Subcritical Water Extraction and Characterization of Polysaccharides and Phenolic Compounds from *Inonotus obliquus*

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Subcritical Water Extraction and Characterization of Polysaccharides and Phenolic Compounds from *Inonotus obliquus*

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Abstract

Inonotus obliquus or chaga, a kind of traditional fungi belonging to the family of *Hymenochaetaceae*, *Basidiomycetes*, has been used as a folk remedy in Asian countries, such as China, Japan, Korea and Russia for five hundred years. This fungus is usually found as a sterile conk (sclerotia) called 'Chaga' on Betula species in nature. In our previous studies, aqueous extracts of *Inonotus obliquus* possessed antioxidant activity and exhibited potent anticancer ability. Recently, extracting bioactive compounds to utilize in food and medicine industries has attracted much attention due to their biological activities, including anti-tumor, immunostimulation, antioxidation, etc. Generally, hot water treatment used for traditional extraction of polysaccharides has several drawbacks like long extraction time and low extraction yield. Subcritical water extraction (SWE) based on the use of water as solvent at temperatures of 100 - 374 ℃ and a pressure (4×10^6 Pa) that is sufficiently high to maintain its liquid state has been reported to be effective to selectively extract a variety of polar or non-polar organic compounds, avoiding the disadvantages of hot water extraction.

To the best of our knowledge, there are few investigations available regarding the optimization on the yield and bioactivity of SWE polysaccharides (IOP) and phenolic compounds (total phenolic content, TPC and total flavonoid content, TFC) from *I. obliquus*. Thus, the purpose of the present study is to optimize the SWE conditions (extraction temperature, residence time and liquid-solid ratio) using response surface methodology, identify the components of extracts and analyze their bioactive characteristics. And all the results will be compared with conventional extraction methods.

1. A series of single factor experiments was conducted to identify the independent variables and investigate the preliminary BBD range of extraction factors including extraction temperature ranging from 160 to 200 ℃, residence time ranging from 0 to 20 min and liquid-solid ratio ranging from 30 to 80 mL/g. According to the

experimental design and analysis of the Box–Behnken design (BBD), the experimental data obtained were fitted to a second-order polynomial equation using multiple regression analysis. The optimum operation parameters for the extraction of polysaccharides were determined as follows: extraction temperature 193.98 ℃, residence time 5.36 min and liquid-solid ratio 52.98 mL/g with predicted value for IOP yield of 168.65 mg/g. Considering the feasibility for operating the SWE treatment device, the parameters were modified slightly in the verification experiment as follows: extraction temperature of 194 ℃, residence time of 5.36 min, and liquid-solid ratio of 53 mL/g. The experimental extraction yield was 168.80 ± 0.59 mg/g, well in agreement with the predicted value (168.65 mg/g) of the model with a desirability value of 0.984 being predicted. The experimental extraction yield was 168.80 ± 0.59 mg/g, about 5.5 times of that extracted by HWE (30.71 ± 0.43 mg/g).

2. Physicochemical characteristics of IOP extracted by the two different methods, SWE and HWE, were compared. The FT-IR spectra of these two polysaccharides reflecting all of the typical absorption peaks of a polysaccharide were almost same. Peaks at different wavenumbers can be attributed to various stretching vibrations including O-H, C-H, C=O, -OH and C-O. Besides, monosaccharide compositions were determined. As the involvement of galacturonic acid and glucuronic acid, IOP was acidic polysaccharides. The main monosaccharide compositions of HWE-IOP were glucose (60.18%) and galactose (18.02%), whereas those of SWE-IOP were glucose (82.49%), xylose (8.60%) and mannose (6.71%). Eight main monosaccharides including rhamnose, galactose, arabinose, glucose, xylose, mannose, galacturonic acid and glucuronic acid in HWE-IOP and SWE-IOP were 1.33: 4.75:1.13: 16.37: 1.85: 3.38: 0.48: 1.51 and 0.02: 0.27: 0.24: 35.53: 5.29: 4.61: 0.15: 0.78, respectively. All the results identified that IOP was a typical hetero-polysaccharide fraction, regardless of extraction methods. The average *Mw* value of HWE-IOP was 66.51 kDa whereas the average Mw value of SWE-IOP failed to acquire, possibly due to its average weight was lower than 10kDa, the low limit of

elution range. SWE-IOP exhibited stronger antioxidant activity than HWE-IOP, with IC_{50} being 0.86, 0.039 and 0.13 mg/mL respectively in DPPH radical scavenging activity, SOD-like activity and Hydroxyl radical scavenging activity assays in comparison to 1.78, 0.077 and 0.41 mg/mL for HWE-IOP, most probably due to the changes of monosaccharide compositions and their molecular weights. The results also indicated that IOP extracted by SWE and HWE at the concentration of 5 mg/mL, had little toxicity on human normal fibroblast (TIG-3) cells with cell viability of 84.73 ± 5.41% and 89.73 ± 3.97% for SWE-IOP and HWE-IOP, respectively. Meanwhile SWE-IOP exhibited a slightly stronger anti-proliferation effect on human alveolar basal epithelial (A549) cells with inhibition ratio of 89.76 ± 3.97% in comparison to 85.83 ± 5.08% for HWE-IOP.

3. According to the results of single factor experiments, the effects of extraction variables (extraction temperature, residence time and liquid-solid ratio) on the extraction yields were determined. Extraction temperature 190 - 230 ℃, residence time 0 - 10 min and liquid-solid ratio 80 - 120 mL/g were employed for RSM. The optimum SWE conditions for TPC were as follows: temperature 207.78 ℃, residence time 5.07 min and liquid-solid ratio 107.41 mL/g, yielding a predicted value of 480.52 mg GAE/g. Also, an estimated yield of TFC was 971.81mg RE/g under the conditions of temperature 209.79 ℃, residence time 4.78 min and liquid-solid ratio 97.90 mL/g. According to the analysis of RSM, the mutually optimal extraction conditions for TPC and TFC were identified. Experimental results showed that the experimental TPC of 477.58 ± 0.59 mg GAE/g dry chaga, TFC of 971.13 ± 0.42 mg RE/g dry chaga and ABTS radical scavenging activity of 178.34 ± 1.23 mM AAE/g were achieved at 209 ℃ for 4.60 min with a liquid-solid ratio of 110.50 mL/g. In comparison, TPC of 121 ± 1.06 mg GAE/g dry chaga, TFC of 240 ±1.43 RE/g dry chaga and ABTS radical scavenging activity of 62.83 ± 0.76 mM AAE/g were determined at 40 ℃ for 4 h with an ethanol concentration of 75%. TPC and TFC obtained by SWE were more than 4 times than those by EE. ABTS radical scavenging activity of the extract by SWE was over 2.5 times than that by

EE. Besides, the amount of individual phenolic compounds in the extract obtained by SWE were determined as follows gallic acid of 0.24 mg/g, epigallocatechin of 8.57 mg/g, catechins of 17.84 mg/g, chlorogenic acid of 1.99 mg/g, vanillic acid of 1.07 mg/g, epicatechin of 13.33 mg/g and ferulic acid of 1.60 mg/g..

In conclusion, results from this study firstly revealed polysaccharides and phenolic compounds with high antioxidant activities could be extracted effectively and efficiently by using SWE from *Inonotus obliquus*, which could be further developed as a potential bioactivity resource for dietary supplement of functional food.

Keywords: subcritical water extraction; optimization; *Inonotus obliquus*; polysaccharides; phenolic compounds; bioactivity.

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Acronyms and abbreviations

2D	Two dimension
3D	Three dimension
BBD	Box–Behnken design
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
C.V.	Coefficient of variance
DC	Dielectric constant
DMEM	Dulbecc's modified eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
FB	Fruiting body
FT-IR	Fourier transform infrared spectroscopy
GAE	Gallic acid equivalent
H_2O_2	Hydrogen peroxide
HO•	Hydroxyl radical
HPAEC	High performance anion exchange chromatography
HPLC	High performance liquid chromatography
HWE	Hot water extraction
IOP	*Inonotus obliquus* polysaccharides
KBr	Potassium bromide
Kw	Ionization constant
MALLS	Multi-angle static light scattering
NaOH	Sodium hydroxide
OD	Optical density
PAD	Pulsed amperometric detection
RE	Rutin equivalent
RID	Refractive index
ROS	Reactive oxygen species
RSM	Response surface methodology
SOD	Superoxide dismutase
SWE	Subcritical water extraction
TBHQ	Tert-butylated hydroxyquinone
TFA	Trifluoroacetic acid
TFC	Total flavonoid content
TPC	Total phenolic content
Vc	Ascorbic acid

Chapter 1 Introduction

1.1 *Inonotus obliquus* and its bioactivity

1.1.1 *Inonotus obliquus*

Inonotus obliquus (persoon) Pilat, also known as chaga mushroom (a Latinisation of the Russian term 'чага'), is a black parasitic fungus that belongs to the Hymenochaetaceae family of Basidiomycetes and subdivided from Aphyllophorales of Polyporaceae (Lee et al., 2008). It usually inhabits selectively as parasitism on living trees, dead standing trees, and fallen trunks of Betula (Niu et al., 2016). The location of this mushroom is mainly distributed in Russia Siberia, Far East, Nordic, Japan Hokkaido, North Korea, northern China (Heilongjiang and Jilin Changbai Mountain), the northern United States, and the North Carolina mountains of the United States, where are located at latitudes of 45 ℕ - 50 ℕ because its mycelium can tolerant low temperature of up to -40 ℃ (Ju et al., 2010). After 10 - 15 years' continuously absorbing nutrients from host plants, it forms sclerotia at the cost of exhausting all essence of the birch (Ham et al., 2009). Generally, mature *Inonotus obliquus* (*I. obliquus*) is comprised of two eye-distinguishable parts, black (mainly outside) and brown (mainly inside) that were previously classified as Sclerotium and Fruiting body (FB), respectively (Chung et al., 2010), but most of Chaga products in Japan are processed without distinguish them (Nakajima et al., 2007). That is, not only fruiting body of the fungus but also a sclerotia or mass of mycelium are mostly

black due to the presence of massive amounts of melanin. Apart that, as a kind of black sterile conks, *Inonotus obliquus* has some own unique morphological characteristics, such as a shape of wedge, the appearance of burnt charcoal with irregular groove marks, hardened surface but tender inside as well as varying in size from approximately 5 to 40 cm in diameter (Lee et al., 2008).

1.1.2 Bioactivity of *I. obliquus*

Traditionally, *I. obliquus* becomes recognized and used as a folk medicine in Russia, Poland and most of the Baltic countries since the 16th century (Cui et al., 2005). Up to now, a variety of products of *I. obliquus*, including tea decoction, extracts, syrups, injections (injections), hip bath agent, aerosol for the treatment of neoplastic diseases have been developed in various countries (Zhao et al., 2004). For example, the Medical Academy of Science in Moscow and the government approved the development of *I. obliquus* as anticancer substance in 1955; a report from Australia showed the ability on cancer cure was sent to the U.S. National Cancer Institute in 1960; a kind of brown liquid called “Befungin” is still widely used for treating cancer in Poland (Zhukovich et al., 2010).

In recent years, more than 20 different kinds of various functional components have been found in *I. obliquus* (Song et al., 2008), such as phenolic compounds (Nakajima et al., 2007), hydrolysable tannins, flavonoids (Zheng et al., 2007b), polyphenols (Lee & Yun, 2007) and

melanins (Babitskaia et al., 2000), lanostane-type triterpenoids (Niu et al., 2016), inotodiol and trametenolic acids, β-glucan (Rhee et al., 2008), peptides (Hyun et al., 2006), polysaccharides (Zhang et al., 2007), and steroids (Cui et al., 2005). According to previous studies, *I. obliquus* exhibits beneficial effects on a variety of diseases including anti-viral (Ichimura et al., 1998), anti-fungal (Kahlos & Tikka, 1994) and antitumor activities (Kahlos et al., 1987), DNA protection, hypertension (Kwon et al., 2008), neurodegenerative (Alzheimer's and Parkinson's diseases) (Heo & Lee, 2005), anti-nociceptive effects (Hyun et al., 2006), autoimmune diseases (Apel & Hirt, 2004), hypoglycaemic activity (Kim et al., 2006), hepatic protection (Wasser & Weis, 1999) as well as anti-tuberculosis (Saar, 1991). Additionally, different biologically active fractions or extracts such as water extract (Youn et al., 2008), methanol extract (Ham et al., 2009), ethanol extract (Sun et al., 2011) had been found to have effective anti-proliferative effects on cancer cells *in vitro* and *in vivo*.

1.2 Subcritical water extraction and its mechanisms

1.2.1 Subcritical water extraction

Extraction is the crucial step for sample preparation prior to the analysis of solid samples. Typical sample preparation techniques depends upon extraction with solvents, including liquid-liquid extraction, Soxhlet extraction, sonication, and other methods have been used. However,

the above conventional methods have obvious drawbacks in terms of extraction efficiency, processing duration, as well as process sustainability.

In recent years, with the development of novel sample extraction techniques, a growing interest in the conversion of biomass, carbohydrates, and other organic materials for producing useful chemical compounds and energy has led to extensive researches on subcritical water. Among them, subcritical water extraction (SWE, also called hot-compressed water or hydrothermal extraction), has become a popular environment-friendly extraction method for different classes of valuable compounds present in numerous kinds of matrices like environmental, food and botanical samples. According to the definition of this terminology, subcritical water denotes the condensed phase of water region between the temperature range from 100 ℃ (boiling point of water) to 374 ℃ (the critical point of water) under a sufficient pressures (1 - 22.1 MPa) that is high enough to maintain its liquid state, such as 15 bar at 200 ℃ and 85 bar at 300 ℃ (Ramos et al., 2002). Superheated steam will be formed once the pressure is lower than the boiling point at any pressure.

The versatility for both polar and non-polar compounds of subcritical water is correlated with the tunable polarity of water, directly depending upon the temperature. Specifically, along with the temperature of water is increased, the polarity of water decreases, which can enhance the solubility of non-polar organics and reduce the solubility of polar organic (Anekpankul et al., 2007). As a result, low temperature water could extract more water-soluble organic

compounds, whereas higher temperature water could extract less soluble organic compounds (Huang et al., 2010). Ionization constant of subcritical water is another important character which is influenced by the temperature. It is often associated with chemical reactions including hydrolysis and degradation without any additional catalyst (Öztürk et al., 2010). Additionally, some other characteristics like water density, viscosity, diffusivity, electric conductance, and solvent ability are altered (Chao et al., 2013). For example, more chemical ingredients are released into the extra-cellular medium due to the destruction of the cellular structure of plant tissues under subcritical water temperature. Hence, SWE has steadily become an efficient, environment-friendly and low cost method of extraction for less-polar organic components from environmental soil, sediments and plant materials (Yang et al., 2013).

1.2.2 Mechanisms

From a kinetics standpoint, four sequential procedures generated in the extraction cell filled with sample materials and high portion of sands under elevated temperature and the pressurized conditions can be used to interpret the mechanisms of the extraction procedure (Teo et al., 2010). The first step is the solutes desorption from the various active sites in the sample matrix bound targets under the pressurized and elevated temperature conditions; the second step is the diffusion of subcritical water into the organic matrix; the third step is the dissolution of the compounds from original binding sites in sample matrix into subcritical water; and the forth

step is the chromatographical elution of the extract-laden solution from the extraction cell to the collection vial. It is difficult to quantify the effect of each step above on extraction efficiency based on extraction yield data alone, whereas it is clear that, in all of the above kinetic steps, higher temperature will lead to improved extraction efficiency.

