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Extraction of Adenosine from *Ganoderma lucidum* Using Novel Extraction Technologies

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The aim of this research is to extract adenosine from *Ganoderma lucidum* (GL) using novel extraction techniques, including high hydrostatic pressure (HHP), ultrasonic assisted extraction (UAE), supercritical carbon dioxide ($ScCO_2$) in comparison with maceration and Soxhlet. The results showed that the highest adenosine extraction could be achieved using HHP (1.86 mg/g dry sample at 2000 bar, 60 ºC sample to solvent ratio of 1:60 and very short extraction time of 5 min) followed by Soxhlet (1.77 mg/g dry sample, 3 h extraction time). The effect of pressure and temperature during supercritical carbon dioxide extraction of adenosine were distinct at pressure higher than 400 bar and moderate temperature (0.40 mg/g dry sample at 500 bar and 60 ºC). The combination of maceration and ultrasonic at moderate temperature (60 ºC) improved the extractability of adenosine up 1.01 mg/g dry sample.

Key Words: Adenosine, *Ganoderma lucidum*, High hydrostatic pressure extraction, Supercritical carbon dioxide extraction, Ultrasonic assisted extraction.

INTRODUCTION

The most important pharmacologically active constituents of *G. lucidum* are triterpenoids and polysaccharides. Triterpenoids have been reported to posses hepatoprotective, antihypertensive, antitumor hypocholesterolemic and antihistaminic effects and antiengiogenic activity, effects on platelet aggregation and complement inhibition. Polysaccharides, especially β-d-glucans, have been known to possess antitumor effects through immunomodulation and antiangiogenesis. In addition, polysaccharides have a protective effect against free radicals and reduce cell damage caused by mutagens[1]. Adenosine decreases blood pressure, heart rate and renal sympathetic nerve activity[2]. Adenosine is inactivated either by extracellular metabolism *via* adenosine deaminase (producing ammonia as a secound product) or following uptake by nucleoside transporter, *via* adenosine deaminase or adenosine kinase[3].

High hydrostatic pressure (HP) is applied for extraction of lycopene from tomato as well as flavonoids components (chrysin and galangin) of propolis[4,5]. It is found

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that lycopene can be extracted from tomato paste waste in only one min. at room temperature without any heating process. The highest recovery (92 %) was obtained by performing the extractions at 5000 bar pressure, 1 min duration, 75 % ethanol concentration and 1:6 (g/mL) solid/liquid ratio. In the case of flavonoid extraction using high pressure the extraction yield of flavonoids compounds for 1 min were even higher than those obtained with extraction at room temperature for 7 days. Eshtiaghi[6] have investigate the effect of high pressure (at 5000 bar and 20 °C) as pretreatment on cell permeabilization and leaching of red beet. The mass transfer (leaching of colourant and minerals) will be increased after high pressure treatment.

The use of ultrasound within the food industry has been a subject of research and development for many years. Ultrasound is an effective novel technology for extraction of components from raw materials. Ultrasonic cavitation creates shear forces that break cell walls mechanically and improve material transfer. The size reduction by ultrasonic cavitation increases the surface area in contact between the solid and the liquid phase (solvent), significantly. The extraction of organic compounds contained within the body of plants and seeds by a solvent could be significantly improved by the use of power ultrasound. Ultrasonically assisted extraction can also be applied to the production of medicinal compounds such as helicid, berberine hydrochloride and bergenin from Chinese plants. The efficient cell disruption and effective mass transfer are cited as the major factors leading to extraction enhancement[7].

Extraction using organic solvent has limitations in obtaining solvent-free extracts. Compressed and supercritical carbon dioxide has been widely applied to the extraction of compounds such as oil, oleoresins, vitamins, colourants and antioxidants substances from different plant products[8,9]. This separation technique offers extraction yield very similar to those obtained by conventional extraction processes using solvents. Its advantages, compared to organic solvents, are that CO_2 is non-toxic, non-flammable and that it is cheap and readily available in bulk quantity, with a high degree of purity. In processing terms, CO_2 has a low critical temperature and pressure (31 °C and 73.8 bar, respectively), which makes it the ideal solvent for natural products without thermal degradation during extraction process[10]. Moreover, the absence of light and air during extraction reduced the risk of degradation reactions. Since CO_2 is non-polar it is not a good solvent for polar substances. Consumption of ethanol is not in large amounts as in conventional solvent extraction and it is easily eliminated from the extract by evaporation at room temperature[10].

