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Bioassay Guided Isolation of Polysaccharide Rich Fraction from Myrmecodia Platytyrae and its Bile Acid Binder Property

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ABSTRACT

Bioassay guided isolation was done on the *Myrmecodia platytyrae* (*MyP*) extracts in order to find the best extract that can bind with bile acid. The function of bile acid binder is to reduce the reabsorption of bile acid in the intestine so that it will cause the decrease of cholesterol in the blood circulation. The extraction process was started by using different organic solvent such as hexane, toluene, ethyl acetate, ethanol and water. All extracts were tested its bile acid binding properties and the extract gave out highest activity was selected to be fractionated. The result showed that water extract has highest bile acid binding property (75%). Then, fractionation process was performed in order to find the best fraction from water extract that have bile acid binding property. The sub-fractionation processes were continued and the process only focussed to the fraction that has highest bile acid binding property until a white powder with 97.02 % carbohydrate was obtained. The powder was subjected to FTIR and was found to have glycosidic bond as shown by bands at 1141 until 1018 cm^{-1} which is a character of polysaccharide. The polysaccharide rich fraction was hydrolysed and subjected to HPLC to identify its monosaccharide groups. The result showed the presence of glucose and fructose were found to be among the major monosaccharides. This result suggested that the polysaccharide from *MyP* water extract with high bile acid binding property was gluco-fructan.

Key words: Myrmecodia platytyrae, Polysaccharide rich fraction, bile acid binding, gluco-fructan

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INTRODUCTION

Myrmecodia plant or ant-nest plant is from Rubiaceae family (Huxley, 1978). Rubiaceae are mainly tropical woody plants and consist mostly of trees and shrubs. Rubiaceae (subfamily Rubioideae) can be found in temperate regions (Mongrand *et al*., 2005). Rubiaceae consist of five genera and out of five, there are only two of which have the association with ants. They are Myrmecodia (45 species) and Hypnophytum (26 species) (Soeksmanto *et al*., 2010). From these species, *M. pendans*, *M. tuberose*, *Hydnophytum formicarum* and *M. platytyrea* are believed to have medicinal value (Soeksmanto *et al*., 2010; Hamsar and Mizaton, 2012). Recently, Roslizawaty *et al*., (2015) reported that *Myrmecodia sp.* has anticholesterol effect. The anticholesterol effect of a herb is depending on its capability to control the cholesterol production and absorption (Yao *et al*., 2014; Rajput *et al*., 2014). Therefore, it is important to determine the potential of herbal extract for the discovery of bioactive compounds and development of potent drugs.

One of the strategies to decrease cholesterol in the blood circulation is through bile acid binding (Chiang, 2013). Bile acids facilitate digestion and absorption of lipids in the small intestine (Staels and Fonseca, 2009). It also involved in regulating cholesterol homoeostasis (Chiang, 2013). Bile acids represent the primary pathway for cholesterol catabolism and account for 50 percent of the daily turnover of cholesterol. Manipulation of the bile acid pool through bile acid sequestration to alter bile acid metabolism has been used to treat dyslipidemia (Hjerpsted *et al*., 2016).

To date, the bile acid resins or sequestrants are among the preferable lipid-lowering agents which have a very good effect in decreasing LDL cholesterol. This type of cholesterol lowering agent also reported as less effective compared to statin family drug (Pahan, 2006). The mechanism that is involved in bile acid sequestrants actions is highly positively charged molecules that bind to the negatively charged bile acids in the intestine. Thus, this action inhibiting bile acid activity which is to solubilize lipid and fat before it can be digested by lipase enzyme. Indirectly, cholesterol digestion and absorption in the intestine is blocked (Pahan, 2006). Bile acid resins or sequestrants drug also was reported to have the ability to inhibit the reabsorption of bile acids which involve in the enterohepatic circulation in the intestine (Staels and Fonseca, 2009). Hence, this causes a reduction of the bile acid pool which leads to increased bile acid synthesis that participates in cholesterol synthesis in the liver. Eventually, these events contribute to the lowering of LDL cholesterol levels.

In this research, bioassay guided isolation was done on *Myrmecodia platytyrea* (*MyP*) in order to determine the efficacy of *MyP* to act as bile acid binder. All the experiments were conducted in order to investigate the chemical content of *MyP* and identification of the bioactive compound that involved in bile acid binding. In addition, *MyP* water extract was fractionated into several fractions. The purification process was done to get the pure compound. The compounds were subjected to HPLC and FTIR in order to identify the structure.

