

Extraction of bioactive components from *Centella asiatica* using subcritical water

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ARTICLE INFO

Article history:

Received 30 May 2008

Received in revised form 9 October 2008

Accepted 9 November 2008

Keywords:

Subcritical water

Asiatic acid

Asiaticoside

Centella asiatica

Extraction

Particle formation

ABSTRACT

Bioactive components, asiatic acid and asiaticoside, were extracted from *Centella asiatica* using subcritical water as an extraction solvent. Extraction yields of asiatic acid and asiaticoside were measured using high-performance liquid chromatography (HPLC) at temperatures from 100 to 250 °C and pressures from 10 to 40 MPa. As temperature or pressure increased, the extraction yield of asiatic acid and asiaticoside increased. At the optimal extraction conditions of 40 MPa and 250 °C, the extraction yield of asiatic acid was 7.8 mg/g and the extraction yield of asiaticoside was 10.0 mg/g. Extracted asiatic acid and asiaticoside could be collected from water as particles with a simple filtering process. Dynamic light scattering (DLS) was used to characterize particle size. Particles containing asiatic acid were larger (1.21 μm) than particles containing asiaticoside (0.76 μm). The extraction yields of asiatic acid and asiaticoside using subcritical water at 40 MPa and 250 °C were higher than extraction yields using conventional liquid solvent extraction with methanol or ethanol at room temperature while the subcritical water extraction yields were lower than extraction yields with methanol or ethanol at its boiling point temperature.

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1. Introduction

Bioactive natural products have an enormous economic importance as specialty chemicals. Bioactive natural products can be used as drugs, biological or pharmacological ingredients, nutraceuticals, and raw materials for the production of drugs [1]. *Centella asiatica* is a tropical medicinal plant with a long history of therapeutic uses for conditions such as dermal disorders, venous insufficiency, and microangiopathy [2]. Previous studies demonstrated that *C. asiatica* extracts can enhance collagen synthesis *in vitro* and extracellular matrix accumulation *in vivo* [3], and can enhance tensile strength in wound tissue and facilitate the wound healing process [4]. Four main bioactive compounds in *C. asiatica* are asiatic acid (1), asiaticoside (2), madecassic acid (3), and madecassoside (4) as shown in Fig. 1 [5]. Among them, asiaticoside is the principal bioactive ingredient in *C. asiatica* since asiaticoside retains the most profound effect on antibacterial and fungicidal activity against pathogens and fungi [6]. Asiatic acid also exhibits bioactive

efficacy [7]. For example, asiatic acid is known to control cell division in human hepatoma, colon cancer, breast cancer, melanoma cells and cytotoxic activity on fibroblast cells [8]. Thus, the synthesis and pharmacological mechanism of asiatic acid derivatives have drawn considerable interest.

Typically, bioactive compounds in herbal plants are present in low concentrations. Thus, it is very important to develop more effective and selective extraction methods for the recovery of the desired bioactive compounds from the herb materials. Traditional organic solvent-based extraction often suffers from low extraction yields, long extraction times, and residual toxic organic solvents in final products. The residual solvents are problematic because residual toxic organic solvents in extracts can deteriorate the quality of the extracts and can cause serious health problems when the extracts are taken into the human body. Hence, high level of vacuum under heating, that is a high energy-consuming evaporation process, is often needed to remove the residual solvents in the extracts to permitted levels. Supercritical fluid extraction (SFE), especially supercritical carbon dioxide (scCO₂) extraction, of bioactive materials from herb plants is a potential alternative to conventional liquid solvent extractions [9]. The major disadvantage of scCO₂ extraction, however, is that extraction of polar components is highly limited by the poor solvent power of scCO₂ for the polar components.