Several attempts including (1) an improvement in the solubility and mass transfer effects and (2) an increased disruption of surface equilibria contribute to the enhancement on SWE extraction efficiency (Ong et al., 2006). As the features of water is changed at elevated temperatures, the ability of subcritical water on dissolution analytes is promoted. A reduced viscosity but improved diffusivity of water can be more permeable to the matrix particles. Besides, extraction rate can be accelerated by the improvement of the mass transferthrough continuous introduction of fresh water during a dynamic extraction. Generally, van der Waals force, hydrogen bonding, dipole attraction of the solutes molecules and active sites in the matrix can lead to the solute–matrix interaction. However, the elevated temperature can overcome the aforementioned interaction by disrupting the surface equilibria. Thus, the thermal energy supplied can disrupt cohesive (solute-solute) and adhesive (solute-matrix) interaction by decreasing the activation energy required for desorption process. Moreover, sufficient pressure exerted on water could facilitate extraction from samples, in which analytes are trapped in the matrix pores.

Based on these features, SWE, as an extractant instead of organic liquid solvent extraction methods, can produce high extraction yield and fast extraction process for a number of organic ingredients.

1.3 Polysaccharides from *I. obliquus*

As one of the vital natural antioxidants, polysaccharides have been attracting great attention in medicine and food industries because of their biological activities, such as antibacterial activity, anticancer activity, and immune-enhancing effects, and so forth (Song et al., 2015). It is well known that polysaccharides are an important class of polymeric carbohydrate molecules composed of long chains of monosaccharide units joined together by glycosidic linkages. Particularly, many natural polysaccharides and polysaccharide-protein complexes derived from fungi have been proven relatively nontoxic and have no adverse effects (Novak & Vetvicka, 2008). Studies showed that polysaccharides from *I. obliquus* possess a wide range of pharmacologic and health-promoting properties including immune enhancement (Kim et al., 2005), anti-oxidation (Huang et al., 2012), anti-diabetes (Zhang et al., 2008), and anti-tumor (Chen et al., 2010b).

1.4 Phenolic compounds from *I. obliquus*

Phenolic compounds are plant secondary metabolites, the derivatives of the pentose phosphate, shikimate, and phenylpropanoid pathways (Balasundram et al., 2006). They possess considerable physiological and morphological effects as one kind of the most widely occurring groups of phytochemicals. Specifically, these compounds are important determinants in the growth and reproduction, providing protection against pathogens and predators, as well as contributing towards the color and sensory characteristics of fruits and vegetables (Song et al., 2015).

Phenolic compounds have a variety of physiological activities, such as antioxidant, anti-mutagenic, anti-allergenic, anti-inflammatory, and anti-microbial effects (Martins et al., 2011). In the last decade, the search for health-related phenolic compounds from various fruits, vegetables, herbs, cereals as well as other plant materials has gained great attention in food industry, and increasing number of research studies have investigated the kinetics and mass transfer mechanism for extraction of phenolic compounds from various plants, and obtained efficient processes for extraction of these compounds (Al-Farsi & Lee, 2008). In this regard, *I. obliquus* fungus has been reported to exhibit antioxidant activity *in vitro* and antitumor activity *in vivo* due to their abundance of phenolic acids and flavonoids (Park et al., 2004).

1.5 Targets and structure of the thesis

I. obliquus has been recognized as a folk medicine. Since a variety of its products has been applied in various countries, it is necessary to improve the extraction yield of antioxidant compounds from *I. obliquus* in terms of production cost, production efficiency as well as bioactivities. Nowadays, SWE, a novel environment-friendly extraction method, has become an interesting alternative in the extensive research activities about the conversion of biomass, carbohydrates and other organic materials in the production of useful chemical compounds and energy. In this study, subcritical water extraction was firstly employed to obtain polysaccharides, total phenolic content as well as total flavonoid content and to investigate the effects of SWE parameters on the yields. Then physicochemical characteristics of polysaccharides extracted by SWE (SWE-IOP) and some phenolic ingredients were identified and its antioxidant activity *in vitro* was also evaluated. Specifically, this thesis consistes of five chapters in which the major points are listed as follows:

In chapter 1, the basic information of the raw material (*I. obliquus*), extraction method (SWE) as well as target compounds (polysaccharides, total phenolic content and total flavonoid content) were introduced. Also, the targets and the structure of the thesis were discussed.

In chapter 2, the extraction conditions were investigated to extract polysaccharides from *I. obliquus* using SWE to achieve the maximum yield of IOP by using RSM with a BBD design.

In chapter 3, extraction from *I. obliquus* extracts at different elevated temperatures was

conducted to detect the effects of thermal processing at different subcritical water conditions on the change of specific constituents and their antioxidant activities.

In chapter 4, the optimum conditions of TPC, TFC and IC_{50} values on DPPH radicals of extracts by using SWE from *I. obliquus* were analyzed by single factor experiment and response surface methodology, according to their extraction temperature, residence time and liquid-solid ratio.

Eventually, all the above researches were compendiously concluded and future works were prospected in chapter 5.

Chapter 2 Optimization of SWE of polysaccharides from *Inonotus obliquus* by response surface methodology

2.1 Introduction

Nowadays, natural antioxidants are considered as functional ingredients for pharmaceuticals, functional foods, dietary supplements, animal feed, cosmetics and other products. Therefore, the interest in natural antioxidants is getting momentum due to not only the generalizability of their action in various redox systems and consequently broad spectra of possible applications (Augustyniak et al., 2010). On the other hand, synthetic antioxidants are speculated to have possible toxicity and causes of health complications. Moreover, with the establishment of healthy concept, consumers are more inclined to opt for natural food additives (Getachew & Chun, 2017).

Response surface methodology (RSM), as an effective statistical analysis technique, has been employed to optimize complex process parameters, including the extraction of polysaccharides, by evaluating interactions among various factors, as well as simultaneously estimating the effects of several process variables and their interactions with response variables (Maran et al., 2013). RSM comprises several statistical designs such as Box–Behnken Design (BBD), Central Composite Design (CCD), Optimal Design and other statistical procedures. Among these designs, the BBD -based statistical modeling represents an independent quadratic design that does not contain an embedded factorial or fractional factorial design (Qu et al., 2016). It has been distinguished as a simplified design to cover three levels of experimental factors with less number of experiments (Zhao et al., 2011).

To the best of our knowledge, little information is available on the *Inonotus obliquus* polysaccharides (IOP) through subcritical water extraction. Hence, the objective of this chapter

was to optimize the operational parameters (extraction temperature, residence time and liquid-solid ratio) of SWE to achieve the maximum yield of IOP by using RSM based on a BBD design.

2.2 Materials and methods

2.2.1 Chemicals and reagents

Hydrogen peroxide, sodium hydroxide, sodium acetate trihydrate, chloride ferric, ferrous sulphate, dibasic sodium phosphate, sodium dihydrogen phosphate, sodium salicylate, ethanol, chloroform, n-butano, potassium persulphate, phenol, and D-glucose were purchased from Wako Pure Chemical (Osaka, Japan). All the chemical reagents were of analytical grade.

2.2.2 Preparation of *I. obliquus*

In this study, *I. obliquus* was obtained from the same batch in the manufacturing process. Samples were dried at 60 ℃ for 5 h to remove residual water and then ground in a high-speed disintegrator (IFM-800, IWATANI, JAPAN) to obtain a fine powder (250 μm). Ground *I. obliquus* was refluxed twice with ethanol (volume fraction 85%) at 70 ℃ in a water bath for 4 h to defat, deactivate enzymes and remove some interference materials (colored materials, oligosaccharides, and some small molecule materials). The ethanol mixture extract was centrifuged (1000 *g*, 20 min). After removing supernatant, precipitation was vacuum dried at 50 ℃ for 12 h and vacuum packed to decrease lipid oxidation.

2.2.3 Subcritical water extraction

Subcritical water extraction (SWE) process was performed in a subcritical device (MMS-200, OMLABO, JAPAN) equipped with a temperature controller and a pressure gauge (Fig. 2-1). Sample (1.0 g) was extracted with distilled water under the predesigned conditions. The

extracts were centrifuged at 2000 *g* for 20 min and filtrated (0.45 μm, Millipore, USA) to collect the supernatant. The supernatant were concentrated by a rotary evaporator into a certain volume under reduced pressure at 60 ℃, and then precipitated by the addition of absolute ethanol to a final concentration of 80% (v/v) overnight at 4 ℃. The precipitates were collected as crude IOP after centrifugation at 2000 *g* for 15 min, washed triple times using dehydrated ethanol. After being re-dissolved in ultrapure water, the aqueous solution was subjected to remove proteins by using Sevag reagent (chloroform and n-butanol in a 4:1 ratio), dialyzed with deionized water for 72 h, concentrated under reduced pressure. Finally, the IOP product was collected after lyophilization.

2.2.4 Determination of IOP yield

The obtained crude polysaccharides were re-dissolved in doubly distilled water for polysaccharides yield and antioxidant capacity determination. The polysaccharides content of crude IOP was determined based on the phenol-sulfuric acid method with some modifications (Zhang et al., 2013). Briefly, 1 mL of dilute polysaccharides sample solution was mixed with 0.5 mL 5% phenol solution and 2.5 mL 98% H_2SO_4 and the mixture was incubated in a 100 ℃ water bath for 15 min. After cooling to ambient temperature, the optical density (OD) of the mixture was determined measured with a spectrophotometer at 490 nm and the polysaccharides content was calculated with D-glucose as the standard (R^2= 0.9991). The results were expressed as the polysaccharides content in per gram of dry *I.obliquus* using the following equation:

$$\text{IOP yield (mg/g)} = \frac{C \times N \times V}{W} \qquad (2\text{-}1)$$

where *C* is the concentration of polysaccharides as calculated from the calibrated regression equation (mg/mL); *N* is the dilution factor; *V* is the total volume of extraction solution (mL); and *W* is the weight of the sample (g).

2.2.5 Single-factor experimental design

A series of single factor experiments was conducted to identify the independent variables and investigate the preliminary BBD range of extraction factors including extraction temperature ranging from 160 to 200 ℃, residence time ranging from 0 to 20 min and liquid-solid ratio ranging from 30 to 80 mL/g. The extraction yield of IOP was the dependent variable. Each experimental factor was determined separately as others were kept constant. Each experiment was performed in triplicate.

2.2.6 Experimental design of RSM

On the basis of results of the former single-factor experiments, BBD with RSM was applied to optimize the extraction conditions of IOP. Three independent variables (X_1, extraction temperature; X_2, residence time and X_3 liquid-solid ratio) and their levels followed in the second stage have been shown in Table 1. For the statistical calculation, process factors are coded according to Eq. (2-2):

$$x_i = (X_i - X_0)/\Delta X \quad i = 1, 2, 3 \qquad (2\text{-}2)$$

where x_i is the coded value of the independent factor; X_i is the actual value of the independent variable, X_0 is the actual value of X_i at the central point, and ΔX is the step change value.

The whole design consisted of 17 experimental points, including 12 randomized points of the independent variables with their responses of the dependent variable (yield of the extracted IOP) and five replicates at the center point to evaluate the pure error. All these runs were measured in a random order to maximize the effect of unexplained variability in the observed response due to extraneous factors and minimize the effects of non-controlled variables. Data from three repeated experiments were analyzed by multiple regressions to fit into the general form of a quadratic polynomial model as shown in the following Eq. (2-3),

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j \quad (2\text{-}3)$$

where Y is the predicted response variable associated with each three levels combination (IOP yield), B_0 is the constant coefficient, B_i is the liner coefficient, B_{ii} is the quadratic coefficient, and B_{ij} is the two-factor interaction coefficient. X_i and X_j are the levels of the independently coded variables ($i \neq j$).

The experimental design, data analysis, and model building of the previous study were performed using Design-Expert software (8.0.5 Statease Inc., Minneapolis, USA). All trials were performed in triplicate.

2.3 Results and discussion

2.3.1 Single factor experimental analysis

(1) Effect of extraction temperature on the extraction yield

The extraction temperature is a crucial parameter affecting the extraction efficiency and selectivity. Generally, target compounds are tied on the sample matrices by means of physical adsorption and chemical interactions (Zhang & Wang, 2017). At appropriate temperature, two aforementioned styles were retrained, thereby facilitating the leaching-out of target compounds in extraction solvents (Qi et al., 2015). Moreover, elevated extraction temperature can modify the characteristics of solvent like decreasing the viscosity and increasing the diffusion, which all make great contributions to the mass transportation of targeted ingredients. However, if the temperature is extremely high in extraction processing, structural degradation and the inactivate activity of polysaccharides may occur (Thirugnanasambandham et al., 2015).

The effect of temperature (160, 170, 180,190 and 200^{o}C) on the IOP yield is shown in Fig. 2-2a when the other factors, residence time and liquid-solid ratio, were fixed at 20 min and 80

mL/g, respectively. As shown when as the temperature increased from 160 to 190°C, the extraction efficiency increased rapidly and the polysaccharides yield reached a maximum of 132.62 ± 2.63 mg/g at 190°C. Upon further increase in temperature, the polysaccharide yield decreased sharply, indicating that excessive temperature may lead to degradation and decomposition of polysaccharides induced by the browning reaction, and its chemical products are not detected in a phenol–sulphuric assay, thus decreased the concentration of IOP yield (Xu et al., 2016). In terms of extraction efficiency and energy consumption, an extraction temperature of 190°C was employed in subsequent extraction experiments.

(2) Effect of residence time on the extraction yield

Extraction time is another important parameter influencing the extraction rate and quality of yielded compounds (Tan et al., 2013). It usually takes polysaccharides some time to contact the release medium, where solvent can permeat into the dried materials so that they could be dissolved and diffused out subsequently from the materials (Zhu et al., 2015). Apart from that, extraction time is crucial in minimizing energy and production cost, which is especially obvious in the extraction process (Chew et al., 2011). Thus, considering economic efficiency and energy consumption, the effect of the extraction time on the IOP yield was investigated and the extraction process was performed for different extraction time from 0 to 20 min when the other factors were as follows: liquid to solid ratio 80:1 mL/g and temperature 190°C, respectively. As illustrated in Fig. 2-2b, the IOP yield was positively correlated with an increased duration, and it increased up to a maximum amount of 151.09 ± 2.77 mg/g in 5 min, which meant a great number of IOP was released to exterior solvent in this early extraction stage. Thereafter, IOP began to continually decrease, which can be attributed to the degradation and hydrolysis of polysaccharides exposed to high temperature for a long extraction time, especially under subcritical water conditions (Liu et al., 2009). For this reason, the extraction time was controlled in the range of 5 min.

(3) Effect of liquid-solid ratio on the extraction yield

In classical extraction methods, solid-liquid ratio is recognized as one of the major parameters for affecting the effectiveness of the procedure and the quality of the extracts (Ozcan, 2006). Various liquid-solid ratio can directly impact the concentrations between the interior plant cells and the exterior solvent, significantly affecting the yield of the extracted polysaccharides (Chen et al., 2012c). An inappropriate liquid-solid ratio may result in the incomplete dissolution of polysaccharides or solvent waste (Thirugnanasambandham et al., 2015). Therefore, different liquid-solid ratio including 30, 40, 50, 60, 70 and 80 mL/g were tested while the other two extraction variables (extraction temperature and residence time) were set in the central points as follows: 190^{o}C and 5 min, respectively. As presented in Fig. 2-2c, the extraction yield of IOP initially increased slightly with increasing liquid to solid ratio and reached a maximum yield (157.17 ± 0.65 mg/g) when the liquid-solid ratio was 50 mL/g. Thereafter, as the liquid-solid ratio continued to increase, the yield tended to decrease. The reason is that some level of liquid-solid ratio can promote diffusivity of solvent into cells and enhance the leaching-out rates of polysaccharides therefrom. However, an excess of solvent could consume cavitation energy from the extraction system and complicate extraction procedure, resulting in a lower IOP yield (Samavati, 2013). Hence, to minimize electricity and time costs, a liquid-solid ratio of 50 mL/g was selected to ensure the extraction yield.

