EXTRACTION OF BIOACTIVE COMPOUNDS FROM GANODERMA LUCIDUM

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ABSTRACT

Ganoderma lucidum (reishi), a polypore group fungus, provides bioactive compounds in which several triterpenoids and polysaccharides are core structures that claim to posses cancerous and immunomodulatory properties. The objective of this work has been to establish a method of extraction from grinded form of *Ganoderma lucidum* with water in sub-critical conditions and with $SCCO_2$ at distinct temperatures to obtain extracts rich in water-soluble organic compounds (WSOC), including mostly beta glucans and triterpenoids including ganoderic acids and alcohols respectively. $SCCO_2$ experiments were carried out at pressures of 10, 20, 30 and 40 Mpa and temperatures ranging from 40 to 60 ^oC with and without modifier. Ethanol was used as modifier at flow rates of 0.1, 0.2 and 0.4 mL/min. The CO_2 flow rate was maintained at 4 mL/min for 2 hours extraction time. The extracts were analyzed by HPLC. Temperatures for experiments that were carried out by batch-scale sub-critical water extraction kept constant at different time intervals starting from 373 up to 573 K. For semi-continuous scale experiments, samples were extracted once with water at 373, 423, 448 and 473 K, respectively, working at pressure around 10 Mpa to keep the water in the liquid state. The flow rate used was 1 mL/min. The extracts were analysed by TOC for water soluble organic compound recovery. The highest WSOC recovery yield was obtained at 473 K as 78.1 % and as 57.4 %, also the highest values for extracted amount of total water-soluble organic carbon (WSOC) were 328 and 241 mg WSOC / g dry sample for batch- and semi continuous scale experiments respectively.

Keywords: Ganoderma lucidum, polysaccharides, beta-glucans, triterpenoids, subcritical water, water soluble organic compounds

1. INTRODUCTION

Ganoderma lucidum has been treasured in China and Japan for many thousands of years. Ganoderma species are one of the most widely researched fungi because of their reported potent bioactive properties. So, it is know to have various medicinal properties such improving ones constitution, increasing the body`s healing ability, and maintaining healthy body. Ganoderma lucidum is one of the focal points in the development of Chinese herbs. Ganoderma lucidum contains several triterpenoids and polysaccharides, which have been investigated in relation to their physiological effects [1, 2]. Those components can be used to cure various human diseases. These substances may be useful as starting materials for the development of chemical therapeutic agents in cancer treatment and for other ailments [3]. Many investigations are currently underway to maximize the production and utilization of these functional molecules. Polysaccharides, tripenes, sterols, lectins and proteins are some of the major active constituents that have been isolated from *G. lucidum* and its closely related species, with the first two compoundsbeing the most extensively investigated. The major bioactive polysaccharides isolated from *Ganoderma* species are glucans, β-1-3 and β-1-6 D-glucan. The basic structure is β-1-3 D-glucopyronan with 1 to 15 units of β-1-6 monoglucosyl side chains [4].

Up to now, numerous methods of extraction have been developed with the objective of obtaining extracts with higher yields and lower costs. Such is the case of extraction with organic

solvents, such as methanol [5, 6], ethanol [7], and acetone [8]. A preliminary procedure for extraction of polysaccharides from *Ganoderma lucidum* involves solvent extraction and solvent adsorption [9]. As a general procedure, there is a broad similarity in the various methods that have been developed to extract the polysaccharides from mushroom fruit-bodies, mycelium and liquid media.

These methods, however, leave residual solvent in the product, which is unacceptable for use on humans. Most of the studies have always focused researcher`s attention on exploring ways to maximize the efficacy of the two valuable herb *Ganoderma lucidum*. Recently, supercritical CO_2 ($SCCO_2$) and pressurized hot water or sub-critical water has become of great interest as an alternative solvent for extraction of natural active compounds. Supercritical CO_2 ($SCCO_2$) was found to be selective in the separation of desired compounds without leaving toxic residues in extracts and without the risk of thermal degradation of processed products such as herbs, spices, fruits and vegetables. Because of having special characteristics, sub- or supercritical water has been widely applied to various applications. It was found that by employing hot water extraction, the most advanced technique in Japan to prepare extracts of herbs, the best quality end-products. So, water has been shown to be capable of extracting different classes of compounds depending on the temperature used. This justifies the interest in its study as a technique of extraction of water soluble organic compounds (WSOC) from *Ganoderma lucidum*. This technique can extract the majority of the active elements in *Ganoderma lucidum* most effectively and retain their efficacious values. For instance, the genuine essence of *Ganoderma lucidum* contains not less than 60% of polysaccharides.