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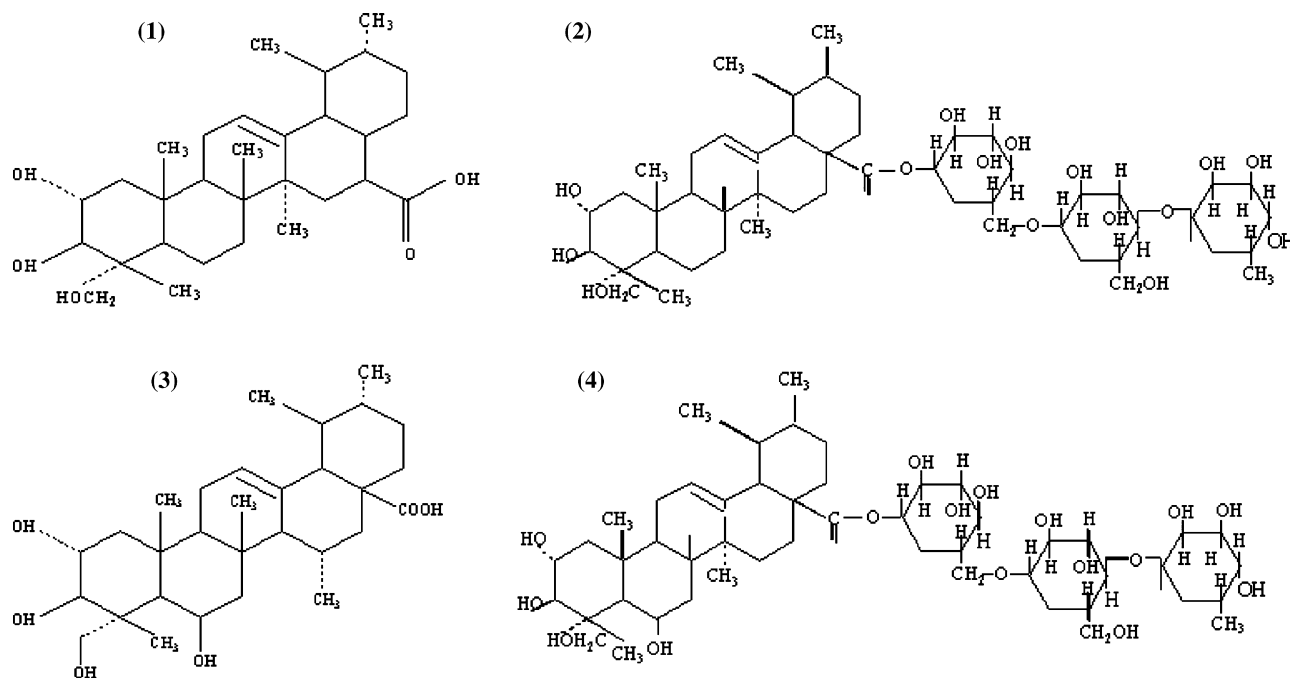


Fig. 1. Chemical structures of asiatic acid (1), asiaticoside (2), madecassic acid (3) and madecassoside (4) from Ref. [5].

Subcritical water extraction, using water under external pressurization above its boiling point as an extraction solvent, received much attention to extract desired polar compounds from herbs or plants [10]. Subcritical water extraction offers an efficient, non-toxic, and environmental-friendly alternative to conventional organic liquid solvent extraction techniques. Other important advantages of subcritical water extraction over the organic liquid solvent extraction include shorter extraction time, higher quality of extracts, and lower solvent extraction costs [11]. Subcritical water as an extraction solvent has been explored to extract polar, bioactive components from herbs and foods. It has been shown that 80% of oxygenates from savory and peppermint can be extracted with subcritical water at 6 MPa and 100 °C [12], 54% of nutraceuticals from oregano can be extracted at 10 MPa, 200 °C [13], and 90–95% of lignans from whole flaxseed can be extracted at 5.2 MPa and 140 °C [14].

In the past, organic solvents such as methanol [15], ethanol [16,17], or methanol–water mixture [18] were used to obtain extracts from *C. asiatica*. This study describes extraction of asiatic acid and asiaticoside from *C. asiatica* with subcritical water. We demonstrate that the extracted asiatic acid and asiaticoside can be collected as a particle form using a simple filtering process after the subcritical water extraction. In addition, we demonstrate that the particles containing asiatic acid and the particles containing asiaticoside can be separated from each other based on their particle size.

2. Material and methods

2.1. Materials

Dried *C. asiatica* were obtained from Dexa Medica Pharmaceutical Company (Jakarta, Indonesia). The sample contains leaves, nodes, petioles and roots of *C. asiatica*. The *C. asiatica* were ground to an average particle size of 520 μm . Asiatic acid standard was purchased from Sigma Aldrich Co. (St. Louis, MO, USA) and asiaticoside standard (purity of 98.5%) was purchased from Fluka (Oakville, Ontario, Canada). Acetonitrile (HPLC grade) and methanol (HPLC grade) were obtained from J. T. Baker (Phillipsburg, NJ, USA) and

ethanol (purity of 99.5%) was obtained from Junsei Chemical Co. (Tokyo, Japan). Deionized water was prepared using a Milli-Q Ultra-pure water purification system with a 0.22- μm filter (MA, USA). 8 μm filter papers, 0.4 μm Nylon membrane filters, and 0.4 μm polycarbonate membrane filters were purchased from Whatman (Maidstone, UK).