2.3.2 Box–Behnken design (BBD) for optimization of extraction conditions

(1) Statistical analysis and the model fitting

Based on single factor experiments mentioned above and according to BBD design matrix, a number of 17 experimental runs (12 runs for various conditions and 5 center point runs for measurement of process stability and inherent variability) with three factors and three levels

were performed to study the reciprocal influence of three independent variables (extraction temperature, residence time and liquid-solid ratio) on IOP extraction yield and establish the optimal SWE conditions. The variables of the actual and coded levels and the experimental results performed three times are shown in Table 2-1 and Table 2-2, respectively. As it can be seen, the IOP yield ranged from 83.21 to 165.73 mg/g. According to multiple regression analysis of the experimental data using Design Expert software version 8.0.5, the data in Table 2-2 were carried out. The fitted model for the IOP yield (mg/g) was correlated according to the following second-order polynomial equation:

$$Y = 164.64 + 14.17X_1 + 10.48X_2 + 5.46X_3 - 20.12X_1X_2 + 1.82X_1X_3$$

$$+ 1.21X_2X_3 - 16.67X_1^2 - 19.89X_2^2 - 10.53X_3^2$$

Where Y is the yield of polysaccharides, X_1, X_2, and X_3 are the coded variables for extraction temperature, residence time and liquid-solid ratio, respectively.

Analysis of variance (ANOVA) and F-test were employed to evaluate the significance and to analyze the adequacy and the goodness-of-fit of the response surface quadratic model (Table 2-3). The high quadratic regression model significance was identified by its high F-value (F = 2408.99) and low p-value ($p < 0.0001$). The goodness-of-fit can be demonstrated by the determination coefficient (R^2), adjusted determination coefficient (R_{adj}^2) and coefficient of variance (C.V.) (Zhang & Wang, 2017). R^2 offers the proportion of the total variation in the response predicted by the model and indicates ratio of sum of squares because of regression to total sum of squares (Zhang et al., 2016). R^2 coefficient highly closed to unity indicates a satisfactory adjustment of the quadratic model to the experiment statistics (Bezerra et al., 2008). In this study, the determination coefficient (R^2) was 0.9997, implying that 99.97% of the variations could be interpreted by the fitted model. Besides, a high degree of correlation between the observed and predicted values was confirmed since the adjusted determination coefficient ($R_{adj}^2 = 0.9993$) for the model had no obvious difference with R^2 (Chen et al., 2012a).

Moreover, a low coefficient of variation (C.V. = 1.07) indicated that the degree of precision and reliability of experimental values of regression model were highly significant (Samavati & Manoochehrizade, 2013). The P-value was used as a tool to not only reveal the significance of each coefficient but also explain the interaction pattern of independent variables (Xu et al., 2016). The smaller the value of *p* with respect to 0.05, the more significant the corresponding coefficient.

As seen from all the regression coefficients listed in Table 2-3, it could be concluded that all the linear coefficient (X_1, X_2 and X_3), all the quadratic term coefficients (X_1^2, X_2^2, X_3^2), and all the cross product coefficients (X_1X_2, X_1X_3 and X_2X_3) with small *p*-value ($p < 0.05$) are significant for IOP extraction. This finding manifested that all the independent parameters analyzed in this study were important factors affecting the yield of extracted IOP. In addition, the significance of the model was also judged by a lack-of-fit test, meaning the failure of the model to represent the data in the experimental domain at points that are not included in the regression (Ghafari et al., 2009). The ANOVA showed that lack of fit (F-value = 0.070 and *p*-value = 0.9728) was not significantly relative to the pure error, implying that it was not significant and just a 2.72% chance could occur due to noise. Consequently, it was determined that the proposed mathematical model and extraction parameters could be used to predict the yield of IOP under any combination of the values of different variables under SWE conditions (Gan & Latiff, 2011).

(2) Response surface plot

To represent a visual interpretation of the interactions between the independent and dependent variables, three-dimensional (3D) response surface and two-dimensional (2D) contour plots (Fig. 2-3) were achieved using Design-Expert software. Generally, 3D response surface plot can display the mutual influences of independent variables on the response variable and further assist in finding the maximum, minimum and saddle points of the responses (Matos

et al., 2015). For 2D response contour plots, not only do 2D response contour plots interpret the reciprocal interactions between independent variables, also reflect the significance of mutual interactions between the variables (Guo et al., 2010). In other words, Circular or elliptical patterns in the contour plots implied whether the mutual interactions between the variables are significant or not (Mullai et al., 2010).

The three-dimensional response surface (Fig. 2-3a) and contour plots (Fig. 2-3b) illustrated the effects of extraction temperature as well as residence time on the yield of IOP at a fixed liquid-solid ratio of 50 mL/g. The IOP yield increased significantly as extraction temperature together with residence time increased in the range of 180.00^{o}C - 193.98^{o}C and 0 - 5.36 min, respectively. However, when these two parameters beyond the peak points, the yield decreased gradually, which probably due to excessive extraction time and temperature could lead to the hydrolyzation or degradation of polysaccharides (Chen et al., 2012b). According to the elliptical shape in the contour plot, the relationships between extraction temperature and residence time were significant. Fig. 2-3c and Fig. 2-3d showed the effects of extraction temperature and liquid-solid ratio on IOP yield when residence time was fixed at a zero level. A maximum IOP was obtained at 193.98^{o}C and a liquid-solid ratio of 52.98 mg/L. As shown in Fig. 2-3e and Fig. 2-3f, the IOP yield with varying residence time and liquid-solid ratio at fixed ratio of extraction temperature (0 level) had been evaluated. The elliptical shape of the 2-D contour plot indicated that the mutual interaction between residence time and liquid-solid ratio were significant.

(3) Optimization of extraction parameters and validation of the model

The optimal values of independent variables and response variable for the proposed extraction were analyzed by Design-Expert software and recommended as follows: extraction temperature of 193.98^{o}C, residence time of 5.36 min, and liquid-solid ratio of 52.98 mL/g with the predicted value for IOP yield of 168.65 mg/g. To validate the adequacy of the model equations, a verification experiment should be tested using the estimated optimal conditions.

However, considering the operation conditions of the device in this study, the parameters were modified slightly in the verification experiment as follows: extraction temperature of 194°C, residence time of 5.36 min, and liquid-solid ratio of 53 mL/g. Under these extraction conditions, triplicate confirmatory experiments were carried out and the average extraction yield of IOP was 168.80 ± 0.59 mg/g, which well agreed with the predicted value. A good correlation between the predicted and experimental values revealed that the regression model was accurate and adequate for the extraction of IOP by SWE method. Therefore, the model was suitable for the optimization of SWE extraction procedure for IOP.

2.4 Summary

In the present study, SWE was employed to extract polysaccharides from *I. obliquus*. Box-Behnken design was successfully conducted to optimize extraction conditions. Based on the response surface methodology test and adjustment of the actual procedure, the optimal IOP extraction parameters are as follows: extraction temperature 194°C, residence time 5.36 min, and liquid-solid ratio 53 mL/g. Under these optimized extraction conditions, the experimental yield agreed closely with the predicted yield. These results indicated that SWE might be applied as a convenient, effective, and environment friendly method for extraction of polysaccharides from natural source *I. obliquus*.

Table 2-1. Independent variables and their levels used in the response surface design (BBD).

Independent variables	Symbol	Levels		
		-1	0	1
Temperature (℃)	X_1	180	190	200
Residence time (min)	X_2	0	5	10
Liquid-solid ratio (mL/g)	X_3	40	50	60

Table 2-2. Box-Behnken design matrix of the three variables (coded and uncoded units) and results for IOP extraction.

Run	Temperature (℃)	Residence time (min)	Liquid-solid ratio (mL/mg)	Polysaccharide content (mg/g)	
				Experimental	Predicted
1	-1 (180)	-1 (0)	0 (50)	83.21	83.32
2	1 (200)	-1 (0)	0 (50)	151.98	151.89
3	-1 (180)	1 (10)	0 (50)	144.41	144.52
4	1 (200)	1 (10)	0 (50)	132.72	132.62
5	-1 (180)	0 (5)	-1 (40)	119.66	119.63
6	1 (200)	0 (5)	-1 (40)	144.14	144.32
7	-1 (180)	0 (5)	1 (60)	127.08	126.91
8	1 (200)	0 (5)	1 (60)	158.86	158.89
9	0 (190)	-1 (0)	-1 (40)	119.57	119.49
10	0 (190)	1 (10)	-1 (40)	138.09	138.02
11	0 (190)	-1 (0)	1 (60)	127.91	127.98
12	0 (190)	1 (10)	1 (60)	151.29	151.38
13	0 (190)	0 (5)	0 (50)	163.67	164.64
14	0 (190)	0 (5)	0 (50)	164.36	164.64
15	0 (190)	0 (5)	0 (50)	165.73	164.64
16	0 (190)	0 (5)	0 (50)	165.05	164.64
17	0 (190)	0 (5)	0 (50)	164.36	164.64

Table 2-3. Analysis of variance (ANOVA) of the response surface quadratic model for the effects of extraction temperature (X_1), residence time (X_2) and liquid-solid ratio (X_3) on IOP extraction.

Source	Sum of Squares	Df	Mean Square	F-value	*p*-value	Significant
Model	8019.19	9	891.02	2408.99	< 0.0001	**
X_1	1605.59	1	1605.59	4340.93	< 0.0001	**
X_2	878.95	1	878.95	2376.36	< 0.0001	**
X_3	238.63	1	238.63	645.17	< 0.0001	**
X_1X_2	1618.54	1	1618.54	4375.92	< 0.0001	**
X_1X_3	13.29	1	13.29	35.92	0.0005	**
X_2X_3	5.90	1	5.90	15.96	0.0052	*
X_1^2	1169.43	1	1169.43	3161.70	< 0.0001	**
X_2^2	1665.12	1	1665.12	4501.85	< 0.0001	**
X_3^2	467.17	1	467.17	1263.06	< 0.0001	**
Residual	0.22	7	0.03			
Lack of Fit	0.13	3	0.043	0.070	0.9728	
Pure Error	0.06	4	0.01			
Cor Total	69.23	16				
SD	1.78					
Mean	166.76					
C.V. (%)	1.07					
Press	267.87					
R^2	0.9997					
Adj. R^2	0.9993					
Pred. R^2	0.9993					
Adequate precision	43.0765					

*Significant, $p < 0.05$

**Highly significant, $p < 0.001$

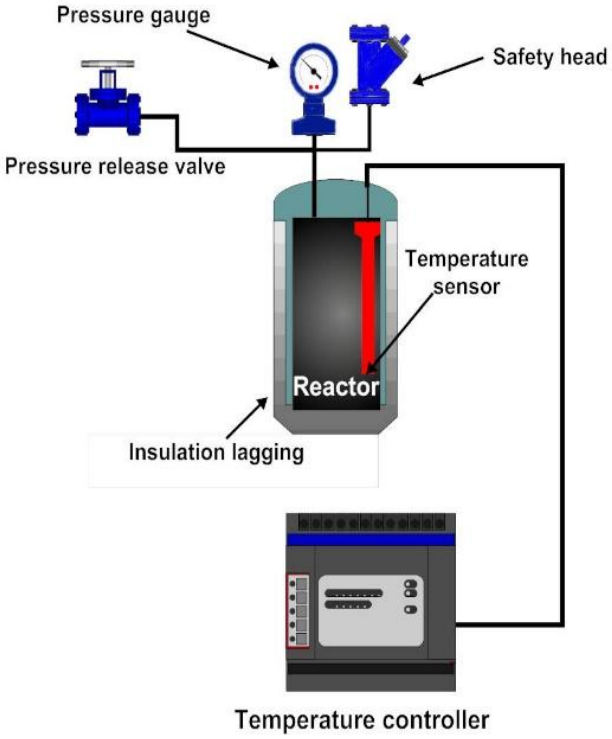

Fig. 2-1. Experimental schematic diagram of subcritical water extraction of IOP.

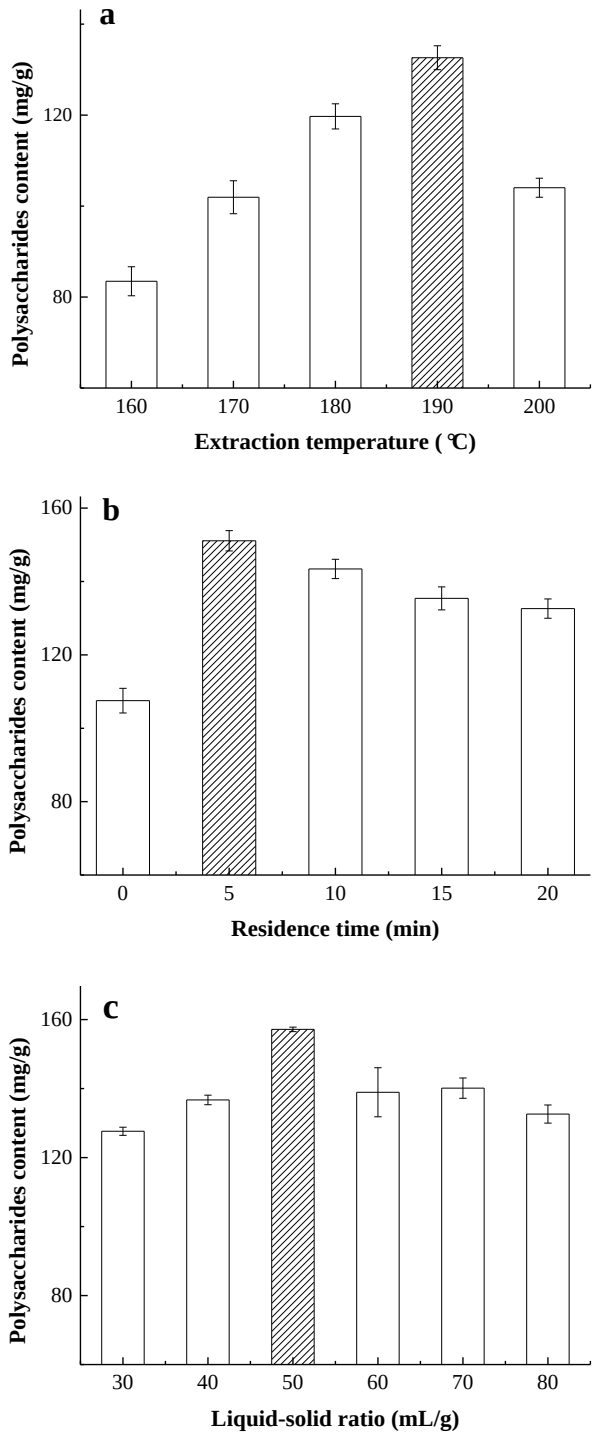

Fig. 2-2. Effect of extraction temperature (a), residence time (b), and liquid-solid ratio (c) on the extraction yield of IOP. (a) 20 min of residence time and 80 mL/g of liquid-solid ratio. (b) 190 ℃ of temperature and 80 mL/g of liquid-solid ratio. (c) 5 min of residence time and 190 ℃ of temperature. Values are means ± S.D. (n = 3).