The use of these types of extractive technologies has a series of advantages: they are clean, quick, cheap, efficient, it has the possibility of automation and good selectivity due to the facility of modifying the polarity of the extraction medium.

In a search for alternative solvents a number of workers have used water in liquid state under pressure above 373 K, but below its critical temperature of 647 K. In these conditions, it is referred to as superheated water or subcritical water [10]. The subcritical water behaves as a nonpolar solvent that is available for the extraction of hydrophobic substances. Because of such characteristics, sub- or supercritical water has been widely applied to various applications. So, water has been shown to be capable of extracting different classes of compounds depending on the temperature used [11]. The use of this type of extractive technology has a series of advantages: it is clean, quick, cheap, efficent, it has the possibility of automation and good selectivity due to the facility of modifying the polarity of the extraction medium. This justifies the interest in its study as a technique of extraction of water soluble organic compounds (WSOC) from *Ganoderma lucidum*.

To summarize, the objective of this work has been to establish a method of extraction from grinded form of *Ganoderma lucidum* with water in sub-critical conditions and with $SCCO_2$ at distinct temperatures to obtain extracts rich in water soluble organic compounds (WSOC), including mostly beta glucans and triterpenoids including ganoderic acids and alcohols respectively. The analysis has been focused towards the determination of recovery efficiency of WSOC including beta glucans among polysaccharides by doing TOC analysis.

Now, while this mushroom certainly won't make you live forever, scientists have been investigating the medicinal properties of Ganoderma and have been astounded by the results yielded so far. According to researchers, Ganoderma contains natural chemicals called triterpenoids, which possess an important anti-cancer action. They are able to inhibit the blood supply to cancerous cells - preventing oxygen and other nutrients from feeding them [12]. Nonetheless, it must be borne in mind that, in *Ganoderma lucidum*, the content of beta glucans

is generally more abundant than that of the other polysaccharides. Nevertheless, they are still poorly characterised, due basically to them being difficult to isolate and identify.

2 EXPERIMENTAL

2. 1 Material

Grinded form of dried *Ganoderma lucidum* which was used in this study was supplied by Refarmer Co., Ltd., Japan. *Ganoderma* contains 6.52 % hydrogen, 1.20 % nitrogen, 50.26 % oxygen and 42.02 % carbon (values are given by Kumamoto University Elemental Analysis Center). They were stored at room temperature keeping away from humidity until they used and did not undergo any further pretreatment. Polystyrene standard kits were acquired from Shodex Company (shodex STANDARD S Series- SM 105). GPC-grade solvent, tetrahydrofuran (THF) was purchased from Wako Chemicals (Japan).

2. 2 Subcritical Water Extraction (SWE)

Subcritical water extraction, that is, extraction using hot water under pressure sufficient to maintain water in the liquid state, has demonstrated its ability to selectively extract different classes of compounds depending on the temperature used, with the more polar extracted at lower temperatures and the less polar compounds extracted at higher temperatures. The selectivity of subcritical water extraction allows for manipulation of the composition of the extracts by changing the operating parameters. Two different extraction mechanisms were used named as batch type and semi-continuous type subcritical water extraction. These kinds of water treatments were done in order to emphasise the effect of water mobility in the subcritical extraction process.

Batch Scale Subcritical Water Treatment

Experiments were done in reaction chamber (electric furnice) with a single cap stainless steel tube reactor (5 ml). 7 experiments were done for each of temperatures of 373 K, 423 K, 473 K, 523 K and 573 K. 0.05 g of *Ganoderma lucidum* (1.25 w/v %) was inserted into the reaction tube for decided time durations starting from 5 minutes up to 60 minutes in 10-minute intervals for individual temperature values. After reaction, the treated sample was taken from the tubular extractor by washing totally 5 ml of pure water, homogenized using ultrasonic cleaner for 15 minutes and centrifuged for 30 minutes. As a following step, filtration (0.45 μm pore size) was done and liquid fractions were separated in order to use for the analyses.