METHOD

Extraction of *MyP* and Selection of Bioactive Extract

Five different types of solvents were used in the experiment including hexane (Hex), toluene (Tol), ethyl acetate (EtOAc), ethanol (EtOH) and water as depicted in Figure 1. Fifty grams of *MyP* dried sample was soaked with 100 ml of solvent at 40°C for 20 min. The extract was filtered through filter paper (Whatman No. 1). The filtrate was taken and the solvent was removed by using rotary vacuum evaporator. Meanwhile, the *MyP* insoluble residue that attached to the filter was taken and let dried in a fume hood for 48 h in order to remove the solvent. The residue was subjected to extraction process again using other organic solvents according to their polarity index. The extracts from organic solvent were recovered using rotary vacuum evaporator. The evaporation process was conducted at 40°C to reduce any possible degradation of the phytochemical in the samples. Each extract was analysed by HPLC experiment to determine their chromatogram. The dried extract was subjected to bile acid binding assay to determine its bioactivity. The best extract that gave out high bioactivity was selected for bioassay-guided fractionation. In this experiment, *MyP* water extract showed higher bioactivity. Water extract of *MyP* was subjected to bioassay-guided fractionation in order to identify the single bioactive compound with the highest activity.

Bile Acid Binding Assay

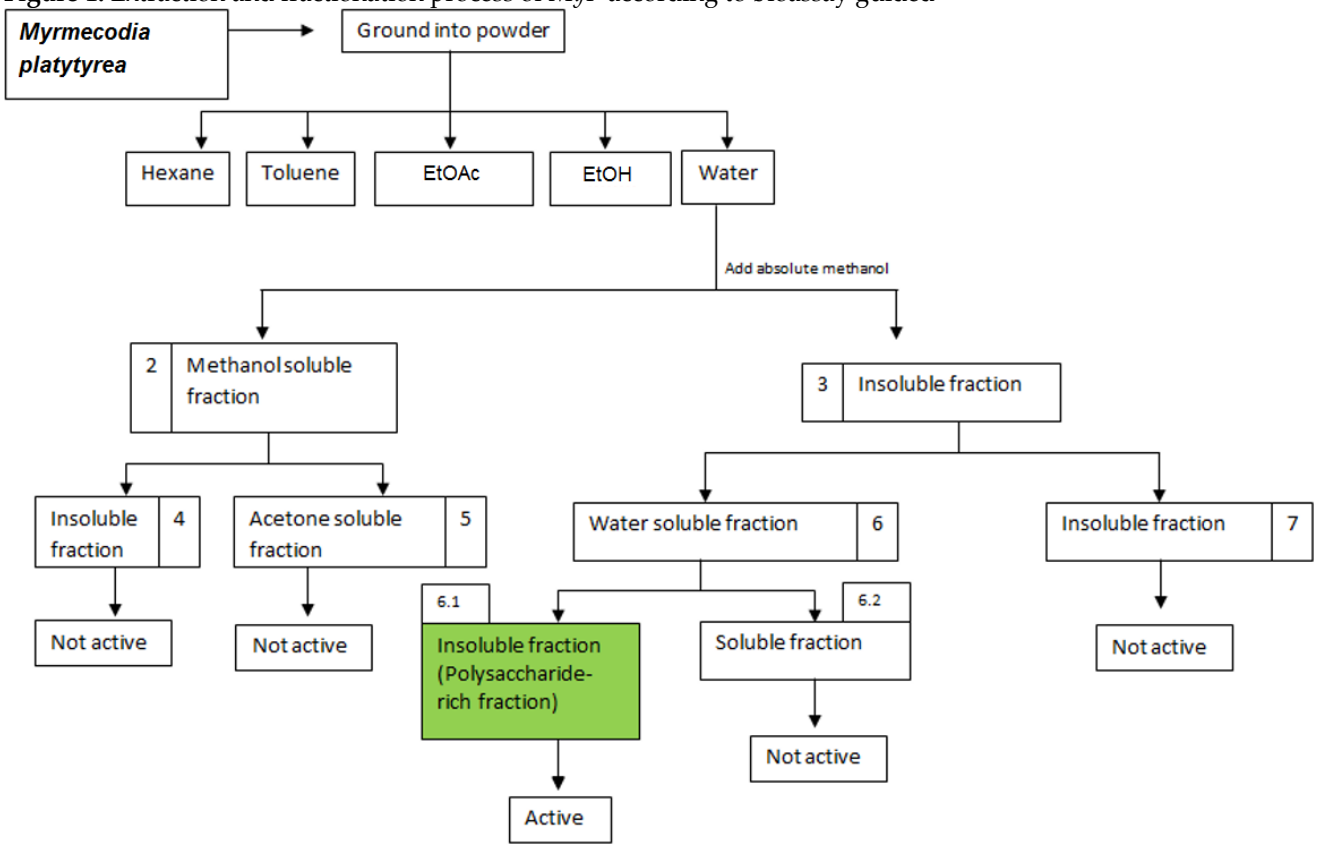
The bile acid binding capacities were detected following a laboratory protocol reported previously (Niua *et al*., 2011). In this experiment, Colesevalam was used as the positive control. The levels of unbound bile acids were calculated according to the standard curves of two pure bile acids, cholic and chenodeoxycholic acids with a concentration of 0, 0.05, 0.1, 0.5, 1 and 2 mg/ml. For imitation of the gastric condition, 25 mg of each sample from bioassay-guided isolation process was treated with 0.25 mL of 0.01 mol/L HCl and incubated for 60 min at 37 °C with continuous shaking. Then 25 µL of 0.1 mol/L NaOH was added to the solution to bring pH to 7 and mixed with 1.25 mL 400 µmol/L bile acid stock solution dissolved in 0.01 mol/L phosphate buffer (pH 7.0) to simulate the intestinal condition. The mixture was incubated at 37 °C for another 60 min and then centrifuged at 6000 rpm for 10 min.