2.2. Extraction apparatus and procedure

The extraction experiments were conducted using a custom-built, subcritical water extraction apparatus. Fig. 2 shows a schematic diagram of the extraction apparatus. The extraction vessel (4) was made of SUS 316 with an internal diameter of 28 mm and a height of 377 mm, giving an internal volume of 232 ml. The preheater (3) was a 80 cm length SUS 316 coil with an internal diameter of 0.635 cm. The extraction vessel and the preheater were manufactured at the Korea Institute of Science and Technology machine shop. The temperature of the extraction vessel and the preheaters were controlled using a furnace (5, Daepoong Industries, Korea). The temperatures of the extraction vessel were monitored by inserting type-K thermocouples (model TJ36CAXL, Omega Engineering, Inc., USA) with a probe diameter of 0.16 cm inside the extraction vessel. The thermocouple has 0.3 s of response time with uncertainty measurement of 1 °C. The thermocouples were connected to a multichannel recorder (model DR 240, Yokogawa, Japan). The system temperature was controlled by a PID temperature controller (model DX 7, Hanyoung Industries, Korea). The high-pressure pump (2) was a model Palsa 608 diaphragm metering pump, manufactured by Palsa Feeder. Co. (NY, USA). This pump could generate pressures up to 50 MPa and could produce a maximum flow rate of about 1.2 l/h. The pressure of the extraction vessel was controlled using a back-pressure regulator (7), manufactured by Tescom (Model 44-2300 Series, MN, USA). The back-pressure regulator was rated to 100 MPa.

The procedure for extraction of asiatic acid and asiaticoside from *C. asiatica* consisted of several steps. *C. asiatica* (50 g) was charged into the extraction vessel. Deionized water was then introduced into the extractor using the high-pressure pump at an experimentally desired pressure. The pressure of the extractor was controlled

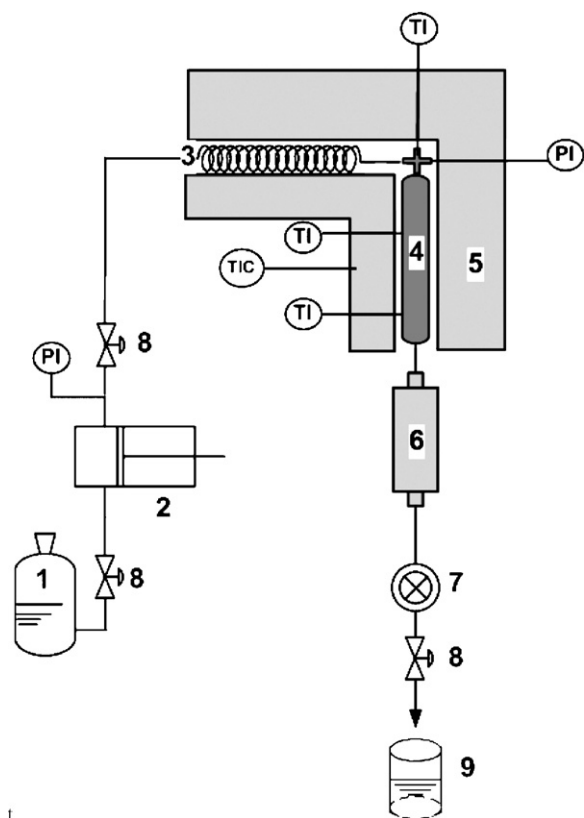


Fig. 2. Schematic diagram of subcritical water extraction apparatus. Components: (1) deionized water reservoir, (2) high-pressure pump, (3) preheater, (4) high-pressure extraction vessel, (5) heat furnace, (6) heat exchanger, (7) back-pressure regulator, (8) isolation valve, and (9) extract collecting bottle.

by the back-pressure regulator. The temperature of the extraction vessel was then increased to an experimentally desired temperature using the furnace. Static extraction was then carried out for 5 h. The extraction pressure was varied from 10 to 40 MPa and the extraction temperature was varied from 150 to 250 °C. The temperature of the extraction vessel was controlled within ± 1 °C and the pressure of the extraction vessel was controlled within ± 0.5 MPa during the extraction. After the extraction, the extracts were collected in the bottle (9) by opening the back-pressure regulator. The temperature of extracts in the water was cooled for 30 min and the extraction yield of asiatic acid and asiaticoside were analyzed. Each subcritical water extraction was carried out in triplicate.

2.3. Conventional liquid solvent extraction

Three different liquid solvents, water, ethanol and methanol, were used to extract asiaticoside and asiatic acid from *C. asiatica*. Asiatic acid and asiaticoside were extracted by charging 3 g of *C. asiatica* in a 250 ml round bottom flask (Pyrex, USA) with 100 ml of either water, ethanol, or methanol. The extractions were carried out at 25 °C for 24 h or at the solvent boiling point for 5 h, while stirring at 1200 rpm using a magnetic stirrer. The mixture was filtered through a 0.4 μ m Nylon membrane filter to obtain crude extracts. Each solvent extraction was carried out in triplicate.

2.4. Analytical method

The concentration of asiatic acid and asiaticoside in *C. asiatica* crude extract was quantitatively determined with HPLC analysis. The HPLC system was comprised of a solvent delivery pump (Young-Lin M930, Seoul, Korea), a column (Optimapak C18 5 μ m,

25 cm \times 0.46 mm, Daejeon, Korea), an absorbance ultra-violet (UV) detector (Young-Lin M720, Seoul, Korea) and built-in software (Autochro 2000, Seoul, Korea). Chromatographic separations were performed using a mobile phase of water and acetonitrile with a gradient of acetonitrile: 20–55% (30 min), 55% (5 min), 55–20% (5 min) at a flow rate of 1.4 ml/min [18]. The amount of sample injection was set at 20 μ L.