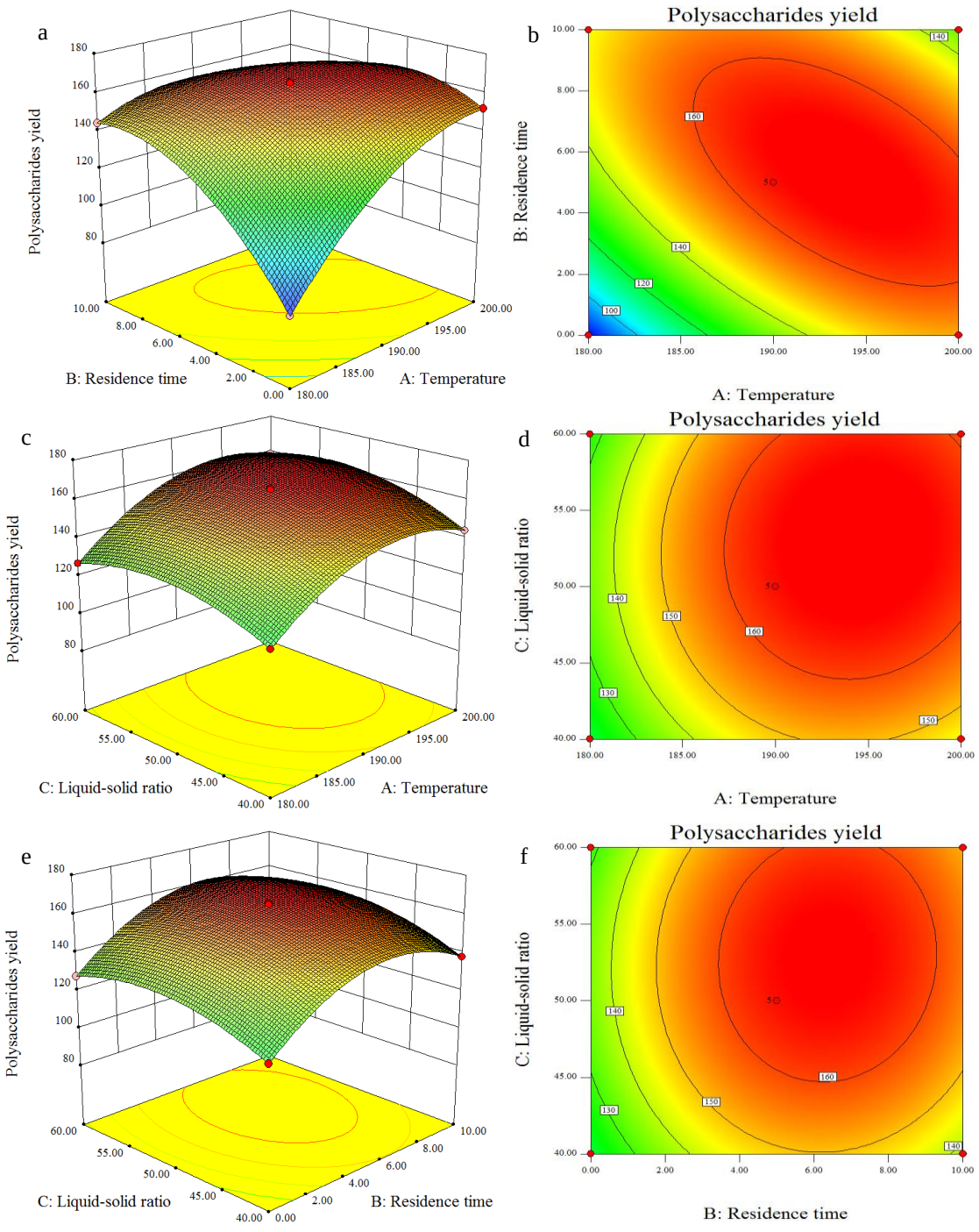

Fig 2-3. Response surface plots (a, c, and e) and contours plots (b, d, and f) for the effects of extraction temperature (X_1), residence time (X_2) and liquid-solid ratio (X_3) on the yield of IOP.

Chapter 3 Comparison of SWE and HWE for polysaccharides extraction from *Inonotus obliquus*

3.1 Introduction

Reactive oxygen species (ROS) are chemically reactive species generated by a partial reduction in oxygen or from exogenous factors and agents, containing oxygen free radicals such as hydroxyl and superoxide as well as non-oxygen radicals such as hydrogen peroxide (Mu et al., 2012). However, it would be hostile and damaged for cells and their physiological functions if the production of ROS and internal reactions initiated by free radicals are out of control (Huang et al., 2012). It is expected that there will be a chain reaction resulting to the multiplication of new free radicals and severe damage including interference and manipulation of protein, tissue loosening, genetic damage and the promotion of disease and aging will happen (Wu & Hansen, 2008). To avoid these potential threats, diverse synthesized antioxidants like butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and tert-butylated hydroxyquinone (TBHQ) are widely spread (Mu et al., 2012). Nonetheless, there have increasing researches concerning their hidden hazards related to health such as liver damage and carcinogenesis (Cheung et al., 2003). Hence, it is of essentiality to promote and apply no-toxicity natural antioxidants so as to eliminate the free radicals and retard the progress of chronic diseases (Singh & Rajini, 2004).

Recently, natural products produced by plants and their synthetic derivatives are expected to play an important role in the development of innovative antioxidants. According to chemical analysis, there are 200 kinds of active substances involving in *I. obliquus* (Xu et al., 2011), among which *I. obliquus* polysaccharide (IOP) possesses comprehensive biological properties, especially antioxidant (Liang et al., 2009) and anti-inflammatory activities (Ma et al., 2012). For instance, IOP could ameliorate the diabetic symptoms induced by streptozotocin (Diao et al., 2014).

Despite polysaccharides from *I. obliquus* might be a good candidate as an antioxidant agent, the realities including that the limited natural resource and difficult artificial cultivation of *I. obliquus* to obtain fruit body make it impossible to obtain a large quantity of natural polysaccharides (Chen et al., 2010a). In general, HWE is still the most common and convenient industrial extraction method for obtaining water-soluble polysaccharides, notwithstanding the drawbacks like consuming time, energy dissipation and inefficiency in large-scale production (Dorta et al., 2012). Therefore, it is a promising alternative that finding a novel method to extract IOP efficiently and economically that could avoid the aforementioned drawbacks of HWE. As mentioned in Chapter 2, SWE could enhance mass transfer between the plant and solvent and improved extractability of polysaccharides by the destruction of cell walls and change of solvent polar. However, it is inevitable that elevated temperature exposure could modify the physicochemical properties of polysaccharides, such as chemical composition,

molecular weight distribution, viscosity, conformation (Zhang et al., 2004). It is generally admitted that biological activities of polysaccharides depended on its molecular structure including monosaccharide composition, glycosidic bond of the main chain, degree of substitution, degree of branching, sugar component and conformation of the main chains (Ma et al., 2012). In previous studies on the structure–activity of polysaccharides, spatial structures may hinder the antioxidant activity so that high molecular weight polysaccharides might be less effective (Chen et al., 2009). Take tea polysaccharides as an example, a relatively low molecular weight and a high content of protein are shown to contribute to the increase of antioxidant activities (Chen et al., 2008).

However, there was no information about the comparisons including physicochemical properties and antioxidant activities of polysaccharides obtained by subcritical water and hot water methods from *I. obliquus*. Thus, the aim of this chapter is to determine the chemical composition, molecular weight distribution and antioxidant activities of IOP extracted by SWE and HWE.

3.2 Materials and methods

3.2.1 Chemicals and reagents

Ascorbic acid, hydrogen peroxide, sodium hydroxide, sodium acetate trihydrate, chloride ferric, ferrous sulphate, dibasic sodium phosphate, sodium dihydrogen phosphate, sodium

salicylate, ethanol, chloroform, n-butano, potassium persulphate, phenol, and D-glucose were purchased from Wako Pure Chemical (Osaka, Japan). Trifluoroacetic acid, monosaccharide standards and 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) were purchased from Sigma-Aldrich, Inc. (Saint Louis, MO, USA). All the chemical reagents were of analytical grade.

3.2.2 Subcritical water extraction and hot water extraction

Based on the results of Chapter 2, IOP was extracted under the optimum SWE conditions (extraction temperature of 194 ℃, residence time of 5.36, liquid-solid ratio of 53 mL/g). The extract was filtered, removed proteins, and then concentrated using a rotary evaporator at 60 ℃. Finally, the extract was stored at -20 ℃ until laboratory study.

For comparison, in HWE, a 5.0 g ground *I. obliquus* was extracted using 150 mL of distilled water at 90 ℃ for 2 h.

3.2.3 Fourier transform infrared spectroscopy of IOP

FT-IR was also used to identify the obtained IOP, which was carried out with the KBr-disk method (Li & Shah, 2014) using a Jasco FTIR 3000 spectrometer (Jasco, Wakayama, Japan). The dried IOP was mixed thoroughly with potassium bromide (KBr) powder, ground and pressed into 1-mm pellet for spectrometric measurement at a frequency range of 400 - 4000 cm^{-1}.

3.2.4 Monosaccharides composition of yielded IOP

The composition of monosaccharides in yielded IOP was firstly determined by using high performance anion exchange chromatography (HPAEC) with pulsed amperometric detection (PAD) as described previously with some modifications (Cai et al., 2016). Briefly, approximately 10 mg of lyophilized polysaccharides sample extracted under the optimum conditions was hydrolyzed in 4 mL of 4 mol/L trifluoroacetic acid (TFA). After incubation at 120 ℃ under nitrogen for 4 h and then cooling to ambient temperature, the hydrolysate was centrifuged and evaporated to remove the rest TFA. The resultant solution, after being added with ultrapure water to 5 mL, was diluted for 50 times. Being filtered through 0.2 μm pore membrane filter, the derivatives were isolated on a Dionex ICS-3000 system (Dionex, Sunnyvale, CA, USA) with an advanced gradient pump and an eluent degas module. Chromatographic column used in this study was a Carbo PAC™ PA10 column (4 × 250 mm). 10 μL of the resultant solution was employed to elute at a flow rate of 1 mL/min at a constant temperature of 35 ℃ to get the ion chromatographs. Various concentrations of NaOH were tested in this work. H_2O (eluent A), 200 mM NaOH (eluent B) and 1 M CH_3COONa (eluent C) were used as the mobile phase. A gradient elution of 0 - 100% by mobile phase A to C for 0 - 20 - 20 was performed for 1 - 35 min. The elution program was as follows: 0 - 20 min (91% A, 9% B and 0% C), 20 - 20.1 min (86% A, 9% B and 5% C), 20.1 - 35 min (71% A, 9% B and

20% C). The retention time of each monosaccharide standard in the mixtures under different elution conditions was confirmed by the analysis of the corresponding monosaccharide.

3.2.5 Molecular weight measurement

The molecular weight of IOP was measured by the method described by Yang et al. (2016b) with some modifications. A high performance size elusion chromatography coupled with multi-angle static light scattering with and refractive index (HPSEC-MALLS-RID) system was used. The HPSEC-MALLS-RID system consists of a pump (e2695,Waters, USA), a HPSEC columns (TSKgel SuperMultipore PW-M,TOSOH, Japan), a MALLS detector (DAWN HELEOSII, Wyatt Technology, Santa Barbara, CA, USA), and a RI detector (OPtilab T-rex, Wyatt Technology, Santa Barbara, CA, USA). Other parameters are as follows: collection Astra Version 5.3.4.20, cell type fused silica, laser wavelength 658.0 nm, calibration constant $2.9267e^{-5}$ 1/(V cm), UV Instrument is Generic UV instrument at 280 nm with Refractive index of 1.331. Mw, Mw/Mn and Rg of the polysaccharide fraction were determined under light scattering intensity of different angles at 25 ℃. IOP (10 mg/mL) was filtered through a 0.45 μm pore membrane before injection (20 μL) and eluted with water (0.5 mL/min).

3.2.6 Evaluation of antioxidant capacity

(1) DPPH radical scavenging activity assay

DPPH radical scavenging activity of extractions was evaluated according to a literature procedure with slight modifications (Li et al., 2013b). Aliquots (0.5 mL) of various concentrations (0.3125 - 10.00 mg/mL) of solutions were mixed with 3 mL (25 μg/mL) of a MeOH solution of DPPH and then were shaken vigorously and allowed to stand in the dark for 30 min. The absorbance was measured with a spectrophotometer at 517 nm against a blank. The decrease of the DPPH solution absorbance indicated an increase in the DPPH radical scavenging activity. Ascorbic acid was used as the positive control. The DPPH radical scavenging activity was calculated according to the following equation:

$$\text{Scavenging activity (\%)} = (1 - A_1/A_0) \times 100\% \quad (3\text{-}1)$$

where A_0 is the absorbance without samples and A_1 the absorbance containing the samples.

(2) SOD-like activity assay

SOD-like activity of the extracts was measured by the SOD Assay Kit-WST according to the technical manual provided by Dojindo Molecular Technologies, Inc. Firstly, 20 μL of the sample solution was added to blank 2 well and each sample, meanwhile, 20 μL of double distilled water was added to each blank 1 and blank 3 well in a 96-well plate. After that, 200 μL of WST working solution was added to each well, mixed waiting for further processing. Sequentially, 20 μL of dilution buffer was added to each blank 2 and blank 3 well, and 20 μL of enzyme working solution was added to each sample and blank 1 well. The plate was incubated at 37˚C for 20 min and the optical density (OD) was measured at 450 nm using a microplate reader (Bio-Rad Model 550, USA). The SOD-like activity was calculated by the following equation:

$$\text{SOD activity (inhibition rate \%)} = \left\{ \frac{(A_{blank1} - A_{blank3}) - (A_{sample} - A_{blank2})}{(A_{blank1} - A_{blank3})} \right\} \times 100\% \quad (3\text{-}2)$$

A_{blank1}, A_{blank2}, A_{blank3} and A_{sample} were the absorbances of blank 1, blank 2, blank 3 and sample wells. 1 Unit of SOD activity was defined as the amount of enzyme having a 50% inhibitory effect on WST-1.

IC_{50} value (mg/mL) was the effective concentration at which SOD activity was scavenged by 50% and was obtained by interpolation from linear regression analysis.

(3) Hydroxyl radical scavenging activity assay

Hydroxyl free radicals generated from $FeSO_4$ and H_2O_2 were detected by their ability to hydroxylate salicylate. The reaction mixture (2.5 mL) contained 0.5 mL of $FeSO_4$ (1.5 mM), 0.35 mL of H_2O_2 (6 mM), 0.15 mL of sodium salicylate (20 mM), and 1.0 mL of different concentrations of the IOP. Ascorbic acid was used as the positive control. The absorbance of the hydroxylated salicylate complex was measured at 562 nm after incubation at 37 ℃ for 1 h. The scavenging effect on hydroxyl free radicals was calculated as Eq. (3-3).

$$\text{Scavenging activity (\%)} = [1 - (A_1 - A_2)/A_0] \times 100\% \quad (3\text{-}3)$$

where A_1 is the absorbance of the sample or ascorbic acid, and A_0 is the absorbance of the solvent control, whereas A_2 is the absorbance of the reagent blank without sodium salicylate.

3.2.7 Cell assays

Human normal fibroblast cells (TIG-3) and human alveolar basal epithelial cells (A549) obtained from Japanese Collection of Research Bioresources (JCRB) Cell Bank were employed to evaluate the nontoxicity and anti-proliferation activity of IOP. Both cells were maintained in Minimum Essential Medium Eagle medium (Sigma Chemical Co., Saint Louis, MO) containing 10% (v/v) fetal bovine serum and antibiotics (consisting of 100 U/mL penicillin and 100 μg/mL

streptomycin) at 37 ℃ in 5% (v/v) CO_2 atmosphere. Cells were cultured for 2 - 3 days to reach the logarithmic phase and used for experiment.

TIG-3 and A549 were grown in DMEM medium at 37 ℃ in a 5 % CO_2 atmosphere to logarithmic phase. Cells were harvested, and an aliquot (100 μL) of cell suspension (5×10^4 cells/mL) were dispensed into a 96-well plate (2×10^3 cells/well) and pre-incubated at 37 ℃ in a 5 % CO_2 atmosphere for 24 h. Then cells were exposed to various concentrations (0.15625 - 5 mg/mL) of IOP for 48 h. After drugs exposure, 96-well plates were removed from incubator and 20 μL MTT stock solution was added to each well incubated at 37 ℃, 5% CO_2 for 4 hours. Afterwards, 96-well plates were removed from incubator and aspirated the solution and further added 100 μL DMSO to each well and rotated the plate for 5 min to distribute evenly. Ultimately, absorbance was measured with an ELISA reader at 540 nm.