EXPERIMENTAL

Ganoderma lucidum (GL) was purchased from Alunyig community, Bangkok, Thailand. Carbon dioxide (99.95 % purity) was supplied by Thai Industrial Gases, Thailand. Ethanol, adenosine and methanol were purchased from Merck, Germany. High hydrostatic pressure (HHP) equipment and supercritical carbon dioxide ($ScCO_2$) extractor were designed and made by SIB Foodtech, Germany and established

in Thailand, Ultrasonic homogenizer (Sonoplus, UW3200, 20 khz, BANDELIN, Germany), high performance liquid chromatography (HPLC) (Perkin-Elmer, type LC-235, diode array detector, C-18 column) were applied in this study.

Sample preparation: The dried *Ganoderma lucidum* were pulverized and sieved (particle size < 3 mm).

Maceration: *Ganoderma lucidum* powders (5 g) were added to 300 mL of 95 % ethanol in a 500 mL flask and mixed on a magnetic stirrer at 50 rpm for 0.5, 1.0, 2.0, 3.0 h at room temperature (28 ºC). The sample was filtered through a Whatman No. 1 filter, then the filtrate was evaporated by a rotavapor (Buchi, Switzerland) under vacuum at 60 ºC. The volume of sample adjuest to 25 mL using HPLC grade ethanol at and stored at 4 ºC in refrigerator until HPLC analysis.

Ganoderma lucidum powders (5 g) were added to extraction thimble and soak in 300 mL of 95 % ethanol in a 500 mL round bottom flask for 1, 2, 3 h. Temperature during soxhlet extraction was set at 70 ºC. The extracted sample was evaporated and prepared for HPLC analysis same as Maceration.

High hydrostatic pressure technique: *Ganoderma lucidum* powders (5 g) were put in a plastic bag and filled with 100, 200, 300 mL of 95 % ethanol, respectively, the bag was sealed using sealing machine. The sealed sample was fixed into the high hydrostatic pressure vessel and treated for 3 min to 3 h at 25 to 60 ºC and 500 to 3000 bar, respectively.

Ultrasonic assisted extraction: *Ganoderma lucidum* powders (5 g) were added to 300 mL of 95 % ethanol in a glass jacket cylinder, dipped ultrasonic probe under 1 cm liquid surface and mixed with a magnetic stirrer. The extraction was conducted at 150 W/cm^2 power density and various treatment temperatures of 25, 45, 60 ºC and 1 to 3 h treatment time, respectively.

Supercritical carbon dioxide extraction: *Ganoderma lucidum* powders (5 g) were filled in a filter paper and put into the extraction vessel. The extraction conditions during supercritical carbon dioxide were 300, 400 and 500 bar and temperature ranging from 25 to 60 ºC. The carbon dioxide flow rate was 2 kg/h constant for all supercritical carbon dioxide experiment. For supercritical carbon dioxide extraction using ethanol as co-solvent were 10 to 30 % ethanol (on sample weight basis) added to the sample and then the sample were fixed in supercritical carbon dioxide extractor.

RESULTS AND DISCUSSION

Maceration and Soxhlet: Adenosine extraction during macereation was less effective compare to soxhlet method at given extraction time. Increasing the extraction time during soxhlet extraction have distinct effect on extraction of adenosine (Table-1). Table-1 demonstrated that soxhlet extraction efficiency was higher than maceration at any time because of higher extraction temperature.

High hydrostatic pressure technique: The effect of pressure and times for high hydrostatic pressure extraction at 25 ºC showed in Fig. 1.