Then, 100 µL of each supernatant and bile acid standards were mixed with 125 µL of 1.22 mmol/L nicotinamide adenine dinucleotide, 5 mmol/L NBT, 100 µL of 625 units/L diphorase and 100 µL of 3-α hydroxysterol dehydrogenase solution (625 units/L). The mixture was incubated at room temperature for 60 min and 100 µL 1.33 mol/L phosphoric acid was added to terminate the reaction. The absorbance of the mixture was recorded at 530 nm. The bile acid binding capacity was determined against a reagent blank. Triplicate tests were conducted for each sample.

Bioassay-guided Fractionation and Isolation of Bioactive Fraction from *MyP* Water Extract for Bile Acid Binding Activity

The powder of *MyP* was boiled for 15 min at 100°C and filtered. Insoluble part was discarded. The filtrate was freeze dried. Then, prior to be fractionated as depicted in Figure 1, *MyP* water extract was added with methanol. The soluble part was filtrated. The bile acid binding test was performed to both sediment (No 3) and methanol soluble part (No 2). Based on the biological activities, the sediment was diluted in water. Water soluble (No 6) and water insoluble (No 7) were tested on their bile acid binding properties. Water soluble part was selected as it has better bile acid binding properties. Water soluble fraction was concentrated. Then, it was added with EtOH 3 times to get sediment (6.1) and EtOH soluble fraction (No 6.2). Again, based on the biological activities, fraction 6.1 was concentrated and freeze-dried to get powder. The powder was identified by testing the total carbohydrate content.

Figure 1: Extraction and fractionation process of *MyP* according to bioassay guided



Characterization of Fraction 6.1

The fraction 6.1 was determined based on the sugar content and chemical constituent. Total carbohydrate content and monosaccharide composition were measured in order to calculate the ratio of monosaccharide and type of polysaccharide. Besides that, the FTIR experiment was performed to characterise their structure.

Determination of Total Carbohydrate Content

This method followed Agrawal *et al*., (2015) with minor modification. The fraction 6.1 sample was disrupted into simpler sugar via hydrolysis process. First, the fraction 6.1 (120 mg) was dissolved in 120 ml of 2 M H_2SO_4. The solution was autoclaved for 2 hours at 120°C. After cooling to room temperature, the hydrolysed fraction 6.1 (HF) was neutralised with 4 M NaOH. Then, 800 ml of methanol was added into the solution and white precipitate was formed. The solution was filtered and dried under reduced pressure by using rotary evaporator. Sample preparation was done by added 10 ml of concentrated sulphuric acid and 2 ml of distilled water into 1.1 mg HF. The mixture was shaken vigorously for 30 min. Then, 2 ml of 5% phenol was added and heated for 5 min at 90°C in a water bath. The mixture was spectrophotometrically measured at 490 nm. This experiment was done triplicate. Glucose solution was used as a standard. Glucose standard was weighed 10 mg and dissolved in 14.3 ml distilled water and transferred into 100 ml volumetric flask to give 100 ppm stock solution. Then, 71.4 ml of concentrated sulphuric acid was added and shake for 30 min. Phenol red (5%) with the amount of 14.3 ml was added to the solution and heated at 90°C for 5 min. After the solution was cooled to room temperature, the solution was diluted into 5 different concentrations (10 ppm, 20 ppm, 30 ppm, 40 ppm and 50 ppm) for the standard calibration curve. Reference cell was a mixture of water and sulphuric acid with a ratio of 1:1. This experiment was done triplicate.