3.3 Results and discussion

Compared with the extraction yield (168.80 ± 0.59 mg/g) under the optimum SWE conditions analyzed in Chapter 2, the polysaccharides yield extracted by HWE was much lower (30.71 ± 0.43 mg/g). In the further experiments, physicochemical characteristics were conducted.

3.3.1 FTIR

FT-IR spectroscopy is a practical technique for identification of characteristic organic groups and exhibition of structural features in polysaccharides (Chen et al., 2014). To compare the two kinds of IOP, FT-IR was applied to analyze the IOP and the results are illustrated in Fig. 3-1. The major peaks of two polysaccharides (the black line for HWE and the red line for SWE) were almost same. Given the fact that the signals within the range of 3600-3300 cm^{-1}, 3000-2800 cm^{-1}, and 1100-1000 cm^{-1} are the characteristic absorption peaks of polysaccharides

(Li et al., 2013a), both HWE-IOP and SWE-IOP spectra could fit the typical pattern of polysaccharides well, indicating that the main structure of polysaccharides was not destroyed under subcritical water conditions.

A strong and broad peak at approximately 3400 cm^{-1} (HWE-IOP: 3408.56 cm^{-1}; SWE-IOP: 3383.50 cm^{-1}) corresponded to a characteristic hydrogen bond O-H stretching vibration (Miao et al., 2014). The absorption peak at around 2940 (HWE-IOP: 2956.18 cm^{-1}; SWE-IOP: 2920.66 cm^{-1}) assigned to the C-H asymmetric stretching vibration including CH-, CH_2- and CH_3- (Wang et al., 2012). The bands at approximately 1630 cm^{-1} (HWE-IOP: 1612.20 cm^{-1}; SWE-IOP: 1634.38 cm^{-1}) suggested the presence of the stretching vibration of C=O or C=C group in structure (Shi et al., 2013). A range from 1000 to 1200 cm^{-1} of extensive absorption bands indicated the stretching vibrations of C-O-H side groups, and C-O-C glycosidic band vibrations were observed in the spectra (Kacurakova et al., 2000). The main absorptions of C-O stretching (HWE-IOP: 1047.16 cm^{-1}; SWE-IOP: 1045.23 cm^{-1}) showed that the characteristics of sugar structures were pyranose configuration (Zhu et al., 2014). Overall, these results showed that polysaccharides extracted by HWE and SWE exhibited the typical absorption peaks of a polysaccharide.

3.3.2 Monosaccharides composition

The monosaccharide composition of IOP was analyzed by HPAEC-PAD, and the results are compared and illustrated in Table 3-1. A wide range of monosaccharide compositions was detected in both HWE-IOP and SWE-IOP. As results showed, HWE-IOP was mainly composed of eight monosaccharides including rhamnose, galactose, arabinose, glucose, xylose, mannose, galacturonic acid and glucuronic acid, with a molar ratio of 1.33: 4.75:1.13: 16.37: 1.85: 3.38: 0.48: 1.51. For SWE-IOP, the molar ratio of above eight monosaccharides was 0.02: 0.27: 0.24: 35.53: 5.29: 4.61: 0.15: 0.78. As the involvement of galacturonic acid and glucuronic acid, both

two IOP were acidic polysaccharides, some of which are beneficial for the health of human beings or animals (Singdevsachan et al., 2016). Furthermore, the main compositions of HWE-IOP were glucose and galactose, whereas the main compositions of SWE-IOP were glucose, xylose, and mannose. In other words, thermal treatment under SWE may not only facilitate the hydroxyl or decomposition of galactose but also promote synthesis of xylose and mannose. The monosaccharide composition was similar to a previous report that polysaccharides from *I. obliquus* were mainly composed of glucose and mannose (Fan et al., 2012). The results implied that elevated temperature could change the monosaccharides composition of polysaccharides.

3.3.3 Molecular weight

Based on previous studies, the bioactive activities of polysaccharides are attributed to the structure features of polysaccharides like molecular weight, chemical components, glycosidic bonds, main chain lengths, polymerization, degrees of branching, and 3-D conformations (Chen et al., 2011). Among these characteristics, molecular weight plays an important role in the apparent viscosity, water-solubility, conformations and other properties of polysaccharides (Liu et al., 2010). Furthermore, natural polysaccharides isolated from fungi and plants are usually highly dispersed and have masses ranging from a few kilo- up to mega-Daltons (Chan et al., 2006). Hence, it is crucial to gain knowledge through identifying the molecular weight of polysaccharides. However, the molecular weight of the polysaccharides extracted by SWE from *I. obliquus* has not yet been determined.

In this study, molecular weight distribution of the polysaccharides was analyzed by HPSEC-MALLS-RI system and the results are summarized in Table 3-2. As shown, the crude HWE-IOP displayed the molecular weight distributions of two main fractions with 127.7 (41.59%) and 22.94 kDa (58.41%), respectively. This finding indicated that HWE-IOP exists as a polysaccharide polymer. The average Mw value of HWE-IOP was 66.51 kDa whereas the

average Mw value of SWE-IOP failed to acquire, possibly because the polysaccharides were decomposed into smaller ones (< 10 kDa) under the tested SWE conditions, which cannot be determined by using this measurement system.

3.3.4 DPPH radical scavenging activity

In general, hydrogen atom transfer, single electron transfer and metal chelation are the three primary proposed mechanisms, through which the antioxidants can play pivotal roles in protecting cells against damage caused by free radical-induced oxidative stress. Constituents like IOP possessing the direct or indirect inhibition on mutagenicity can effectively scavenge free radicals (Ham et al., 2009). Thus, the antioxidant activity of IOP was determined by using DPPH radical scavenging activity. The DPPH free radical is a relatively stable free radical that can be reduced after accepting an electron or hydrogen radical in the presence of an antioxidant to become a stable diamagnetic molecule (Chung et al., 2008). According to the characteristic that DPPH exhibits a maximum absorption wavelength at 517 nm with a phenomenon that the solution color turns purple into yellow in the presence of antioxidants (Meir et al., 1995), DPPH assay has been widely used as a substrate to evaluate the antioxidative activity of samples, including natural polysaccharides (Yuan et al., 2008).

The scavenging activity of polysaccharides extracted by SWE and HWE against the DPPH radicals are shown in Fig. 3-3a. Ascorbic acid was as the positive control to verify the validity and authenticity of the assay. In this study, ascorbic acid showed DPPH radical-scavenging activity of 97.80 ± 0.23% at the initial concentration of 0.3125 mg/mL, exhibiting excellent scavenging ability on DPPH radicals. And altered concentrations of all the SWE-extracts (0.3125, 0.625, 1.25, 2.5, and 5 mg/mL) showed DPPH scavenging activities in a dose-dependent manner. As shown, both IOP samples exhibited DPPH radical scavenging activities

at a concentration range of 0.3125 - 5 mg/mL. Specifically, when the sample concentration ranging from 0.3125 - 5 mg/mL, the scavenging activity of SWE-IOP increased from 20.21% to 90.66% whereas the scavenging activity of HWE-IOP increased from 13.57% to 75.78%. The half inhibition concentration (IC_{50}) increased in the order of SWE-IOP (0.86 mg/mL) and HWE-IOP (1.78 mg/mL), which indicated that the thermal processing condition for samples processed under subcritical water conditions significantly improved the scavenging ability on DPPH radicals. Moreover, these findings implied that IOP can donate an electron or hydrogen atom to scavenge DPPH radical.

3.3.5 SOD-like activity

Superoxide dismutases (SODs), as metalloenzymes catalyzing the dismutation of superoxide anion to oxygen and hydrogen peroxide, ubiquitously exist in eukaryotes and prokaryotes (Reactive oxygen species in cancer). SOD-like is recognized as a non-enzyme-small molecule super antioxidant that has a similar antioxidant function with SOD (Yang et al., 2016a). Moreover, SOD-like is conducive to the absorption of the body's normal metabolism. Thus, a quick and simple method for the assay of SOD-like activity, based on the ability to inhibit the auto-oxidation of pyrogallol, is widely used to predict antioxidant capability. Furthermore, it cannot damage human gastric easily and is conducive to the absorption of the body's normal metabolism. Hence, SOD-like activity assay is widely used to determine radical scavenging activity due to its ability to inhibit the auto-oxidation of pyrogallol. In this study, ascorbic acid was used as a positive control for the SOD-like activities assay.

As shown in Fig 3-3b, at the initiate concentration of 0.3125 mg/mL, the activity of SWE-IOP (3.10 ±2.49%) was almost as twice as that of HWE-IOP (1.58 ±0.44%). With the increase of concentrations, SOD-like activity of both fractions increased dramatically, suggesting that

IOP extracted by these two methods were sensitive to influencing the expression of SOD-like. There was no difference between two kinds of IOP (SWE-IOP, 99.54± 0.16%; HWE-IOP, 99.53 ± 0.82%) and ascorbic acid (99.14± 0.42%), indicating that IOP may have potential effects on human cells. Moreover, SOD-like activities were in a dose-dependent manner. As shown in Table 3-3, the IC_{50} values of SWE-IOP and HWE-IOP were 0.039 mg/mL and 0.077 mg/mL, respectively.

3.3.6 Hydroxyl radical scavenging activity

Hydroxyl radical, a dangersome oxidant, can react with biomacromolecules functioning in living cells and induce damage to virtual all types of biomolecules, such as lipids, proteins, carbohydrates and DNA (Yuan et al., 2008). Generally, generation inhibition and scavenging power are two primary aspects of anti-oxidation to control reactive oxygen species for the protection of living systems. For example, hydrogen atoms at all ring C-H bonds of aldoses, uronic acids, and other sites on carbohydrates can potentially contribute to the formation of hydroxyl radicals (Duan & Kasper, 2011). What's worse is that these micro-molecular radicals can cross membranes at specific sites without any restriction and lead to oxidative injury in the biomolecules indirectly by producing hydroxyl radical via Fenton reaction and/or iron-catalyzed Haber–Weiss reaction, so as to easily damage or even kill cells (Erel, 2004). Thus, the removal of hydroxyl radicals is pivotal for the protection of living systems (Luo et al., 2011). Therefore, the removal of hydroxyl radicals is very important to protect the body from oxidization-related injury. Xie et al. (2012) mentioned that polysaccharides can scavenge hydroxyl radicals whereby donating electron or hydrogen donors.

The hydroxyl radicals scavenging abilities of SWE-IOP and HWE-IOP are illustrated in Fig. 3-3c. The results showed that IOP possessed a dose-dependent activity within the concentration of 0.3125 to 5 mg/mL. It can be easily observed that both kinds of IOP had lower

scavenging abilities than ascorbic acid at all tested concentrations. However, when the concentration was 0.3125 mg/mL, SWE-IOP exhibited stronger scavenging activity even than that of ascorbic acid. As for SWE-IOP, the scavenging activity increased significantly from 57.82% to 91.41% when its concentration increasing from 0.3125 to 5 mg/mL. Compared with the results of SWE-IOP, HWE-IOP has a lower inhibition ability, which increased from 32.08% to 85.48% with the increase of sample concentration ranging from 0.3125 to 5 mg/mL. In addition, the IC_{50} values of SWE-IOP and HWE-IOP were 0.13 mg/mL and 0.41 mg/mL, respectively (Table 3-3). The difference of scavenging activities between SWE-IOP and HWE-IOP is in agreement with the aforementioned study that a relatively elevated temperature expose may exert an influence on bioactivity via modifying the molecular weight of polysaccharides (Zhang et al., 2004). There are two possible ways for polysaccharides eliminating radicals: polysaccharides supply the hydrogen to combine with radicals so that the radical chain reaction can be terminated; polysaccharides themselves integrate with radical ions directly. But up till now, the exact mechanism underlying the free-radical scavenging activity exerted by polysaccharides is still not fully understood (Chen et al., 2008). Restated, *I. obliquus* polysaccharides obtained by HWE showed lower DPPH radical scavenging activities than that achieved by SWE, suggesting that elevated temperature might appropriately degrade the high-molecular-weight polysaccharides and enhance their antioxidant activities.

3.3.7 Cell assays

IOP's non-toxicity on TIG-3 cells and the anti-proliferative activity against the growth A549 cells were investigated using the MTT assay. As shown in Fig. 3-4a, cells viability was not dramatically decreased after IOP addition with no change. Less cells death is probably attributed to the normal physiological metabolism of fibroblast cells themselves. At a

concentration of 5 mg/mL, the cell viabilities of TIG-3 cells of SWE-IOP and HWE-IOP were 84.73 ±5.41% and 89.73 ±3.97%, respectively. It can be indicated that IOP extracted by these two methods has no cytotoxicity, and can be a potential bioactive ingredient. The inhibition effect on A549 cells was summarized in Fig. 3-4b. The results showed that both SWE-IOP and HWE-IOP possessed a dose-dependent activity within the concentration of 0.15625 - 5 mg/mL. Specifically, the inhibition rate of SWE-IOP was 89.76 ± 3.97% at the concentration of 5 mg/mL while HWE-IOP's inhibition rate was 85.83 ± 5.08% at the same concentration. It should be noted that the inhibition ability of SWE-IOP was much stronger than that of HWE-IOP at a lower concentration, which is in accordance with the results of antioxidant activity.

3.4 Summary

In this chapter, the polysaccharides yield by using two different extraction methods as well as some basic features of SWE-IOP and HWE-IOP were investigated. Compared with HWE, the proposed SWE realized the significantly increased extraction yield under the higher temperature within a shorter time. Besides, the FTIR spectra analysis indicated that both samples comprise neutral polysaccharides with almost the same function groups. Moreover, monosaccharides composition determination reveals that both polysaccharides are acidic polysaccharides with the major ingredient being glucose. In addition, average molecular weight measurements were implemented. Results showed that the Mw of SWE-IOP is much lower than that of HWE-IOP, thereby effecting the antioxidant activities evaluated by three different antioxidant assays (DPPH, SOD-like, and Hydroxyl).

To sum up, polysaccharides obtained by SWE had stronger antioxidant activities with a higher extraction yield, compared to those extracted by HWE. SWE may provide a promising platform to obtain bioactive polysaccharides from functional foods or medicines. Further studies on the precise chemical structures and biological functions of IOP are still necessary in progress.

Table 3-1. Analysis of monosaccharide composition with HPAEC-PAD method.

`	HWE-IOP		SWE-IOP	
Monosaccharide reference	REL. Area (%)	Molar ratio of monosaccharides (mol/mol)	REL. Area (%)	Molar ratio of monosaccharides (mol/mol)
Rhamnose	3.47	1.33	0.03	0.02
Galactose	18.02	4.75	0.65	0.27
Arabinose	1.95	1.13	0.26	0.24
Glucose	60.18	16.37	82.49	35.53
Xylose	4.77	1.85	8.60	5.29
Mannose	7.66	3.38	6.71	4.61
Galacturonic acid	0.51	0.48	0.10	0.15
Glucuronic acid	3.43	1.51	1.15	0.78

Table 3-2. Results of chromatogram of IOP for molecular weight determination.

	SWE-IOP	HWE-IOP	
Peak	1	1	2
Limits (min)	16.865 - 21.685	11.880 - 17.344	17.427 - 20.283
Calculated mass (g)	3.6083×10^{-5}	1.5182×10^{-5}	2.1320×10^{-5}
Mass fraction (%)	100%	41.59%	58.41%
dn/dc (mL/g)	0.135	0.135	0.135
Mw (kDa)	n/a	127.7	22.94
Polydispersity (Mw/Mn)	n/a	1.295	1.084

Table 3-3. Comparison of antioxidant activities of IOP obtained by HWE and SWE.