HPLC was calibrated with standard solutions of asiatic acid and asiaticoside of known solution concentrations. Standard solutions were prepared by first dissolving 1.5 mg of asiatic acid or asiaticoside in 1 ml methanol. These mixtures were diluted to obtain solutions of desired, known concentrations in the range of 0.5–10 mg/g, and used to construct calibration curves of asiatic acid and asiaticoside. Four different schemes to measure extraction yields of asiatic acid and asiaticoside were performed in this study, as shown in Fig. 3.

2.4.1. Direct analysis

Extracts were sonicated for 10 min to obtain well-dispersed particles in water. During the sonication, 10 ml amount of extracts were collected using a 20 ml syringe. The collected samples were dissolved in 20 ml of methanol and analyzed with HPLC.

2.4.2. Separation scheme 1

Subcritical water extracts were filtered using the 8 μ m size filter paper. Filtrate was analyzed with HPLC. Filter cake on the 8 μ m size filter paper were dissolved in 30 ml of methanol using a shaking sonicator at 50 °C for 5 h and analyzed with HPLC.

2.4.3. Separation scheme 2

After the subcritical water extract was filtered using the 8 μ m size filter paper, the filtrate was filtered again with the 0.4 μ m size polycarbonate membrane filter. The filter cakes on the 8 μ m size filter paper and the 0.4 μ m size polycarbonate membrane filter were dissolved in 30 ml of methanol using a shaking sonicator at 50 °C for 5 h and analyzed with HPLC. The filtrates from the 8 μ m size filter paper and the 0.4 μ m size polycarbonate membrane filter were analyzed with HPLC.

2.4.4. Separation scheme 3

Subcritical water extract was filtered using the 0.4 μ m size polycarbonate membrane filter. Filtrate was analyzed with HPLC. Filter cake on the 0.4 μ m size polycarbonate membrane filter were dissolved in 30 ml of methanol using a shaking sonicator at 50 °C for 5 h and analyzed with HPLC.

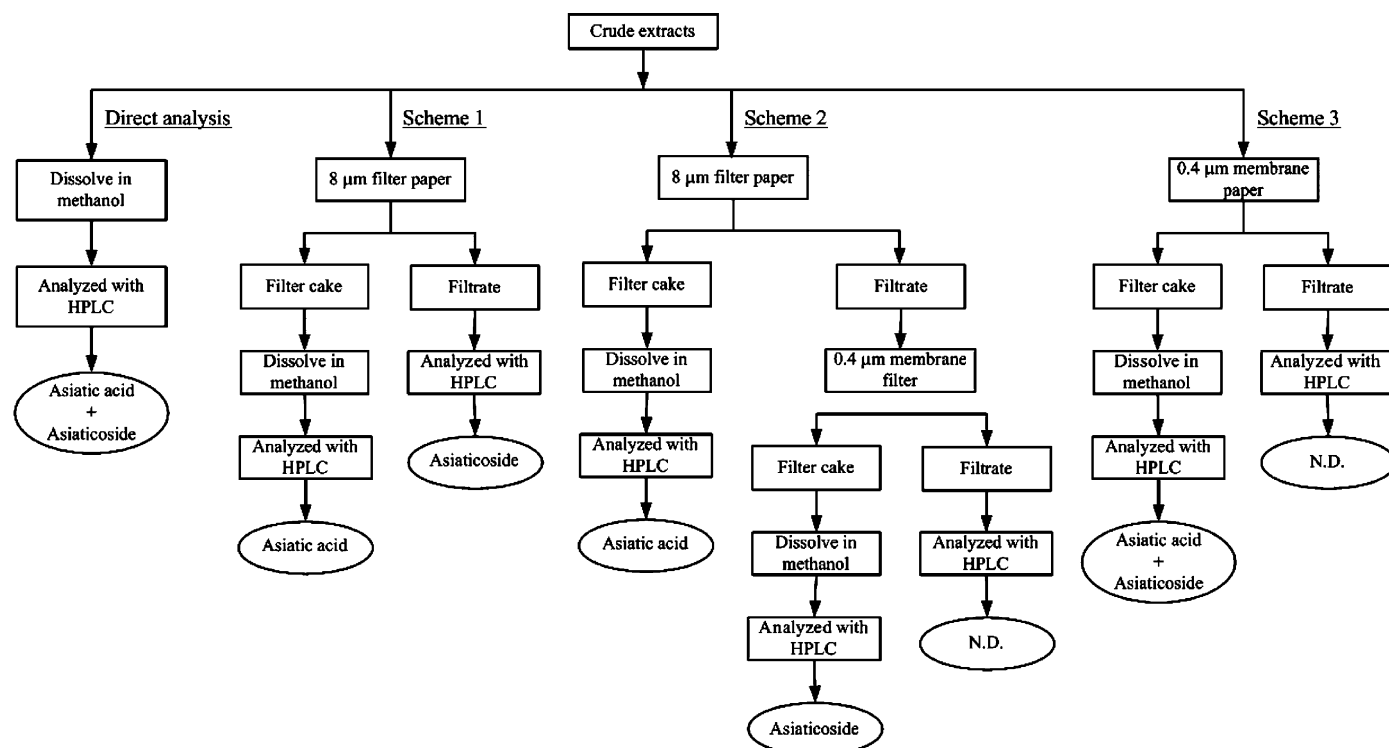
All HPLC analyses were performed at condition as described in Section 2.4.