Monosaccharide Composition Analysis by HPLC

The fraction 6.1 was hydrolyzed into simpler sugar and HPLC analysis method used in this experiment was provided by Zhang *et al*., (2009) with slight modification. The crude HF (33.2g) was mixed with 0.3 M NaOH (1ml) and 0.5 M 3-methyl-1-phenyl-5-pyrazolone (PMP) (1 ml). The mixture was incubated at 70°C for 1 hour. A yellowish solution was formed. Then, 1 ml of 0.3 M HCl and 3 ml of chloroform were added and left for 5 min until 2 layers appeared. This process was repeated 3 times to extract out any contaminant. The solution was centrifuged and the upper layer was collected and filtered using 0.45 µm pore size filter to get uncontaminated HF.

The HF was derivatized with PMP and purified on a LiChroCART, RP-18e column (4.6 x 250 mm) connected to Waters HPLC system equipped with a photodiode array (PDA) detector set at 245 nm. The PMP derivative was injected 10 µl and eluted at a flow rate of 1.0 ml/min at 35°C. The buffer solution for HPLC was prepared by dissolving 3.26 g of KH_2PO_4 and 2.64 g K_2HPO_4 in 2 L Millipore water. The mixture was added with 0.5 ml of 0.025 % triethylamine (TEA) and pH was maintained at 7. The buffer solution was filtered with 0.45 µm Whatman filter. The mobile phase A consisted of acetonitrile and mobile phase B was 0.05% NaH_2PO_4 buffer (pH 7). The gradient elution was 90-87-85 % B by a linear decreased from 0-10-44 min. The same method was used for sugar standards. The working standard consisted of glucose, mannose, galactose, rhamnose, galacturonic acid, xylose and arabinose mixture. The final concentration of each sugar was 2.22 mg/ml and 0.66 mg/ml for glucose. For example, 10 mg of xylose standard was dissolved in 4.5 ml of Millipore water to get 2.22 mg/ml concentration of the standard. This standard was derivatized with PMP. The standard xylose and derivatised HF were mixed according to Table 1.

Volume standard (ml), Vs	0	0.1	0.2	0.3	0.4	0.5
Volume HF (ml), Vo	0.3	0.3	0.3	0.3	0.3	0.3
Volume water (ml)	0.5	0.4	0.3	0.2	0.1	0
Total volume (ml)	0.8	0.8	0.8	0.8	0.8	0.8

RESULTS

Bile-Acid Binding Assay

Fraction Hexane, Chloroform, EtOAc and Water

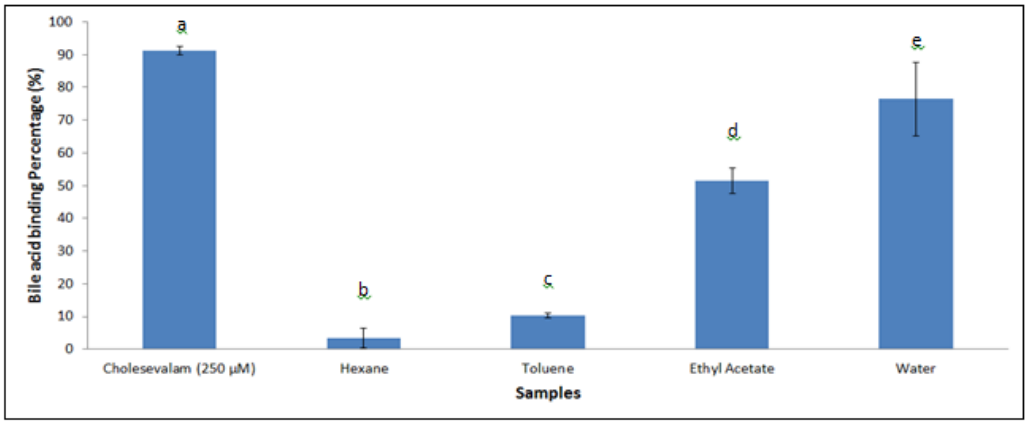


Figure 2: Percentage of bile acid binding activity of hexane, toluene, EtOAc and water *MyP* extract. Colesevelam showed the highest percentage followed by water, EtOAc, hexane and toluene extract. Water extract showed the highest activity compared to other organic solvent extracts. Results are expressed as mean ± S.D. Different superscripted letters on top of the chart indicate statistical differences ($p < 0.05$). n = 8.