Method	Extraction conditions			Extraction yield (mg/g)	Antioxidant assay (IC_{50}, mg/mL)		
	Temperature (℃)	Residence time (min)	Liquid-solid ratio (mL/g)		DPPH	Hydroxyl	SOD-like
HWE	100	120	30	30.71 ±0.43	1.780	0.413	0.077
SWE	194	5.36	53	168.80 ±0.59	0.859	0.129	0.039

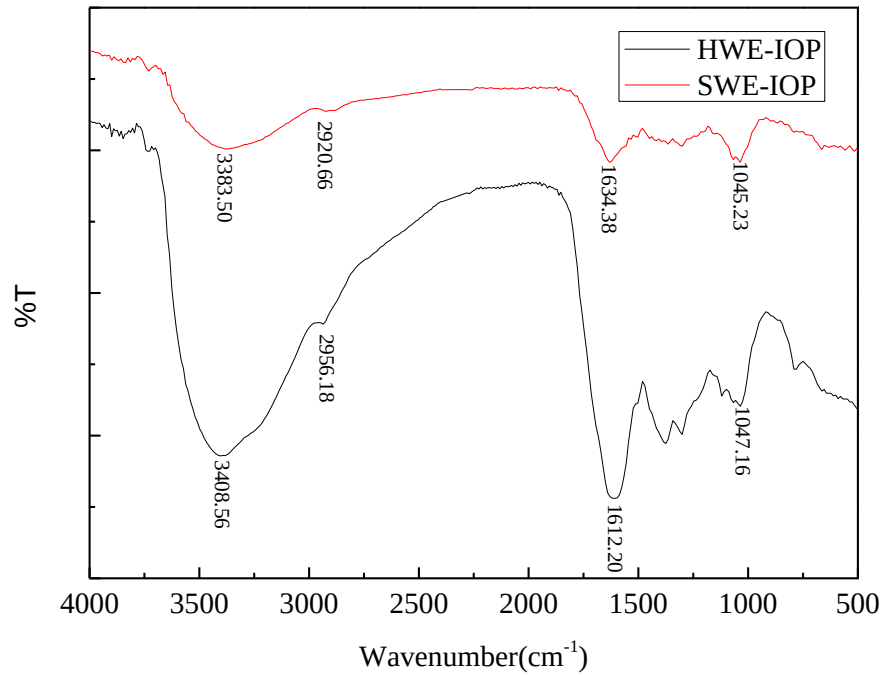

Fig. 3-1. FT-IR spectrum of the polysaccharides extracted from *I. obliquus* by using SWE (SWE-IOP) and HWE (HWE-IOP).

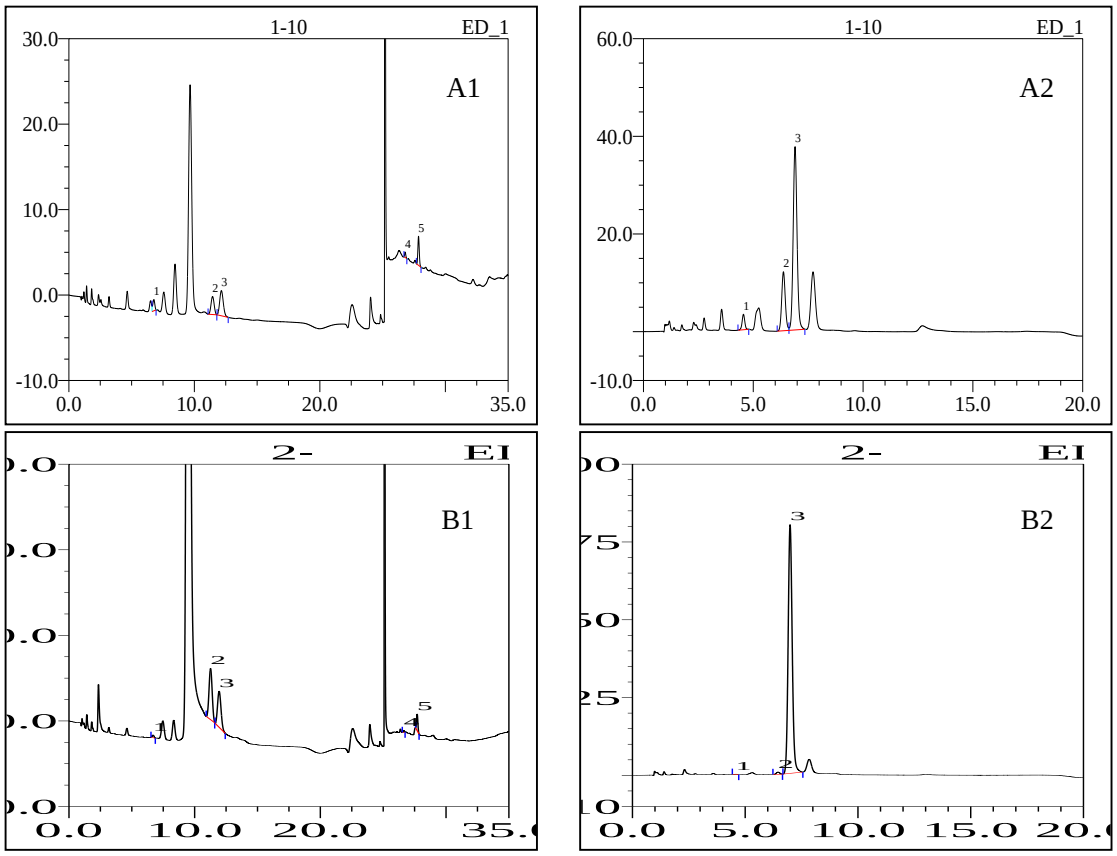

Fig. 3-2. HPAEC-PAD chromatogram profiles of the monosaccharides from (A) HWE-IOP and (B) SWE-IOP. Peak identity (1): 1, Arabinose; 2, Xylose; 3, Mannose; 4, Galacturonic acid; 5, Glucuronic acid; Peak identity (2): 1, Rhamnose; 2, Galactose; 3, Glucose.

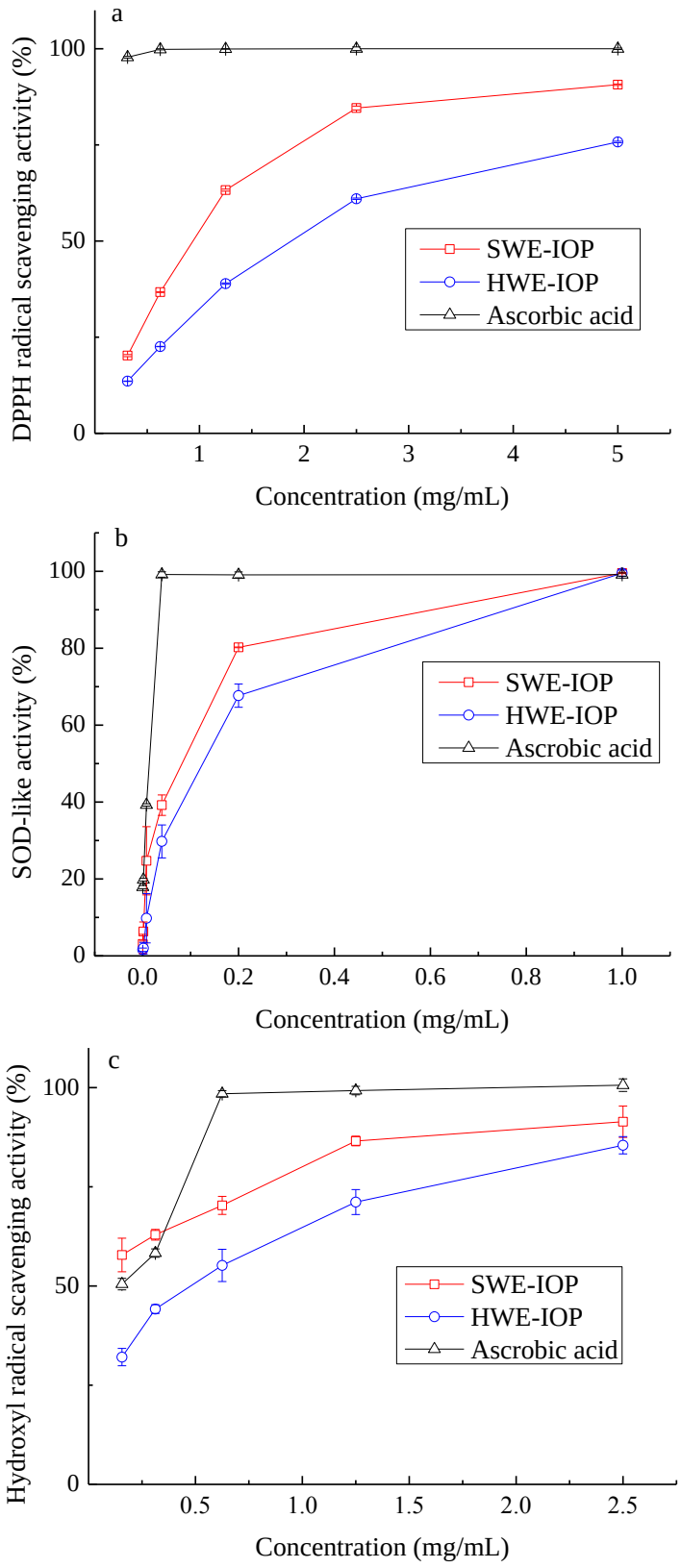

Fig. 3-3. Antioxidant activities of SWE-IOP, HWE-IOP, and ascorbic acid: (a) DPPH radicals scavenging activity, (b) SOD-like activity and (c) hydroxyl radicals scavenging activity. Values are means ± S.D. (n = 3).

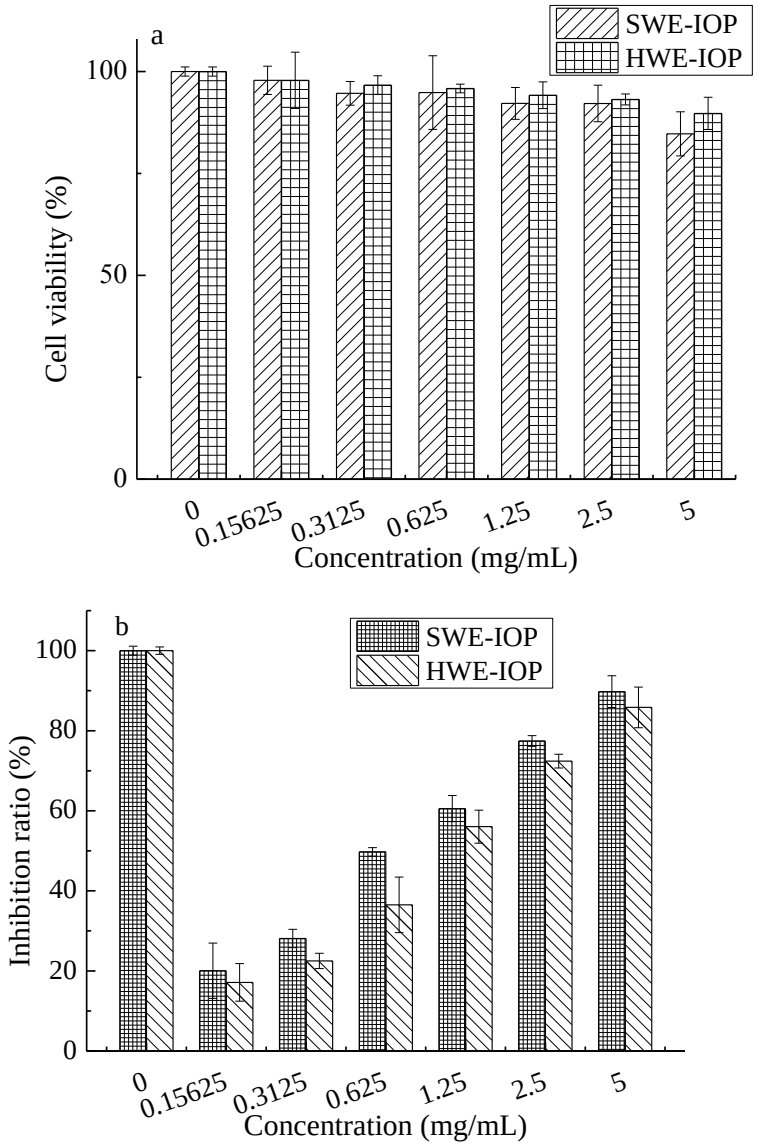

Fig. 3-4. Cell assays of SWE-IOP (under the optimal conditions) and HWE-IOP. (a) The non-toxicity effect on growth of TIG-3 human normal fibroblast cells for 48 hours; (b) The anti-proliferation against the growth of A549 human alveolar basal epithelial cells for 48 hours. Values are means ± S.D. (n = 3).

Chapter 4 Multi-response optimization of phenolic antioxidants from *Inonotus obliquus* and their identification by HPLC

4.1 Introduction

The medicinal fungus *Inonotus obliquus* (Fr.) Pilat, also known as the Chaga Mushroom (CM), is a fungus that belongs to the Hymenochaetaceae family of Basidiomycetes. It has been used as a folk remedy in Russia and Eastern Europe for more than four centuries (Zheng et al., 2007a), where its beneficial influence on the treatment of several human diseases (Chen et al., 2007).

I. obliquus synthesizes a range of phenolic constituents, which include small phenolics (Nakajima et al., 2007), hydrolysable tannins (Yang & Zheng, 2007), flavonoids (Zheng et al., 2007b), polyphenols (Lee & Yun, 2007) and melanins (Babitskaia et al., 2000). These phenolic compounds show a remarkable potential for scavenging free radicals, and thus reduce the incidence of oxidative stress-induced diseases, including cancer (Orzechowski, 2007), hypertension (Kwon et al., 2008) and neurodegenerative (Alzheimer's and Parkinson's diseases) (Heo & Lee, 2005) and autoimmune diseases. In consequence, they have considerable pharmaceutical importance.

Extraction is an important step in isolation and recovery of high value-added compounds, in particular, polysaccharides (mentioned in Chapter 2) and phenolic compounds (Do et al., 2014). Generally, hydrodistillation or solvent extraction including ethanol, ethyl acetate (Kaur et al., 1998), ether (Malekzadeh et al., 2001), methanol (Saleem et al., 2002), and aqueous acid solution have been employed for extraction of phenolic compounds. The release of different phenolic compounds depends greatly on extraction process variables such as method, temperature, matrix particle, solvent to liquid ratio, the solvent's polarity, and extraction time

in terms of the complicated internal structure that phenolic compounds entwining with other plant components. In other words, selection of extraction approach determines both the quantitative and qualitative composition of phenolic compounds.

The main objectives of this chapter were to investigate effects of SWE conditions (temperature, extraction time and liquid-solid ratio), and to apply RSM approach in order to optimize these conditions to obtain the highest phenolic content and highest antioxidant activity of obtained liquid extracts of *I. obliquus*.

4.2 Materials and methods

4.2.1 Chemicals and reagents

Folin-Ciocalteu, ethanol, sodium carbonate, sodium nitrite, aluminum nitrate, sodium hydroxide, disodium hydrogen phosphate, and phenol were obtained from Wako Pure Chemical (Osaka, Japan). Chemical HPLC-grade standards (purity > 95%) of gallic acid, chlorogenic, p-hydroxybenzoic, vanillic, ferulic, and flavonoid standards including catechin, epicatechin epigallocatechin and rutin were purchased from Sigma Co. (St. Louis, MO, USA). Other reagents used in the experiments were all of the analytical grade.

4.2.2 Determination of total phenolic content

Total phenolic content (TPC) was determined according to the Folin–Ciocalteu colorimetric method (Miguel et al., 2010). The sample (0.5 mL) and 2 mL of sodium carbonate (75 g/L) were added to 2.5 mL of 10% (w/v) Folin-Ciocalteu reagent. After 30 min of reaction at room temperature (intermittent shaking for color development), the absorbance was measured at 765 nm. The TPC was determined from the linear equation of a standard curve

prepared with gallic acid. The content of total phenolic content was calculated as mean ± SD (n = 3) and expressed as mg gallic acid equivalents (GAE)/g dry weight.