The particle size in subcritical water extracts was also measured using a dynamic light scattering (DLS) technique with an ELS-8000 instrument (Photal Otsuka Eng., Japan). The DLS measurements were carried out at room temperatures using a He–Ne laser (10 mW) and 90 °C scattering angle. Prior to DLS measurements, the extracted particles in water were dispersed using sonication for 10 min. All DLS measurements were triplicate.

3. Result and discussion

3.1. Conventional liquid solvent extraction

Table 1 lists the extraction yield of asiatic acid and asiaticoside using either water, ethanol or methanol. The extraction conditions were 25 °C and 24 h or the boiling point of each solvent and 5 h. Water is not effective to extract asiatic acid and asiaticoside from *C. asiatica*. When water at 25 and 100 °C was used, asiatic acid was not extracted and only a small amount of asiaticoside (1–1.3 mg/g) was extracted. The low extraction yield may be due



N.D. = Not detectable

Fig. 3. Flow chart of subcritical water extract separation.

Table 1
Comparison of asiatic acid and asiaticoside extraction yields between conventional liquid solvents.

	Extraction yields (mg/g)					
	Water 25 °C ($\epsilon = 80$) ^a	Water 100 °C ($\epsilon = 55$) ^a	Methanol 25 °C ($\epsilon = 33$) ^b	Methanol 65 °C ($\epsilon = 27$) ^b	Ethanol 25 °C ($\epsilon = 25$) ^b	Ethanol 78 °C ($\epsilon = 18$) ^b
Asiatic acid	0	0	2.2 ± 0.05	2.3 ± 0.30	2.0 ± 0.13	10.0 ± 0.26
Asiaticoside	1.0 ± 0.05	1.3 ± 0.09	8.2 ± 0.01	12.4 ± 0.05	5.2 ± 0.21	17.7 ± 0.68

^a Ref. [20].

^b Ref. [19].

to the highly polar nature of water (dielectric constant, $\epsilon = 80$). When relatively less polar solvents, methanol ($\epsilon = 33$) or ethanol ($\epsilon = 25$), was used, higher extraction yields were obtained. When methanol or ethanol at 25 °C was used, the extraction yield of asiatic acid was 2.0–2.2 mg/g and the extraction yield of asiaticoside was 5.2–8.2 mg/g. Extraction yield of asiatic acid and asiaticoside increased as the extraction temperature increased. When methanol or ethanol at their boiling points was used, the extraction yield of asiatic acid was 2.3–10.0 mg/g and asiaticoside was 12.4–17.7 mg/g. Thus higher extraction temperature is beneficial to extract larger amount of asiatic acid and asiaticoside.

3.2. Subcritical water extraction

Fig. 4 shows the effect of subcritical water temperature on the asiatic acid and asiaticoside extraction yield. Extraction yields of asiatic acid and asiaticoside were analyzed with the direct analysis scheme (Fig. 3). The extraction yield of asiatic acid and asiaticoside increased significantly from 0 to 7.0 mg/g and from 1.1 to 8.4 mg/g, respectively, when temperature increased from 100 to 250 °C. The dielectric constant (ϵ) of subcritical water significantly decreases from 57 to 28, and approaches the dielectric constants of methanol ($\epsilon = 33$) or ethanol ($\epsilon = 25$) when the temperature increased from

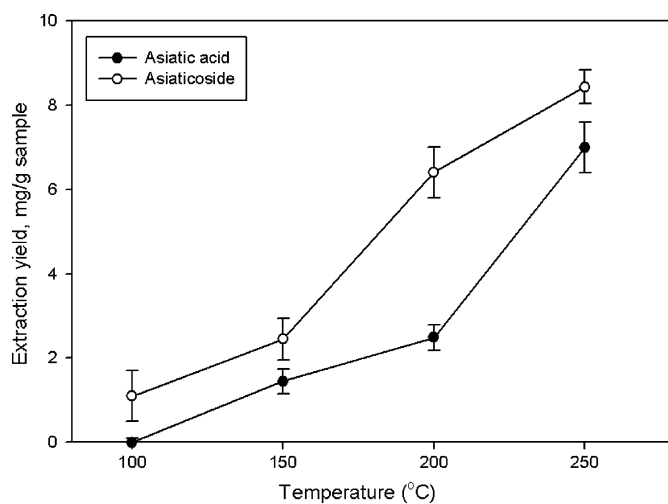


Fig. 4. The effect of temperature on the asiatic acid and asiaticoside extraction yields at a fixed pressure of 20 MPa and a fixed extraction time of 5 h.