Methanol Soluble Fraction (2) and Methanol Insoluble Fraction (3)

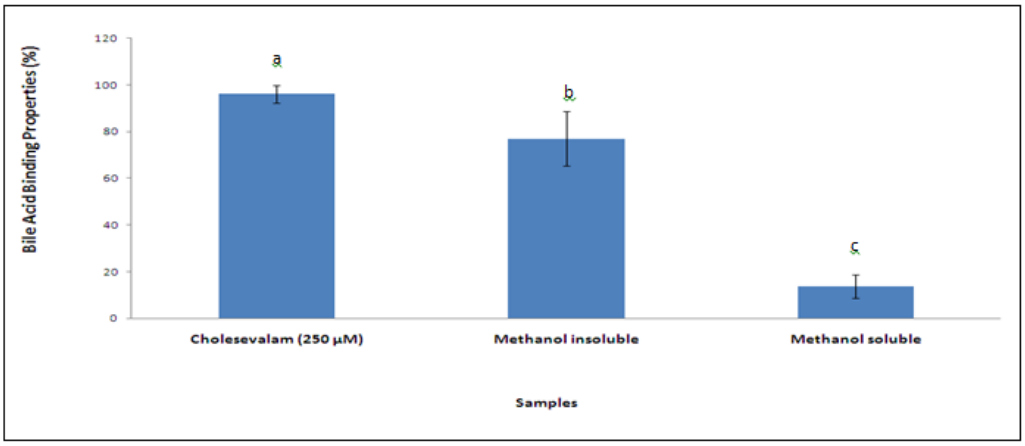


Figure 3: Percentage of bile acid binding activity of methanol soluble fraction (2) and methanol insoluble fraction (3). Fraction 3 showed higher inhibition activity compared to Fraction 2. Results are expressed as mean ± S.D. Different superscripted letters on top of the chart indicate statistical differences ($p < 0.05$). n = 8.

EtOH Soluble (6.2) and EtOH Insoluble Fraction (6.1)