TABLE-1
EFFECT OF TEMPERATURE AND TIMES FOR TWO EXTRACTION METHODS

Extraction method	Temperature (ºC)	Time (h)	Adenosine (mg/g GL)
Maceration	28	0.5	0.16
Maceration	28	1.0	0.21
Maceration	28	2.0	0.23
Maceration	28	3.0	0.23
Soxhlet	70	1.0	0.82
Soxhlet	70	2.0	0.99
Soxhlet	70	3.0	1.77

GL = *Ganoderma lucidum*

It could be seen in Fig. 1 that adenosine extraction increased with increasing the pressure (500 to 2000 bar). The reason was that the higher the hydrostatic pressure is, the more solvent can enter cells and the more bio-active substances can permeate out to solvent[11]. In contrast, increasing the treatment time at given treatment pressure lead to slight decrease of extracted adenosine. This may be because of enzymatic degradation of adenosine during long extraction time.

The effect of combined temperature, pressure and times (at given *Ganoderma lucidum*-solvent ratio of 1:20) on high hydrostatic pressure extraction of adenosine is shown in Table-2.

TABLE-2
EFFECT OF TEMPERATURE, PRESSURE AND TIMES FOR HHP EXTRACTION

Pressure (bar)	Temperature (ºC)	Time (min)	Adenosine (mg/g GL)
2000	25	60	1.01
2000	25	120	0.98
2000	25	180	0.94
2000	45	60	1.29
2000	60	60	1.44
2000	60	3	1.33
2000	60	5	1.61
2000	60	10	1.49
2500	60	5	1.35
3000	60	5	1.40

GL = *Ganoderma lucidum*; HPP = High hydrostatic pressure.

As shown in Table-2, the optimum conditions for adenosine extraction were at 2000 bar and 60 ºC for 5 min. Zhang *et al.*[12] have found that in the case of extraction of bio-active compounds from *Rhodiola sachalinensis* under ultrahigh pressure the leaching out rates of flavones and *salidroside* had no significant increase when the pressure holding time was beyond 3 min. At 2000 bar treatment pressure and 1 h extraction time increasing the treatment temperature lead to increasing the adenosine extractability.

The effect of *Ganoderma lucidum*-solvent ratio at pressure 2000 bar, temperature 60 ºC and time 5 min for extraction showed in Table-3.

TABLE-3
EFFECT OF GL-SOLVENT RATIO FOR HHP EXTRACTION

GL-solvent ratio (g/mL)	Adenosine (mg/g GL)
1:20	1.61
1:40	1.77
1:60	1.86

TABLE 4
EFFECT OF CO-SOLVENT FOR EXTRACTION

Co- solvent (ethanol)	Adenosine (mg/g GL)
None	0.40
10%	0.41
20%	0.67
30%	0.43

GL = *Ganoderma lucidum*; HHP = High hydrostatic pressure

The effect of *Ganoderma lucidum*-solvent ratio was indicated that adenosine increased along with the accretion of ethanol amount. Higher solvent ratio increased the concentration gradient between sample and solvent. This lead to higher leaching-out substances in the extraction solvent.

Ultrasonic assisted extraction: The effect of temperature and time for adenosine extraction by using ultrasonic assisted extraction (*Ganoderma lucidum*-solvent ratio of 1:60) showed in Fig. 2. It was found that extracted adenosine increased with increasing temperature and times. Application of ultrasound at 25 ºC and 1 h increased the extractability of adenosine up to 2 times higher than maceration method (Fig. 2 and Table-1). This may be the mechanical effects of ultrasound (cavitation effect) that provide a greater penetration of solvent into cellular materials and improves mass transfer.