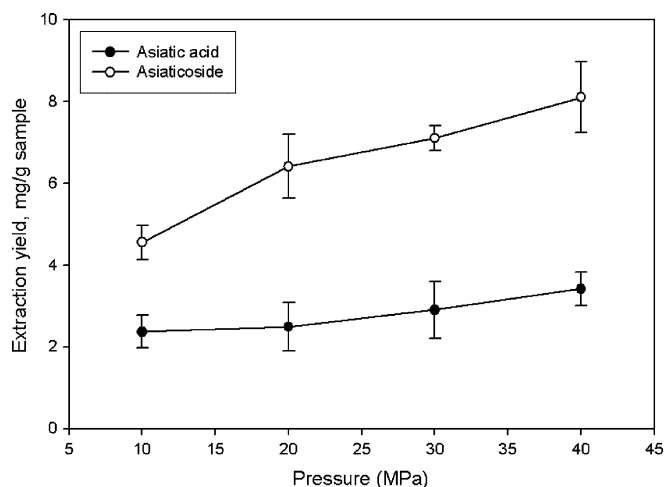


Fig. 5. The effect of pressure on the asiatic acid and asiaticoside extraction yields at a fixed extraction temperature of 200 °C and a fixed extraction time of 5 h.

100 to 250 °C at 20 MPa [19,20], indicating the polarity of subcritical water decreases at a higher temperature compared with the polarity of water at room temperature. This is responsible for the enhancement of asiatic acid or asiaticoside extraction yields in the subcritical water conditions compared with the extraction yield in water at room temperature and atmospheric pressure. Miller et al. measured solubilities of polycyclic aromatic hydrocarbons in subcritical water at 25–250 °C and 3–6 MPa. The solubility of the polycyclic aromatic hydrocarbons, such as chrysene, propazine, and chlorothalonil in the subcritical water (250 °C) is 130,000 times higher than the solubility of polycyclic aromatic hydrocarbons in water at room temperature (25 °C). The high solubility in the subcritical water was attributed to the low polarity, low dielectric constant of the subcritical water relative to those in water at room temperature [21,22].

Fig. 5 shows the effect of subcritical water pressure on the asiatic acid and asiaticoside extraction yield. Extraction yields of asiatic acid and asiaticoside were analyzed with the direct analysis scheme (Fig. 3). The extraction yields were not strongly dependent on the extraction pressure. As pressure increased from 10 to 40 MPa, the extraction yield of asiaticoside slightly increased from 4.6 to 8.1 mg/g, and the extraction yield of asiatic acid slightly increased from 2.4 to 3.4 mg/g. Dielectric constants of subcritical water do not strongly change with pressure. As pressure increased from 10 to 40 MPa at 200 °C, the dielectric constant of subcritical water increased slightly from 35.6 to 36.8 [20]. Thus, the polarity of the subcritical water does not change much with pressure. As a result, the subcritical water pressure does not have a strong effect on the extraction yield.

3.3. Separation of asiatic acid and asiaticoside

Asiatic acid and asiaticoside extracted using subcritical water precipitated when the extracts were collected from the subcriti-

cal water and cooled to room temperature in water. This section describes the separation of asiatic acid and asiaticoside using a simple filtering process with two different sizes of filters (8 and 0.4 μm). Direct analysis and three separation schemes were used, as described previously in Section 2.4 (see Fig. 3). Table 2 shows the extraction yield of asiatic acid, asiaticoside at an optimized condition (250 °C, 40 MPa, 5 h), and the average particle sizes on the filters and filtrates. The extraction yield of asiatic acid was 7.8 mg/g and asiaticoside was 10.0 mg/g when the direct analysis was used. These extraction yields are higher than the extraction yields with methanol and ethanol at room temperature, but lower than the extraction yields with the liquid solvents at their boiling points (see Table 1). The particle size of the extracts was measured to be 0.81 μm. When the separation Scheme 1 was used, the extraction yield of asiatic acid present on the 8 μm size filter was 7.0 mg/g, which is close to the extraction yield of the direct analysis. In the filtrate solution penetrating through the 8 μm size filter, no asiatic acid was detected while 8.4 mg/g of asiaticoside was present. The size of particles present on the 8 μm size filter was larger (1.10 μm) than the size of particles that were present in the filtrate (0.68 μm). Thus, the larger particles containing most of the asiatic acid can be isolated from the other components in the extract using the 8 μm size filter. When the separation Scheme 2 was used, no asiatic acid was present on the 0.4 μm size filter while 7.3 mg/g asiaticoside was present. In the filtrate, no asiatic acid and no asiaticoside were detected. The size of the particles present on the 0.4 μm size filter was larger (0.80 μm) than the size of particles present in the filtrate (0.40 μm). Thus, the particles containing most of asiaticoside can be isolated from the particles containing asiatic acid as well as the particles containing other components in the extract based on their particle size. When the separation scheme 3 was used, the extraction yield of asiatic acid (7.1 mg/g) and asiaticoside (9.3 mg/g) present on the 0.4 μm size filter was very close to the extraction yields of the direct analysis. In the filtrate, no asiatic acid or a very small amount of asiaticoside (0.1 mg/g) was present. Therefore, the particles containing asiatic acid and asiaticoside can be collected from water using the very simple filtering process in the subcritical water extraction of *C. asiatica*. Thus, extensive evaporation of solvent using heating and/or vacuum, that are typical additional processes necessary to recover dissolved extracts in extraction solvents, is not necessary when asiatic acid and asiaticoside are extracted from *C. asiatica* using subcritical water.