Semi- continuous Scale Subcritical Water Extraction

A schematic diagram of a laboratory built subcritical water extraction system is presented in Figure 1. The system consists of two HPLC pumps (PU-2080-100 MPa, Jasco Co., Japan)**,** an oven, a stainless steel extraction cell (10 ml Vessel, Thar Designs, Inc., USA)**,** and a collecting flask. Water was de-oxygenated for 30 min using ultrasonic cleaner (Honda, W-211) prior to the extraction. With the HPLC pump, the water was then delivered at a constant flow rate to the extractor and the extraction cell was completely filled with plant material (4.46 g of ground *Ganoderma lucidum*) and mounted vertically in the oven with water flowing from top to bottom. The water was brought to a set temperature by means of the preheating coil inside the oven before entering the extractor. The extraction pressure was controlled by adjusting the back-pressure regulator (AKICO) connected to the outlet coil.

A chiller (Sibata Co., Japan) was used to cool the extract from the oven to a constant temperature close to 298 K, thus avoiding losses of the products caused by the hot water. The second pump connected to the outlet coil was used to deliver ethyl alcohol to flush through any organic compounds that were precipitated in the coil as the temperature of the water was cooled. The extract was collected in fractions in collecting flasks.

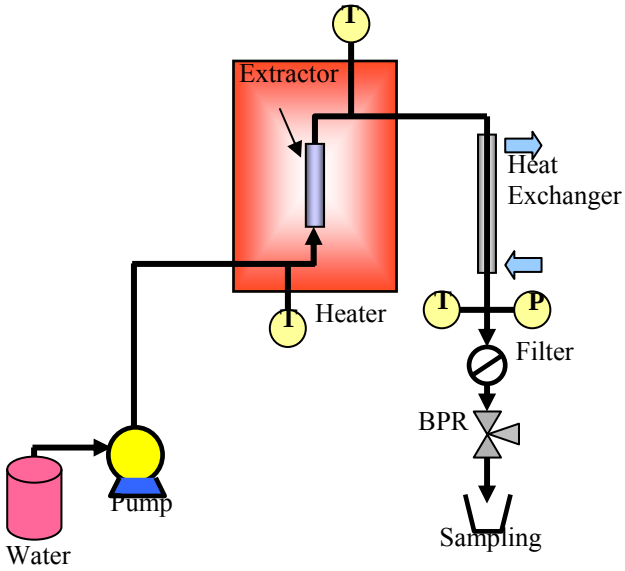

Fig. 1. Subcritical water extraction system

Two different extraction procedures have been used depending on whether individual extractions (at a chosen temperature). The extraction experiments were carried out mainly to determine the effect of temperature holding the pressure and water flow rate constant. The effect of temperature in the extraction process was studied. For this purpose, three different assays were carried out. Among them, samples were extracted once with water at 373, 423 and 473 K, respectively, working at pressure around 10 Mpa to keep the water in the liquid state. The flow rate used was 1 mL/min. All runs were performed at least in duplicate. Any doubtful results were checked and the experiments were repeated up to four times.

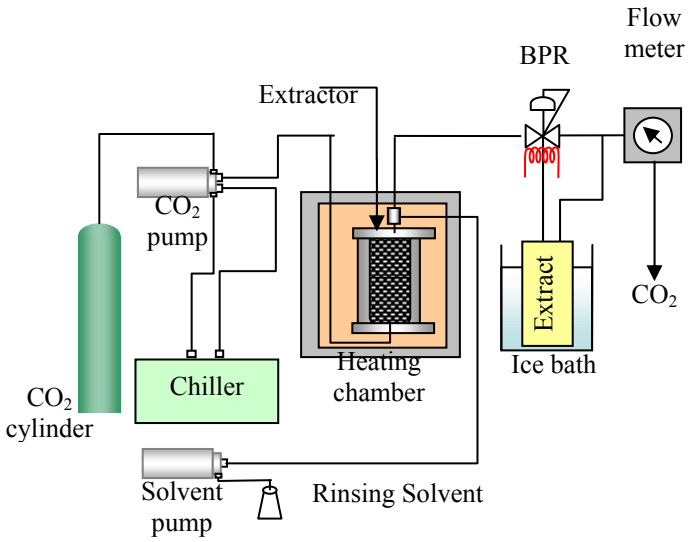

Fig. 2. Diagram of the supercritical fluid extraction apparatus

2. 3 Supercritical Carbon Dioxide Extraction ($SCCO_2$)

The extraction of ganoderic acids and alcohols from reishi with $SCCO_2$ were carried out $SCCO_2$ experiments were carried out using apparatus given in Figure 2 at pressures and temperatures ranging from 10 to 20 MPa and 40 to 100 ^oC, respectively with and without using any modifiers. The flow rate of the CO_2 was maintained at 4 mL/min for selected extraction time. The extracts were analyzed by high-performance liquid chromatography.