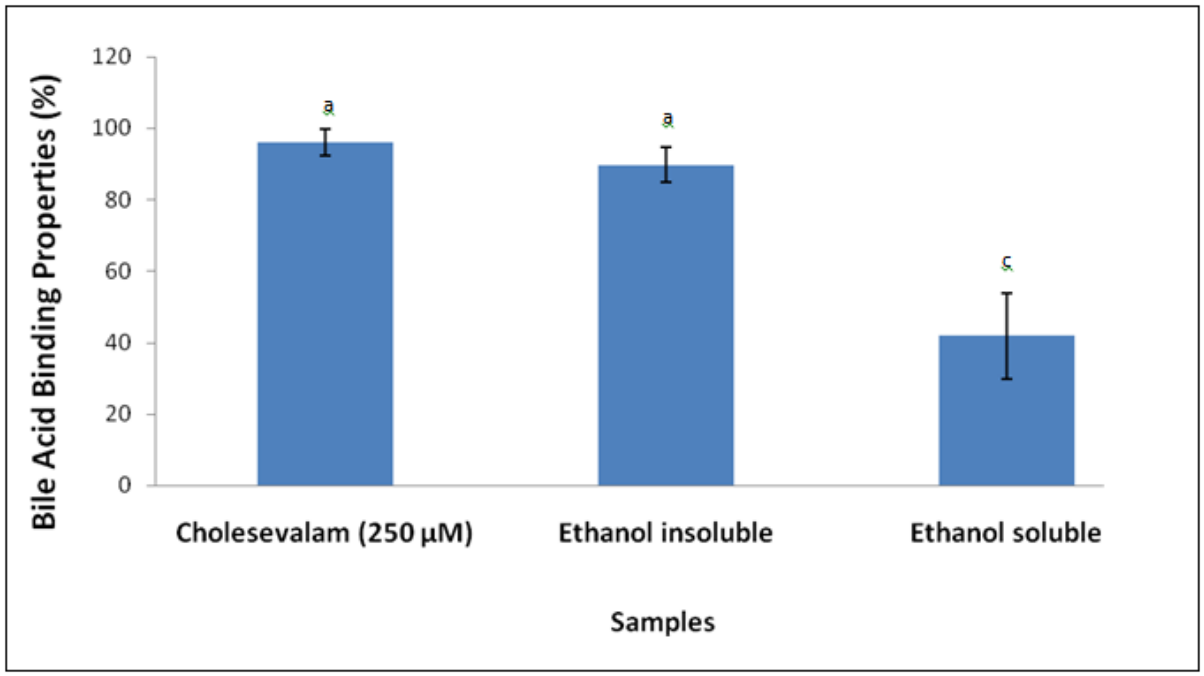


Figure 4: Percentage inhibition of the bile acid binding activity of acetone insoluble fraction (4) and acetone soluble fraction (5). Fraction 5 showed higher activities compared to Fraction 4. Fraction 5 was selected to be fractionated by using liquid-liquid portioning. Results are expressed as mean ± S.D. Different superscripted letters on top of the chart indicate statistical differences ($p < 0.05$). n = 8.

Polysaccharide Rich Fraction (6.1)

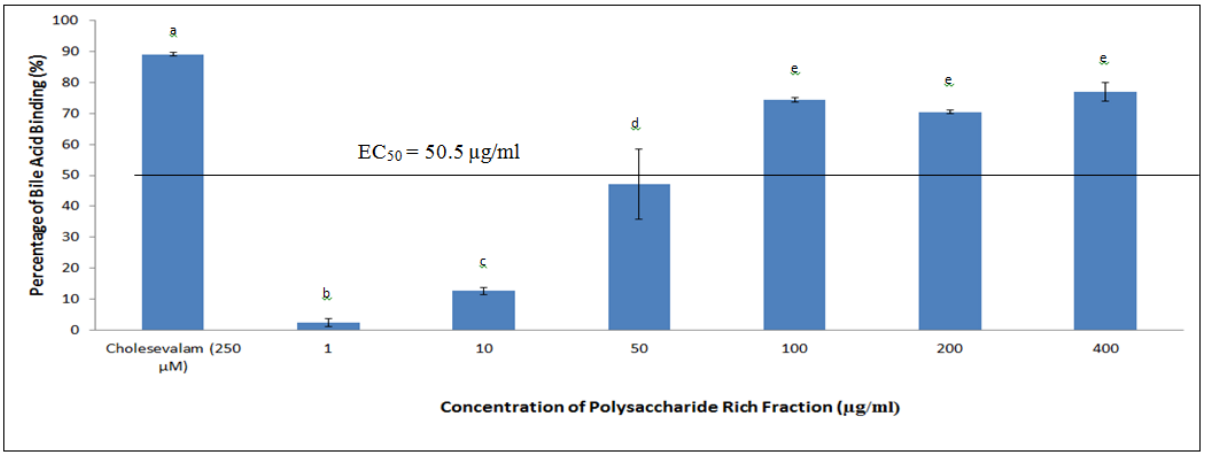


Figure 5: Percentage inhibition of bile acid binding by a polysaccharide-rich fraction. The concentrations of the fraction used in the experiment were 1, 10, 50, 100, 200 and 400 µg/ml. From the graph, it was showed that the fraction can effectively bind with bile acid when the concentration was increased. It was also showed that the binding activity was dose dependent. The effective concentration 50 (EC50) of the fraction was 50 µg/ml. This experiment was done in triplicate. The result showed was mean ± standard deviation. Different superscripted letters on top of the chart indicate statistical differences ($p < 0.05$).

Total Carbohydrate Content

Table 2: Percentage of total carbohydrate content (%) in the sample.

Sample	Percentage of total carbohydrate content in sample (%)
Fraction 6.1	97.02

Note: Total carbohydrate content was performed on sample 6.1. The result showed that 97.02 from the sample is primary metabolite which is carbohydrate. This means the fraction is carbohydrate rich fraction and other 2.98 percent from it is additional contents such as a secondary metabolite.