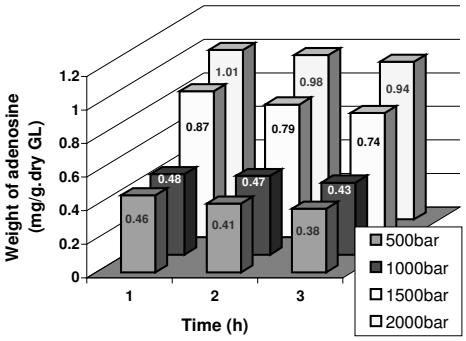

Fig. 1. Comparison of adenosine contents with various pressure and times for high hydrostatic pressure extraction

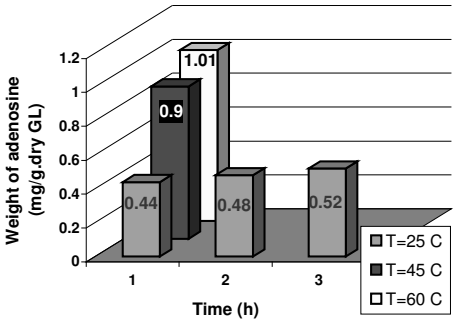

Fig. 2. Comparison of adenosine contents with various temperature and times for ultrasonic assisted extraction

Supercritical carbon dioxide extraction: The effect of pressure and temperature on extraction of adenosine is shown in Fig. 3. Increasing the pressure from 300 to 500 bar and 1 h treatment time has a positive effect on extractability of adenosine at room temperature. Increasing the treatment temperature up to 60 ºC lead to additionally increasing the extractability of adenosine at 500 bar.

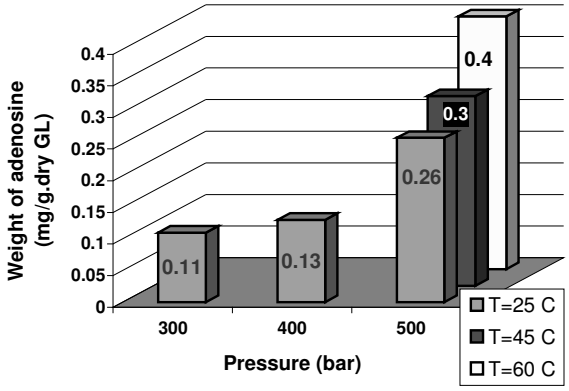

Fig. 3. Comparison of adenosine contents with various temperature and times for supercritical carbon dioxide extraction

Adding ethanol as co-solvent had an distinct positive effect on adenosine extraction up to 20 % ethanol content (on sample weight basis). Addition of organic co-solvents like ethanol, methanol, acetone, increase the solubility power of CO_2 and the yield of extraction of polar substances such as polyphenols[11]. Ethanol is a permitted co-solvent in food industry. Further increasing the ethanol content had a reverse effect on extractability of adenosine. The overall less extractability of adenosine using supercritical carbon dioxide, this may be the polarity of adenosine.

Comparison of extraction methods: Table-5 showed the results for adenosine extraction using different extraction methods (at constant *Ganoderma lucidum* to solvent ratio 1: 60). The best extraction method was high hydrostatic pressure method (at 2000 bar, 60 ºC, 5 min) followed by soxhlet method (70 ºC, 3 h) and ultrasound (60 ºC, 1 h). Supercritical carbon dioxide extraction method was less effective compare to high hydrostatic pressure and ultrasonic assisted extraction but distinct effective than maceration.

TABLE-5
COMPARISON OF ADENOSINE CONTENTS FROM EXTRACTION METHODS

Extraction method	Temp. (ºC)	Time (min)	Adenosine (mg/g GL)
Maceration	28	120	0.23
Soxhlet	70	180	1.77
High hydrostatic pressure (2000 bar)	60	5	1.86
Ultrasonic assisted extraction	60	60	1.01
Supercritical carbon dioxide (500 bar, 20 % ethanol)	60	60	0.67

GL = *Ganoderma lucidum.*

Conclusion

The novel technologies such as high hydrostatic pressure, ultrasonic assisted extraction and supercritical carbon dioxide are suitable extraction techniques for

extraction of bio-active substances such as adenosine. The amount of extracted substances using high hydrostatic pressure, ultrasonic assisted extraction or super-critical carbon dioxide are comparable or distinct higher than conventional Soxhlet and maceration methods. Additionally, because of low processing temperature (at room or moderate temperature up to 60 ºC) no or less thermal degradation of substances will be occur.

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