2. 4. Analyses

The total amount of the total extractable water soluble organic compounds (WSOC) was determined after extractions in both batch and semi- continuous scale subcritical water extractions. The recovery efficiency for WSOC was determined by Total Organic Carbon (TOC) analysis. System was calibrated using glucose solutions prepared at various concentrations before each analysis.

The average molecular weights of the polysaccharide compounds extracted from *Ganoderma lucidum* were estimated by Gel Permeation Chromatography (GPC) equipped with UV-visible detector (UV-970, Jasco, Japan) with TSK Gel GMH_{XL}-L column (Tosoh Corporation) (7.8 mm (ID)*30.0 cm (L)) in reference to calibration curve that was formed using standards of known molecular weights. THF was used as the mobile phase with a flow of 1.0 ml/min (10 µl injection volume) at 298 K column temperature in 25 min detection time; the detection was set at 254 nm for both polysaccharide compounds and polystyrene standards where the molecular weights were previously known.

For determination of triterpene constituents, including ganoderma alcohols and ganoderma acids in the products of *Ganoderma lucidum*, an analytical system was developed using HPLC with an ODS column. The mobile phase was composed of 1 % $AcOH/H_2O$-CH_3CN and 2 % $AcOH/H_2O$-CH_3CN, and the elution profile was monitored at 243 and 250 nm for ganoderma alcohols and acids respectively.

Particle structures of the powder microcapsules were evaluated by JEOL JSM-5200 model (Tokyo, Japan) scanning electron microscope. Powders were attached to SEM stubs using a 2-sided adhesive tape and left in desiccator. Then they were coated with a fine layer of gold through Sputter Coating Attachment of JEOL JFC- 1100 E (Jeol, Tokyo, Japan), in vacuumed evaporators before examination. For observation, a Scanning Electronic Microscopic JEOL JSM-5310LV (Jeol, Tokyo, Japan) working with a voltage of 15 kV. The microphotographs were carried out with a camera coupled to the microscopic. The samples were systematically observed with 1500 of magnification.

3 RESULTS AND DISCUSSION

Each experiment was conducted in triplicates and the relative standard deviation was in a range of 2-7%.

It was demonstrated that the modified $SCCO_2$ extraction is suitable for the extraction of *Ganoderma lucidum*. In addition, it was seen that molecular weights of extracts became smaller for high temperatures having increasing values for $SCCO_2$ extraction in comparison to hydrothermal treatment.

In case of hydrothermal experiments, batch scale hydrothermal treatment of *Ganoderma lucidum* was carried out by contacting the sample material in tubular reactor for a specific contact time. After treatment process, it was seen that the yield of recovery for total water soluble organic carbon (WSOC) was not changed with sample amount, it was greatly effected by temperature. Lowest

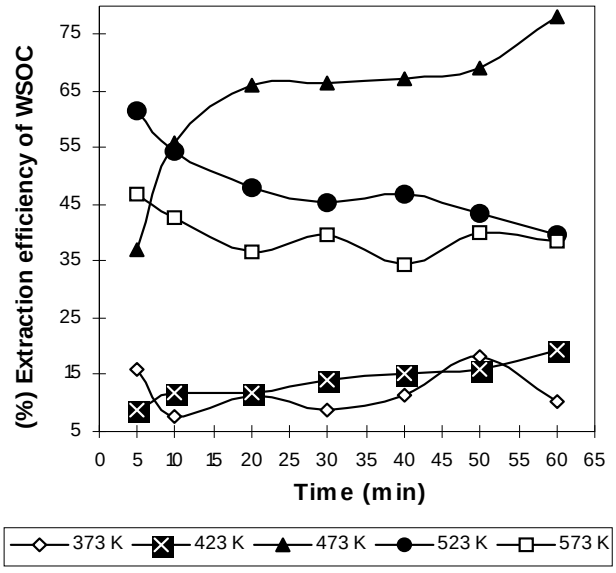

Fig. 3. Percent recovery of WSOC at various temperatures

recovery was recorded as 7.52 % at 100 ^{o}C. Figure 3 shows temperature effect on WSOC recovery efficiency depending on time.