4.2.3 Determination of total flavonoids content

The total flavonoid content (TFC) in extract were measured by a colorimetric assay (Zhishen et al., 1999). The extract (5 mL) was added to a 10 mL flask, and then 5% $NaNO_2$ solution (0.3 mL) was added. After mixed well, the solution was allowed to stand for 6 min at room temperature; and 5% $Al(NO_3)_3$ solution (0.3 mL) was added to the flask, mixed well and kept for 6 min at room temperature. At last 4% NaOH solution (4.4 mL) was added, mixed well and kept at room temperature for 12 min. Absorbance was read at 510 nm on a UV-spectrophotometer, and the TFC (%) was estimated using the calibration curve. A calibration curve ($R^2 = 0.999$) was used to calculate the TFC and the results were expressed as mg of rutin equivalent (RE) per g of dry weight.

4.2.4 DPPH free radical-scavenging assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical-scavenging activity of the extracts was measured by DPPH assay as described in 3.2.6.1. Aliquots (0.5 mL) of various concentrations of solutions were mixed with 3 mL (25 μg/mL) of a MeOH solution of DPPH. Discolorations were measured at 517 nm using an UV spectrophotometer (Shimadzo, Kyoto, Japan) after remaining for 30 min in the dark.

4.2.5 Statistical analysis

A Box-Behnken design (BBD) was constructed using the software Design Expert Version 8.0.5 (Stat-Ease Corporation, Minneapolis, MN, USA). It was used for evaluating the effect of

independent variables on the properties of extracted bioactive compounds. Three extraction variables were considered for this study: temperature, time and liquid-solid ratio (Table 4-1). The design comprises 17 sets of test conditions for each extraction method where three levels were attributed to each factor at high, central, and low levels, with additional four replicated center points. Maximum and minimum treatment levels were selected by carrying out preliminary screening tests according to the literature reports and instrumental aspects.

4.2.6 Phytochemicals quantification by HPLC

The phenolic compounds in lyophilized *I. obliquus* were identified according to the previous method (Chou et al., 2015) with slight modifications. The high performance liquid chromatography (HPLC) system is composed of a Shimadzu LC-20AT HPLC pump system pump system and a Shimadzu SPD-20A UV/Vis detector (Shimadzu SCL-20A system controller module, Kyoto, Japan). A Capcell Pak C18 column (250 × 4.6 mm, 5 m; SHISEIDO Co., Ltd. Tokyo, Japan) and a gradient solvent system consisting of MeOH (solvent A) and deionized distilled water (dd H_2O) with 2% glacial acetic acid (solvent B) (conditions: 5 - 17% A from 0 to 10 min and kept at 17% A from 10 to 40 min; 17 - 60% A from 40 to 120 min; flow rate = 0.4 mL/min) were used for separation of components whose UV spectra were recorded from 220 to 450 nm. Phenolic acid and flavonoid compounds were also run on the HPLC as standards to verify chemical compounds of lyophilized *I. obliquus*.

4.3 Results

4.3.1 Models fitting

In order to the best combination of variables for the total phenolic content and antioxidant capacity from *I. obliquus*, investigating the extraction variables is of necessity. Preliminary

trials enabled the range of extraction temperature (200 - 220 ℃), residence time (0 - 10 min) and liquid-solid ratio (80 - 120 mL/g) to be fixed (Table 4-1). The experimental and predicted data in terms of total phenolic content and total flavonoid content designed by Box-Behnken developing for the process optimization for each response under different SWE conditions are represented in Table 4-2.

Among the 17 experiments containing 5 replicates, the total phenols of the SWE ranged statistically ($p < 0.001$) from 280.07 (assay number 4) to 474.23 mg GAE/g (central point). And the yielded flavonoid content increased from 695.22 to 976.73 mg RE/g. The highest values for TPC and TFC were observed at the central point of the experimental design with a liquid-solid ratio of 100 mL/g for 5 min at 210 ℃ (central point). The lowest values for TPC and TFC appeared at 220 ℃ for 10 min with a liquid-solid ratio of 100 mL/g, suggesting that excessive and prolonged exposure to high temperature can accelerate the decomposition rate of phenolic compounds.

All data were calculated using a Design Expert program (version 8.0.5) and fitted to second order polynomial equations. The analysis of variance (ANOVA) showed that TPC and TFC were well interpreted with quadratic polynomial model. Table 4-3 illustrates all regression coefficients corresponding *p* values, indicating the considerable effect of these coefficients on respective response variables. As a matter of fact, the results showed that for these responses, extraction temperature, residence time and liquid-solid ratio have a significant quadratic effect ($p < 0.05$). All the linear coefficients were significant ($p < 0.05$) on TPC and TFC, whereas no significant effect of liquid-solid ratio was found on TFC. The significance of interaction effects of the investigated operation conditions on TPC were determined and those on TPC were as follows: extraction temperature - residence time and residence time - liquid-solid ratio on TPC as well as extraction temperature - residence time. The interaction effect of extraction temperature - liquid-solid ratio had no significant effects on both TPC and TFC.

In Table 4-4, the validity of models are confirmed using lack of fit testing were summarized. ANOVA for the lack of fit test for all responses was insignificant ($p > 0.05$) indicated that the models adequately fitted the experimental data and were not significant relative to the pure error, thereby exhibiting good reproducibility of the data. Besides, the coefficients of multiple determination (R^2) of 0.9999 and 0.9961 were obtained for TPC and TFC, respectively. It could be reveals that good correlations were established between responses and independent variables.

4.3.2 Response surface analysis of TPC

The effects of extraction parameters such as extraction temperature (X_1), residence time (X_2) and liquid-solid ratio (X_3) were investigated on the SWE of antioxidant compounds from *I. obliquus*. The mathematical model correlating the content of TPC in term of significant independent variables is given below:

$$Y_{TPC} = 473.58 - 35.83X_1 - 23.04X_2 + 7.76X_3 - 58.61X_1X_2 - 7.14X_2X_3 - 44.20X_1^2 - 31.25X_2^2 - 10.73X_3^2$$

It can be easily seen that all parameters were significant (p-value < 0.05) effect on the SWE for TPC values, apart from X_1X_3. For maximum TPC yield (480.52 mg GAE/g), extraction temperature, residence time, and liquid-solid ratio were 207.78 ℃, 5.07 min, and 107.41 mL/g, respectively.

The significance of each coefficient determined by F-values and p-values were demonstrated in Table 4-4. As shown, a strong positive correlation between the TPC and extraction parameters was quadratic with good regression coefficient ($R^2 = 0.9999$). The larger the magnitude of the F-value and smaller the p-value, the more significant the corresponding coefficient (Şahin et al., 2013). According to Fisher's F-test, it can be concluded that the model has a significant effect on the total phenolic content response ($p < 0.0001$).

The adequacy of selected model describing the effect of extraction parameters on TPC response was determined by the lack of fit test. As shown in Table 4-4, the ratio of the mean square of lack of fit was superior to 0.05, indicating that, the model was valid.

The relationship between extraction parameters and TPC was investigated by response surface plots and the effects of (A) temperature and residence time, (B) temperature and liquid-solid ratio and (C) residence time and liquid-solid ratio on the extraction yield of TPC were illustrated in Fig.4-1. As clearly shown from Fig. 4-1A, the yielded TPC increased with extraction temperature until 207.78 ℃ with a fixed liquid-solid ratio of 100 mL/g for 5 min. However, above this extraction temperature (207.78 ℃), the TPC response started to decrease. High temperatures might have increased the diffusion and solubility rate of the many compounds resulting in antioxidant compounds being extracted at a higher rate (Şahin et al., 2013). Nonetheless, elevated temperatures could also affect the activity of the extracts due to the degradation and loss of the phenolic compounds or phenolic compounds reacting with other components of the plant material (Dorta et al., 2012). When extraction temperature was fixed at 210 ℃ and liquid-solid ratio, the extraction yield increased with residence time until 5.07 min and then decreased.

As shown in Fig. 4-1B, an increase in extraction temperature at 100 mL/g of liquid-solid enhanced significantly the TPC extraction when the extraction time was fixed at 5 min. However, there was no significant difference in extraction yield when liquid-solid ratio increased, suggesting the interaction between extraction temperature and liquid-solid ratio is not significant. The effect of residence time and liquid-solid ratio is shown in Fig. 4-1C. It is demonstrated that the TPC value could be superior to 410 mg GAE/g dry matter for a high liquid-solid ratio and a higher level of residence time variable when the extraction temperature was fixed at 210 ℃.

4.3.3 Response surface analysis of total flavonoid content

According to the data listed in Table 4-2, the second-order equation fitting the content of TFC was given below:

$$Y_{TFC} = 970.95 - 31.28X_1 - 20.69X_2 - 86.11X_1X_2 - 87.01X_1^2 - 53.17X_2^2 - 31.39X_3^2$$

Analyzed by F-values and p-values, some significantly influencing are only shown in this equation to avoid unnecessary interference. Specifically, X_1 (extraction temperature), X_2 (residence time), X_1X_2, X_1^2, X_2^2 and X_3^2 are the most significant parameters ($p < 0.05$). However, X_3, X_1X_3 as well as X_2X_3 have less effect ($p > 0.05$) on TFC through SWE. For maximum TPC yield (480.52 mg GAE/g), extraction temperature, residence time, and liquid-solid ratio were 209.79 ℃, 4.78 min, and 97.90 mL/g, respectively.

As shown in Table 4-4, the high F-value (196.87) and low p value ($p < 0.0001$) demonstrated that the quadratic regression model was highly significant. Moreover, determination coefficient R^2 (0.9961) and adjusted determination coefficient Adj. R^2 (0.9910) revealed the excellent correlations between the independent variables. Furthermore, the highly significant degree of precision and reliability of experimental values of regression model were identified by coefficient of variation (C.V. = 0.82). More importantly, lack of fit (F-value = 5.49 and p value = 0.0668) was not significantly relative to the pure error.

Fig. 4-2 illustrates the relationship between extraction parameters and total phenolic content was investigated by response surface plots.

As can be seen from Fig.4-2A, TFC increased dramatically and could reach greater than 860 mg RE/g dry matter with the increase of both extraction temperature and residence time ranging from 200 to 209.79 ℃ and from 0 to 4.78 min, respectively. Then, a decrease in TFC yield was observed when these two variables increased. Fig 4-2B represented the effect of extraction temperature and liquid-solid ratio on TFC yield. As shown, the yielded TPC

increased with liquid-solid ratio until 97.90 mL/g at 210 ℃ when residence time was fixed at 5 min.

As shown in Fig. 4-2C, by setting extraction temperature at the fixed high level (210 ℃), it could be seen that the highest TFC would be obtained on lower levels of aforementioned residence time and liquid-solid ratio, i.e. 4.78 min and 97.90 mL/g.

The optimum SWE conditions for TPC and TFC from *I. obliquus* are summarized in Table 4-5. Extraction temperature in the range of 207.78 - 209.79 ℃, residence time in the range of 4.78 - 5.07 min and liquid-solid ratio in the range of 97.90 - 107.41 mL/g produced the optimal TPC (480.52 mg GAE/g) and TFC (971.81 mg RE/g) from *I. obliquus*. According to the apparatus feature and desirability function approach, the simultaneous optimum conditions for TPC and TFC were as follows: extraction temperature of 209 ℃, residence time of 4.6 min, and liquid-solid ratio of 100.5 mL/g. The DPPH radical scavenging activity of the extract was 0.122 ± 0.59 mg/mL.

The TPC and TFC under above SWE conditions were 477.58 ± 0.59 mg GAE/g and 971.13 ± 0.42 mg RE/g, respectively. The predicted results matched well with experimental results obtained using optimum extraction conditions, which were also confirmed by a good correlation ($R^2 > 0.95$) As a result, the model from central composite design was considered to be accurate and reliable for predicting the total phenolic content and the total flavonoid content of extracts obtained from *I. obliquus* by SWE.

In order to compare, ethanol extraction (EE) method was also employed to obtain TPC and TFC. Their results in addition to ABTS radical scavenging activity are summarized in Table 4-6. According to the table, the TPC and TFC extracted by SWE were as four times as those extracted by EE. More importantly, ABTS radicals scavenging activity was enhanced by the means of SWE, indicating that SWE is a satisfactory extraction method to achieve phenolic compounds in terms of yields and antioxidant activity.

4.3.4 HPLC analysis

Phenolic compounds including gallic acid, epigallocatechin, catechins, p-hydroxybenzoic acid, chlorogenic acid, vanillic acid, epicatechin, ferulic acid, and rutin were investigated in this study. The amounts of phenolic compounds in the extract from *I. obliquus* under the optimum extraction conditions were determined by HPLC with the results shown in Table 4-7. The amount of individual phenolic compounds (expressed as mg/g dried plant) were as follows: 0.24 gallic acid, 8.57 epigallocatechin, 17.84 catechins, 1.99 chlorogenic acid, 1.07 vanillic acid, 13.33 epicatechin and 1.60 ferulic acid, respectively.

4.4 Summary

In this chapter, SWE was successfully employed to obtain phenolic compounds from *I. obliquus*. The extraction conditions (extraction temperature, residence time and liquid-solid ratio) were optimized by response surface methodology (RSM) with a Box--Behnken design. Significant improvement was observed in the yields of phenolics and flavonoids obtained with this technique. The verification test definitely reveals that the regression models were satisfactorily accurate for the production of TPC and TFC. Moreover, six kinds of phenolic compounds (gallic acid, epigallocatechin, catechins, chlorogenic acid, vanillic acid, epicatechin and ferulic acid) were determined by HPLC in the extract obtained under the optimal conditions from *I. obliquus*. This study indicated that *I. obliquus* can be considered a good source of naturally-occurring antioxidant compounds, which could be potentially applied in food industry.

Table 4-1. Independent variables and their levels used in the response surface design (BBD).

Independent variables	Symbol	Levels		
		-1	0	1
Extraction temperature (℃)	X_1	200	210	220
Residence time (min)	X_2	0	5	10
Liquid-solid ratio (mL/g)	X_3	80	100	120

Table 4-2. Combinations of two variables with their coded terms obtained from RSM and observed responses under different experimental conditions.

Std	Temperature (℃)	Residence time (min)	Liquid-solid ratio (mL/g)	TPC (mg GAE/g dry chaga)		TFC (mg RE/g dry chaga)	
				Experimental	Predicted	Experimental	Predicted
1	-1 (200)	-1 (0)	0 (100)	398.96	398.38	794.11	796.63
2	1 (220)	-1 (0)	0 (100)	444.52	443.94	914.11	906.30
3	-1 (200)	1 (10)	0 (100)	468.96	469.54	919.67	927.48
4	1 (220)	1 (10)	0 (100)	280.07	280.65	695.22	692.69
5	-1 (200)	0 (5)	-1 (80)	447.78	447.33	887.73	880.70
6	1 (220)	0 (5)	-1 (80)	374.89	374.44	819.29	822.59
7	-1 (200)	0 (5)	1 (120)	461.18	461.63	890.27	886.97
8	1 (220)	0 (5)	1 (120)	390.74	391.19	812.93	819.96
9	0 (210)	-1 (0)	-1 (80)	438.71	439.74	897.29	901.79
10	0 (210)	1 (10)	-1 (80)	408.07	407.94	869.96	869.18
11	0 (210)	-1 (0)	1 (120)	469.41	469.54	911.60	912.38
12	0 (210)	1 (10)	1 (120)	410.21	409.19	866.73	862.23
13	0 (210)	0 (5)	0 (100)	473.78	473.58	965.32	970.95
14	0 (210)	0 (5)	0 (100)	473.23	473.58	969.38	970.95
15	0 (210)	0 (5)	0 (100)	473.78	473.58	976.73	970.95
16	0 (210)	0 (5)	0 (100)	472.89	473.58	970.31	970.95
17	0 (210)	0 (5)	0 (100)	474.23	473.58	973.00	970.95

Table 4-3. Estimated coefficients of the fitted second-order polynomial model for TPC, TFC.

