To potentially exploit the mushrooms in our study as a good source of GAGs, additional study is needed on how to obtain high yielding mushroom extracts.

GAGs levels measured by the DMMB dye-binding assay from the top two mushroom extracts (log white fungi and bolete) were highly correlated with the highest absorption peak around 190-210 nm from spectrophotometric analysis; this tentatively represented the carboxylate chromophore of iduronate and glucuronate present in the GAGs chains (Lima et al., 2011), as shown in Figure 3 (L for log white fungi; and I for bolete). Although hygroscopic earthstar contained the highest levels of GAGs according to the DMMB dye-binding assay, its absorption (G) peak at around 190-210 nm was shifted toward the absorption peaks of the shitake mushroom (E), milk white russula (H), rosy russula (D), and sajor-caju mushroom (F) extracts. The Jew's ear (C) extract had the lowest absorption pattern, consistent with the DMMB dye-binding analysis. Since DMMB dye reacts with all types of sulfated GAG chains, this assay was not able to distinguish the types of GAGs present in the mushroom extracts. Although our spectrophotometric analysis confirmed the presence of GAG chains at peaks around 190-210 nm (Lima et al., 2011), we also found additional broad peaks around 240-260 nm that were related to other aromatic compounds (de Micalizzi et al., 1998). Some ions, such as calcium or sodium, or the sulfation pattern of GAGs or other contaminants can shift the peaks (McEwen et al., 2009). These factors might have affected the correlation between our spectrophotometric analysis and DMMB dye-binding assay. Taken together, our UV-based spectroscopy confirmed the presence of GAGs, but could not distinguish their types. Further study of the GAGs found here using magnetic resonance spectroscopy (NMR) or disaccharide analyses should be performed (Santos et al., 2017).

## CONCLUSION

Our results found GAGs in edible mushrooms grown in northern Thailand, providing a potentially new source of these important compounds.

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