The particle formation of asiatic acid and asiaticoside after the subcritical water extraction is due to the high solubility of asiatic acid and asiaticoside in subcritical water, while they are not soluble, or are less soluble, in water at room temperature. The low polarity of the subcritical water is suitable for dissolving asiatic acid and asiaticoside. After the extraction, the extracts and the subcritical water were discharged from the extractor by depressurization and were cooled to room temperature. When the subcritical water is changed to water at room temperature, the polarity of the extraction solvent increases and the solubility of asiatic acid and asiaticoside decreases. As a result, asiatic acid and asiaticoside dissolved in the subcritical water phase precipitated in the water phase. It is not clear what is causing smaller particles con-

Table 2

Asiatic acid and asiaticoside extraction yields at an optimized conditions (250 °C, 40 MPa, 5 h) and particle sizes as measured by DLS.

		Asiatic acid (mg/g)	Asiaticoside (mg/g)	Particle size (μm)
Direct analysis		7.8 ± 0.16	10.0 ± 0.48	0.81 ± 0.10
Separation scheme 1	Filter	7.0 ± 0.15	–	1.10 ± 0.15
	Filtrate	–	8.4 ± 0.31	0.68 ± 0.08
Separation scheme 2	Filter	–	7.3 ± 0.24	0.80 ± 0.11
	Filtrate	–	–	0.40 ± 0.07
Separation scheme 3	Filter	7.1 ± 0.04	9.3 ± 0.27	1.00 ± 0.09
	Filtrate	–	0.1 ± 0.03	0.43 ± 0.08

taining asiaticoside compared with the particles containing asiatic acid. The higher solubility of asiaticoside in water at room temperature may be responsible for the smaller particle size. When the subcritical water was changed to water at room temperature, the higher solubility of asiaticoside may lead to a lower value of supersaturation. Particle size is proportional to supersaturation in many systems for crystal growth from liquid solutions [23]. Thus the smaller particle size containing asiaticoside may result from the lower supersaturation value.

4. Conclusion

In the extraction of *C. asiatica* using subcritical water, the extraction yields of asiatic acid and asiaticoside increased with an increase in temperature, while the extraction yields only slightly increased with an increase in pressure. The optimum extraction conditions of asiatic acid and asiaticoside were at 250 °C and 40 MPa. Under these conditions, 7.8 mg/g of asiatic acid and 10.0 mg/g of asiaticoside were extracted. The extraction yields of asiatic acid and asiaticoside, using subcritical water at the optimum conditions, were higher to those using the conventional liquid solvent extraction with methanol or ethanol at room temperature, while its values were lower to those with methanol or ethanol at its boiling point temperature. The extracted asiatic acid and asiaticoside precipitated when the subcritical water containing the extracts were depressurized and cooled to room temperature. This enables the collection of asiatic acid and asiaticoside as particles using the simple filtering process. The size of the particles containing asiatic acid was larger than the size of the particles containing asiaticoside. Thus, separation of asiatic acid and asiaticoside can be achieved when choosing an appropriate size of filter.

Acknowledgements

The authors gratefully appreciate the financial support from PT Dexa Medica. Partial support by Nano R&D program through the Korea Science and Engineering Foundation funded by the Ministry of Education, Science and Technology (2008-02344) was appreciated.