Regression coefficient	Response	
	TPC	TFC
Intercept		
β_0	473.58	970.95
Linear		
β_1	-35.83***	-31.28***
B_2	-23.04***	-20.69***
B_3	7.76***	0.91
Interaction		
β_{12}	-58.61***	-86.11***
β_{13}	0.61	-2.22
β_{23}	-7.14***	-4.39
Quadratic		
β_{11}	-44.20***	-87.01***
β_{22}	-31.25***	-53.17***
β_{33}	-10.73***	-31.39***

Significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Table 4-4. Analysis of variance (ANOVA) of the fitted second-order polynomial models for TPC and TFC.

	Sum of squares	DF	Mean square	F-value	*p*-Value
TPC					
Model	42876.78	9	4764.09	6211.78	< 0.0001
Residual	5.37	7	0.77		
Lack of it	4.27	3	1.42	5.17	0.0733
Pure error	1.10	4	0.28		
Cor total	42882.15	16			
C.V. (%)	0.20	R^2	0.9999		
Adj. R^2	0.9997	*Pred.* R^2	0.9984	Adequate precision	287.247
TFC					
Model	93468.48	9	10385.39	196.87	< 0.0001
Residual	369.26	7	52.75		
Lack of it	297.08	3	99.03	5.49	0.0668
Pure error	72.18	4	18.05		
Cor total	93837.75	16			
C.V. (%)	0.82	R^2	0.9961		
Adj. R^2	0.9910	*Pred.* R^2	0.9481	Adequate precision	49.951

Table 4-5. Estimated optimum conditions, predicted and experimental values of responses under subcritical water conditions.

Response	Extraction conditions			Predicted Value
	Temperature (℃)	Residence time (min)	Liquid-solid ratio (mL/g)	
TPC	207.78	5.07	107.41	480.52 mg GAE/g
TFC	209.79	4.78	97.90	971.81 mg RE/g

Table 4-6

Comparison of ethanol extraction (EE) and subcritical water extraction (SWE)

Methods	Conditions	TPC (mg GAE/g)	TFC (mg RE/g)	ABTS (mM AAE/g)
EE	Temperature of 40ºC, time of 4 h and concentration of 75%	121 ± 1.06	240 ±1.43	62.83 ±0.76
SWE	Temperature of 209ºC, time of 4.6 min and liquid-solid ratio of 100.5 mL/g	477.58 ±0.59	971.13 ±0.42	178.34 ±1.23

Table 4-7. HPLC results of the crude phenolic compounds from *I. obliquus* by using optimum SWE conditions.

Compounds	Content (mg/g dry chaga)
Gallic acid	0.24
Epigallocatechin	8.57
Catechins	17.84
p-Hydroxybezoic acid	N.D.
Chlorogenic acid	1.99
Vanillic acid	1.07
Epicatechin	13.33
Ferulic acid	1.60
Rutin	N.D.

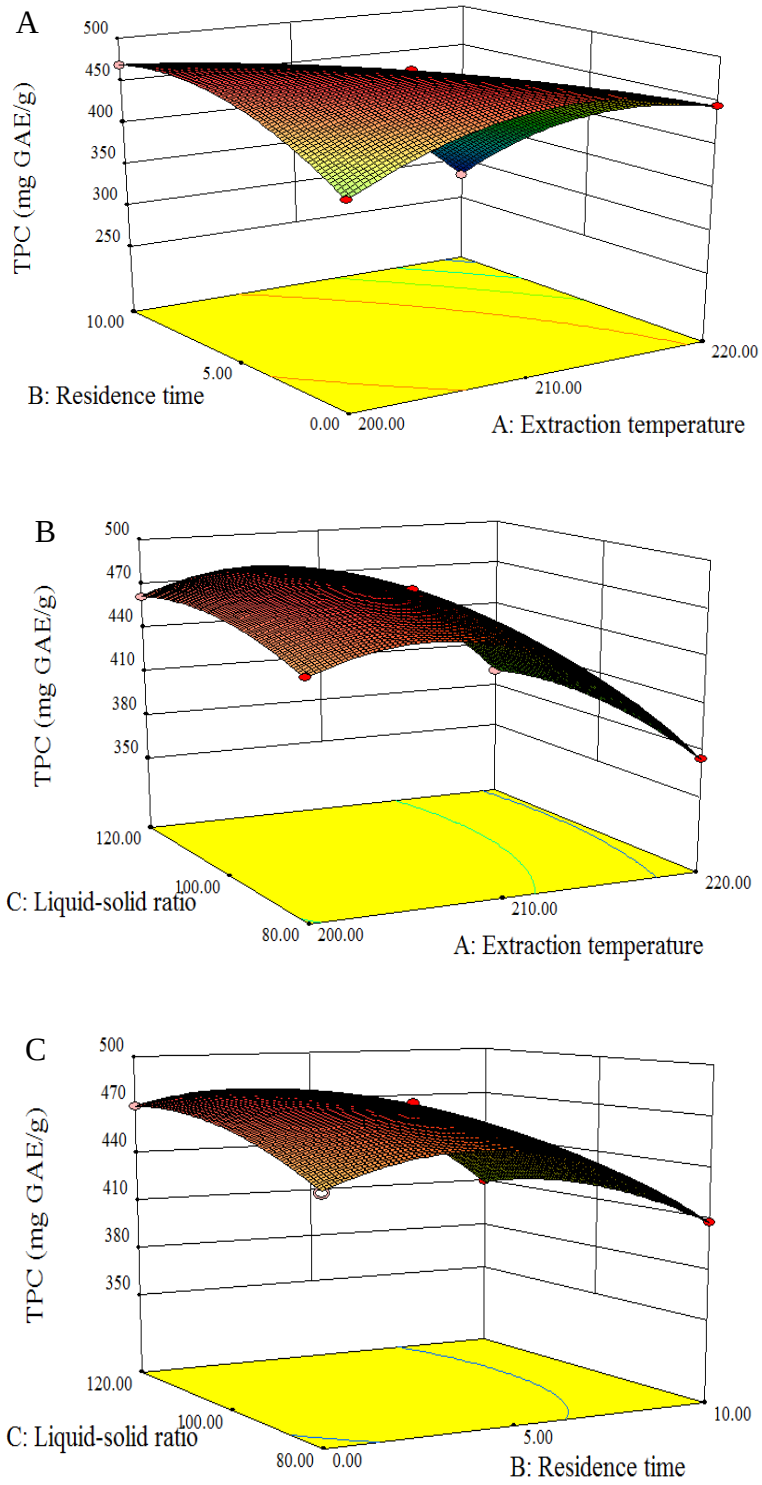

Fig. 4-1. Response surface plots for the effects of (A) temperature and residence time, (B) temperature and liquid-solid ratio and (C) residence time and liquid-solid ratio on the extraction yield of TPC. The value of the missing independent variable in each plot was kept at the centrepoint.

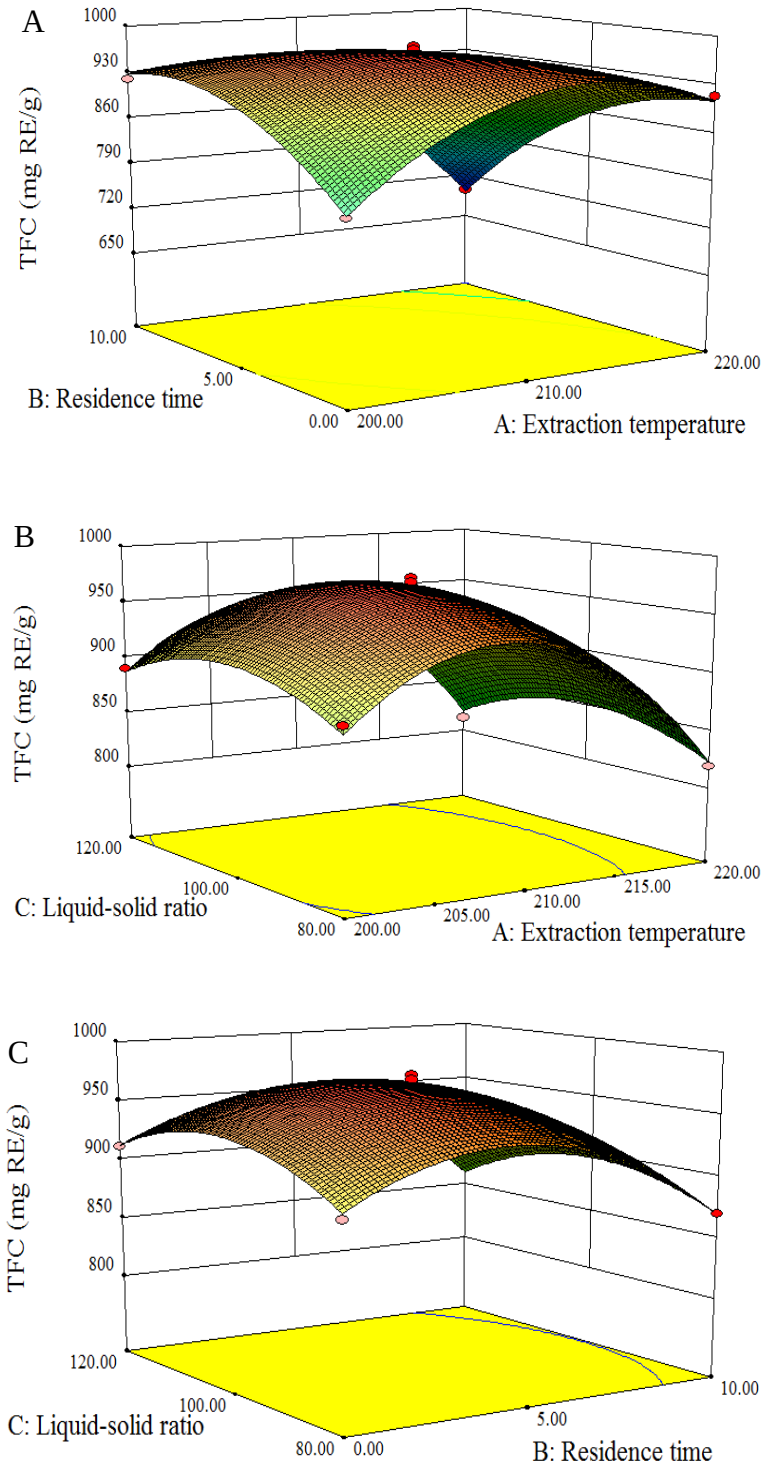

Fig. 4-2. Response surface plots for the effect of (A) temperature and residence time, (B) temperature and liquid-solid ratio and (C) residence time and liquid-solid ratio on the extraction yield of TFC. The value of the missing independent variable in each plot was kept at the centerpoint.

Chapter 5 Conclusions and future researches

5.1 Conclusions

In this study, subcritical water extraction was used to extract antioxidant polysaccharides and phenolic compounds from *I. obliquus*. Response surface methodology was successfully employed to optimize extraction conditions. Physicochemical characteristics of polysaccharides and phenolic compounds were also investigated. Compared with those obtained by HWE, it could be concluded that the application of SWE significantly enhanced extraction yield efficiently, and improved the bioactivity. All the results indicate that SWE is a highly effective and rapid method of extracting bioactive polysaccharides and phenolic compounds which could be potential resources in food and medicine industries.

Main results and conclusions were summarized as follows.

1. It was proved that polysaccharides from *I. obliquus* could be obtained efficiently by using SWE. According to the single factor method and RSM with BBD, a second-order polynomial equation using multiple regression analysis analyzed by ANOVA and the optimum processing parameters (temperature of 194 ℃, residence time of 5.36 min and liquid-solid ratio of 53 mL/g) for IOP were achieved. Under the optimal conditions, the experimental extraction yield was 168.80 ± 0.59 mg/g, in excellent agreement with the predicted value (168.65 mg/g) by the model with a desirability value of 0.984 was predicted.
2. Extraction yield and physicochemical characteristics of IOP extracted by SWE and HWE

were compared. First of all, the yield of SWE-IOP was 5.5 times greater than that of HWE-IOP (30.71 ± 0.43 mg/g). Besides, the FT-IR spectra of two polysaccharides showing the characteristic functional groups of polysaccharides were investigated and several groups like O-H, C-H, C=O, -OH and C-O were identified. Monosaccharides compositions and average molecular weight were compared as well. The average Mw value of HWE-IOP was 66.51 kDa whereas the average Mw value of SWE-IOP was < 10kDa. The main compositions of HWE-IOP were glucose (60.18%) and galactose (18.02%), whereas the main compositions of SWE-IOP were glucose (82.49%), xylose (8.60%) and mannose (6.71%). Furthermore, SWE-IOP exhibited stronger antioxidant activity than HWE-IOP, with IC_{50} being 0.86, 0.039 and 0.13 mg/mL respectively in DPPH radical scavenging activity, SOD-like activity and Hydroxyl radical scavenging activity assays in comparison to 1.78, 0.077 and IC_{50} 0.41 mg/mL for HWE-IOP, most probably due to the changes of monosaccharide compositions and their molecular weights. Cell assays results also indicated that IOPs extracted by SWE and HWE at the concentration of 5 mg/mL, had little toxicity on human normal fibroblast (TIG-3) cells with cell viability of 84.73 ± 5.41% and 89.73 ± 3.97% for SWE-IOP and HWE-IOP, respectively. Meanwhile SWE-IOP exhibited a slightly stronger anti-proliferation effect on human alveolar basal epithelial (A549) cells with inhibition ratio of 89.76 ± 3.97% in comparison to 85.83 ± 5.08% for HWE-IOP.

3. SWE was successfully employed to obtain phenolic compounds from *I. obliquus*, by the means of RSM using a BBD design. The experimental results were adequately fitted with second-order polynomial models which showed significant linear, quadratic and interaction effects of the independent variables. After feasibility and desirability function approach, the highest values for experimental TPC (477.58 ± 0.59 mg GAE/g dry chaga) and TFC (971.13 ± 0.42 mg RE/g dry chaga) were achieved at 209 ℃ for 4.60 min with a liquid-solid ratio of 110.50 mL/g. In comparison, TPC of 121 ± 1.06 mg GAE/g dry chaga, TFC of 240 ±1.43 RE/g dry chaga and ABTS radical scavenging activity of 62.83 ± 0.76 mM AAE/g were determined at 40 ℃ for 4 h with an ethanol concentration of 75%. In addition, the amount of individual phenolic compounds (0.24 mg/g of gallic acid, 8.57 mg/g of epigallocatechin, 17.84 mg/g of catechins, 1.99 mg/g of chlorogenic acid, 1.07 mg/g of vanillic acid, 13.33 mg/g of epicatechin and 1.60 mg/g of ferulic acid) were determined.

Consequently, subcritical water extraction can be a promising alternative for the extraction of antioxidant materials from *I. obliquus* since it not only enhances extraction yield but also promotes bioactivity for targets compounds. Moreover, *I. obliquus* could be further developed as a potential antioxidant resource supplying polysaccharides and phenolic compounds for dietary supplements of functional foods.

5.2 Future work

1. Although the maximum yield conditions of IOP have been determined, isolation and purification of IOP should be also carried out.

2. Structure characteristics of SWE-IOP and HWE-IOP and their comparisons need to be further investigated in the future.

3. Other extractions should be explored and compared with SWE methods in terms of extraction yield, activity *in vitro* as well as activity *in vivo*.

4. In order to comprehensively identify bioactivity effects of SWE-IOP, rat experiments *in vivo* should be introduced in further investigation.

5. Isolation and purification of total phenolic compounds should be further studied.

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