HPLC Profile of Polysaccharide

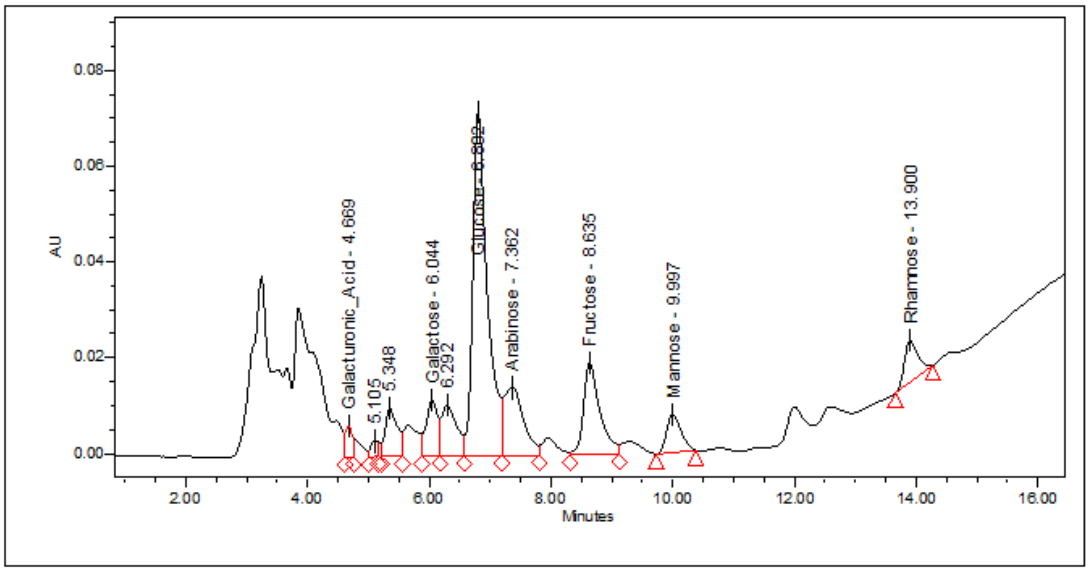


Figure 6: Monosaccharide composition analysis. The polysaccharide sample was hydrolyzed with alkali (NaOH) and neutralised with HCl. The simpler sugar was detected by using HPLC system.

Table 3: Analysis of monosaccharides from HPLC chromatogram.

Name	Area R1	Area R2	Area R3	Mean	SD	RSD(%)
Galacturonic_Acid		115738	122354	119046	4678	3.93
Galactose	155249	181445	177822	171505	14194	8.28
Glucose		786798	816525	801662	21020	2.62
Arabinose		248952	236983	242968	8463	3.48
Xylose		66940	64571	65756	1675	2.55
Fructose	361286	378700	385755	375247	12595	3.36
Mannose	131693	163608	162182	152494	18029	11.82
Rhamnose	132094	159035	158031	149720	15273	10.20

Note: The table above showed glucose and fructose were found to be among the major monosaccharides. This result suggested that the polysaccharide from fraction 6.1 was gluco-fructan. The HPLC standard and standard curve for each monosaccharide for the experiment above can be referred in the appendix.

FTIR of fraction 6.1 (Polysaccharide)

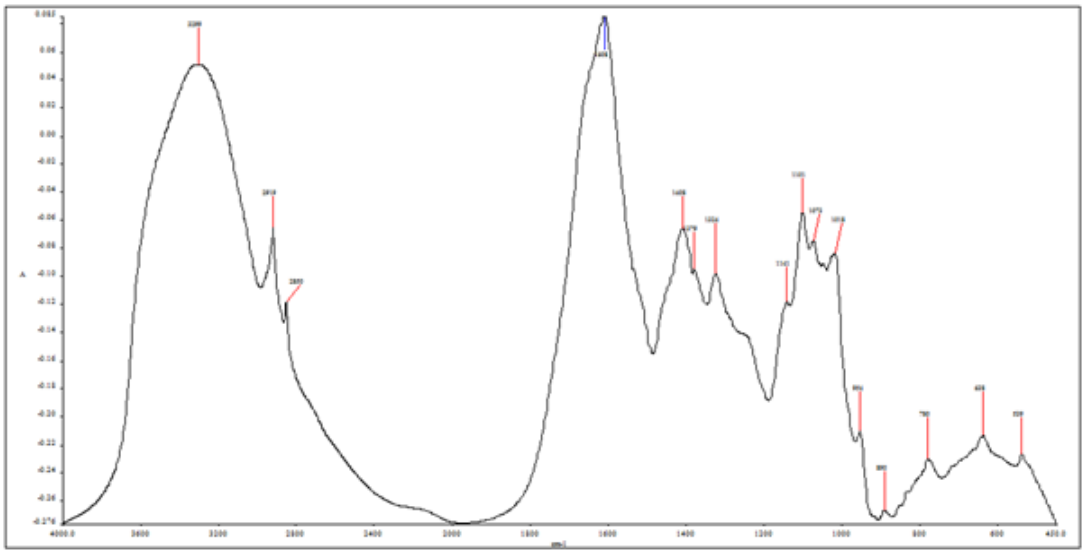


Figure 7: FTIR of fraction 6.1 (polysaccharide-rich fraction). IR spectra supported an alcoholic moiety as shown by strong and broad bands at 3369 cm-1 (OH stretching), 2919 cm-1 (C-H2 bond) and the most important linkage to prove polysaccharide presence which is glycosidic linkage as shown by bands at 1141 until 1018 cm-1 (C-O bond).