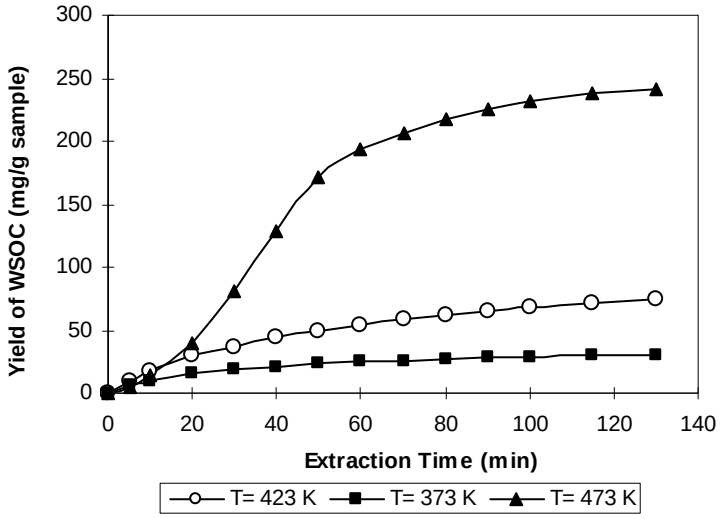

Fig. 4. Effect of temperature on the extraction curve

Temperatures up the 473 K were gradually increased and showed positive effect on the recovery efficiency having highest values of 18.048 %, 19.37 % in individual temperatures for 373 K and 423 K respectively. After 473 K, the efficiency became lower again. The highest yield obtained at 473 K as 78.11 % and decreased to 61.59 % and 46.82 % for 523 K and 573 K respectively.

The semi-continuous scale extraction of water soluble polysaccharides was carried out by contacting the *Ganoderma lucidum* with a continuous flow of pure de-oxygenated water brought to subcritical state. During the extraction process, the solubility of water soluble compounds sharply decreased after expanding the water using back pressure regulator where water was no more in subcritical state. The extraction was carried out at operating conditions; temperature ranging from 373 to 473 K at constant pressure and water flow rate of 10 Mpa and 1 ml/min respectively for 130 min extraction time.

As shown in Figure 4, the extracted amount of total water-soluble organic carbon (WSOC) recovery including polysaccharides increased with an increase in temperature. The highest yield of WSOC extracted with sub critical water at 373, 423, and 473 K were 30.25, 74.72 and 241.05 mg WSOC g^{-1} of dry sample respectively. This temperature dependence of the yield is due to the increased solubility of water-soluble organic compounds in sub critical water as the water temperature increases.

The highest extraction yield (57.37%) was achieved in extracts obtained by individual extractions at 473 K, which are the sum of the yields obtained in sequential extractions at 473 K with a total duration of 130 min. The results suggest that pressurized hot water extraction is greatly affected by extraction temperature. On the other hand, it should be noted that pressure does not affect the performance of sub critical water extraction as long as it is high enough for the water to maintain the liquid state.

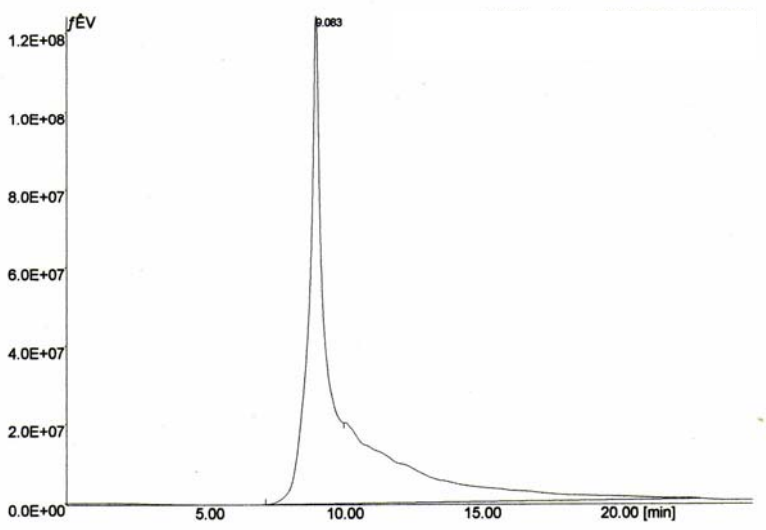

Fig. 5. GPC Chromatogram

Referring to the Gel Permeation Chromatography (GPC) was done after both experiments. For the estimation of average molecular weights of water soluble organic compounds, calibration curve was formed using polystyrene standard kits including 7 portions having the molecular weights from $1.24*10^3$ up to $3.44*10^6$ taking the concentration as 0.05 % for all injected samples.

All analysis conditions were same for the polystyrene standards and the sample material. By the end of analysis it was seen that the elution volume for the samples were too small comparing the values detected for polystyrene standards, changing around 9-10 ml for all extracted samples in both batch and semi-continuous scale extractions where the elution volume was at least 7.25 ml for the smallest size polystyrene standard. This indicates that the long chained structure of the water soluble polysaccharides divided into very small portions in assistance of the high temperature effect so that the exact numerical detection could not be done referring to reference calibration curve. Referring to the representative GPC chromatogram for extracted samples (Figure 5), it can be said that the molecular weights for the extracted water-soluble organic compounds, which are mainly composed of polysaccharides including mostly beta glucans, were very small. In addition, it was also seen that the amount of extract was little bit increased with high temperature. It may be observed from Figure 6 that there are distinctive differences between the molecular structures being getting smaller as a result of treatments at higher temperatures.

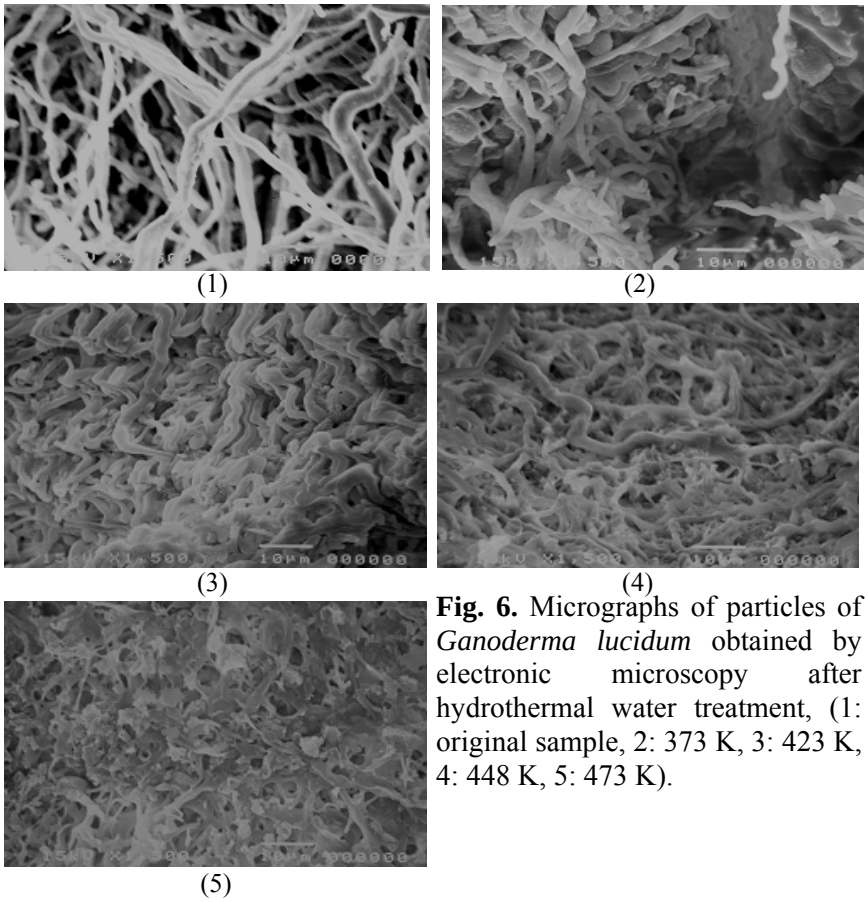

Fig. 6. Micrographs of particles of *Ganoderma lucidum* obtained by electronic microscopy after hydrothermal water treatment, (1: original sample, 2: 373 K, 3: 423 K, 4: 448 K, 5: 473 K).

The order of elution is, in part, determined by the structural characteristics, since those compounds with more units of carbon units forming polysaccharides having larger molecular sizes were eluted first.

4 CONCLUSION

The results indicate that the operating conditions are the crucial points for sub- and supercritical treatment of *Ganoderma lucidum* for recovery of bioactive compounds. In case of hydrothermal treatment, increase in temperatures showed positive effect on the recovery efficiency for water soluble organic compounds. The highest yield obtained at 473 K as 78.1 % and as 57.4 %, also the highest values for extracted amount of total water-soluble organic carbon (WSOC) were 328

and 241 mg WSOC g^{-1} of dry sample for batch- and semi continuous scale experiments respectively.

It was demonstrated that the modified $SCCO_2$ extraction is beneficial for the extraction of Ganoderma lucidum. The advantage of modified supercritical extraction over non-modified supercritical extraction was in the polarity component extraction and enhancement of the fluidity of extracts.

It was also seen that molecular weights of extracts became smaller for high temperatures having increasing values for $SCCO_2$ extraction in comparison to hydrothermal treatment.

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