DISCUSSION

Nowadays, there are three bile acid sequestrants being used which are Cholestyramine, Colesevelam and Colestipol (Pahan, 2006). However, these drugs can cause side effects (Davidson *et al*., 1999). Therefore, research always been done to find a new bile acid sequestrants drug. One of the potent compounds that are primary metabolite from the tuber of *MyP* water extract is a polysaccharide. Water extract showed stronger bile binding properties (Figure 2). As a result, water extract was selected in the next experiment which fractionation and isolation were carried out by using water extract for bile acid binding properties.

The water extract was further fractionated by diluting the water extract powder into methanol which gave out two fractions: (a) methanol soluble and (b) methanol insoluble fraction. According to Figure 3, methanol insoluble fraction showed very good activity of bile acid binding. This indicated that methanol insoluble fraction is containing a compound that can easily bind with bile acid with stronger affinity. The fraction has higher bile acid binding compared to methanol soluble fraction but lower than Colesevelam drug control. Colesevelam drug showed a very good bile acid binding which was 98 % binding property. While methanol insoluble fraction showed 78.5 % bile acid binding property. Therefore, methanol insoluble fraction was selected to be sub-fractionated by adding water to get water soluble fraction. This water soluble fraction was added to absolute EtOH three times than its volume. Precipitation of whitish sediment was formed and called as EtOH insoluble fraction. As shown in Figure 4, the EtOH insoluble has the ability to bind with bile acid and not significantly different ($p<0.05$) compared to Colesevelam drug control. This indicated that this fraction can act as good as Colesevelam drug to bind with bile acid. Therefore, it is important to study this EtOH insoluble fraction (also named as fraction 6.1) as this fraction containing a large compound and has the effect of binding properties with bile acid.

Chemistry identification experiment was done on the fraction 6.1 which was brownish and not soluble in ethanol. A thick viscous solution was formed. The fraction was freeze-dried and carbohydrate content test was performed to identify the composition. Total carbohydrate content test showed that the fraction is containing 97.02 % carbohydrate. This indicated that the fraction is a polysaccharide-rich fraction. Figure 7 showed the FTIR experiment of polysaccharide which proves the presence of glycosidic bond in the polysaccharide powder.

The polysaccharide was hydrolyzed to study its monosaccharide composition. The hydrolyzed monosaccharide was injected into HPLC system. The chromatogram of the sample was used to count its simple sugar quantity. The Table 3 showed that glucose and fructose give out higher reading which 59% and 39.9% respectively. This result suggested that the polysaccharide from *MyP* water extract is a gluco-fructan group type. Glucan and fructan group type always can be found in plant tuber (Li, 2016). There were many previous studies had been done on water soluble polysaccharide and proved that this primary compound has a potency to be developed as cholesterol lowering agent.

The soluble polysaccharide was experimentally proven can bind with the bile acid (Zacherl *et al*., 2011). The proposed action of polysaccharide was it will disrupt the metabolism of cholesterol by decreasing the absorption of bile acid itself. Besides that, reduction of dietary cholesterol absorption and binding with bile acids will increase the transformation of LDLs from blood circulation into bile as it will be taken into the liver (Panith *et al*., 2016). The ability of polysaccharide to bind with bile is depending on its viscosity and structure of the polysaccharide (Zacherl *et al*., 2011). Camire and Dougherty (2003) suggested that polysaccharides with large hydrophobic surface areas have potentially important roles for bile acid binding properties. Besides that, Pigeon *et al*., (2002) reported that the capability of polysaccharide to bind with bile acid is due to its resistance to digestive enzymes and its water-holding capacity. The polysaccharide was proved to have bile acid binding in the previous study. The polysaccharide bound with bile acid and brought it out through defecation (Panith *et al*., 2016). Bile acid binding capability of polysaccharide ensures the cholesterol level decreased in an animal laboratory experiment (de Almeida *et al*., 2013). Polysaccharide also is considered as not toxic as this primary metabolite cannot be absorbed by the intestine. Therefore, acute liver injury case report related to polysaccharide ingestion using animal laboratory is not yet reported.

CONCLUSION

It was concluded that *MyP* water extract containing gluco-fructan polysaccharide which has bile acid binding property. This ability might help in reducing bile acid reabsorption and decreasing digestion of lipid. Thus, *MyP* water extract might be useful for hypercholesterolemia treatment.

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