References

- [1] T. Henkel, R.M. Brunne, H. Muller, F. Reichel, Statistical investigation into the structural complementarity of natural products and synthetic compounds, *Angewandte Chemie-International Edition* 38 (1999) 643–647.
- [2] L. Incandela, G. Belcaro, M.R. Cesarone, M.T. De Sanctis, E. Nargi, P. Patricelli, M. Bucci, Treatment of diabetic microangiopathy and edema with total triterpenic fraction of *Centella asiatica*: a prospective, placebo-controlled randomized study, *Angiology* 52 (2001) S27–S31.
- [3] F.X. Maquart, F. Chastang, A. Simeon, P. Birembaut, P. Gillery, Y. Wegrowski, Triterpenes from *Centella asiatica* stimulate extracellular matrix accumulation in rat experimental wounds, *European Journal of Dermatology* 9 (1999) 289–296.
- [4] L. Sugunam, P. Sivakumar, G. Chandrakasan, Effect of *Centella asiatica* extract on dermal wound healing in rat, *Indian Journal of Experimental Biology* 34 (1996) 1208–1211.
- [5] H. Matsuda, T. Morikawa, H. Ueda, M. Yoshikawa, Medicinal foodstuffs. XXVII. Saponin constituents of gotu kola (2): Structures of new ursane- and oleanane-type triterpene oligoglycosides, centellasaponins B, C, and D, from *Centella asiatica* cultivated in Sri Lanka, *Chemical & Pharmaceutical Bulletin* 49 (2001) 1368–1371.
- [6] B.M. Hausen, *Centella asiatica* (Indian pennywort), an effective therapeutic but a weak sensitizer, *Contact Dermatitis* 29 (1993) 175–179.
- [7] B.C. Park, S.H. Paek, Y.S. Lee, S.J. Kim, E.S. Lee, H.G. Choi, C.S. Yong, J.A. Kim, Inhibitory effects of asiatic acid on 7,12-dimethylbenz[a]anthracene and 12-O-tetradecanoylphorbol 13-acetate-induced tumor promotion in mice, *Biological & Pharmaceutical Bulletin* 30 (2007) 176–179.
- [8] C.D. Coldren, P. Hashim, J.M. Ali, S.K. Oh, A.J. Sinskey, C. Rha, Gene expression changes in the human fibroblast induced by *Centella asiatica* triterpenoids, *Planta Medica* 69 (2003) 725–732.
- [9] E. Reverchon, I. De Marco, Supercritical fluid extraction and fractionation of natural matter, *The Journal of Supercritical Fluids* 38 (2006) 146–166.
- [10] J.W. King, K. Srinivas, Multiple unit processing using sub-, supercritical fluids, *The Journal of Supercritical Fluids* 47 (2009) 598–610.
- [11] M. Herrero, A. Cifuentes, E. Ibanez, Sub- and supercritical fluid extraction of functional ingredients from different natural sources: plants, food-by-products, algae and microalgae—a review, *Food Chemistry* 98 (2006) 136–148.
- [12] A. Kubatova, A.J. Lagadec, D.J. Miller, S.B. Hawthorne, Selective extraction of oxygenates from savory and peppermint using subcritical water, *Flavour and Fragrance Journal* 16 (2001) 64–73.
- [13] I. Rodríguez-Meizoso, F.R. Marin, M. Herrero, F.J. Señorans, G. Reglero, A. Cifuentes, E. Ibáñez, Subcritical water extraction of nutraceuticals with antioxidant activity from oregano. Chemical and functional characterization, *Journal of Pharmaceutical and Biomedical Analysis* 41 (2006) 1560–1565.
- [14] J.E. Cacace, G. Mazza, Pressurized low polarity water extraction of lignans from whole flaxseed, *Journal of Food Engineering* 77 (2006) 1087–1095.
- [15] T.D. Babu, G. Kuttan, J. Padikkala, Cytotoxic and antitumor properties of certain taxa of umbelliferae with special reference to *Centella asiatica* (l) urban, *Journal of Ethnopharmacology* 48 (1995) 53–57.
- [16] K. Punturee, C.P. Wild, U. Vinitketkumneun, Thai medicinal plants modulate nitric oxide and tumor necrosis factor- α in J774.2 mouse macrophages, *Journal of Ethnopharmacology* 95 (2004) 183–189.
- [17] C.L. Cheng, M.W.L. Koo, Effects of *Centella asiatica* on ethanol induced gastric mucosal lesions in rats, *Life Sciences* 67 (2000) 2647–2653.
- [18] P.K. Inamdar, R.D. Yeole, A.B. Ghogare, N.J. deSouza, Determination of biologically active constituents in *Centella asiatica*, *Journal of Chromatography A* 742 (1996) 127–130.
- [19] G. Akerlof, Dielectric constants of some organic solvent–water mixtures at various temperatures, *Journal of the American Chemical Society* 54 (1932) 4125–4139.
- [20] D.P. Fernandez, A.R.H. Goodwin, E.W. Lemmon, J. Sengers, R.C. Williams, A formulation for the static permittivity of water and steam at temperatures from 238 K to 873 K at pressures up to 1200 MPa, including derivatives and Debye–Huckel coefficients, *Journal of Physical and Chemical Reference Data* 26 (1997) 1125–1166.
- [21] D.J. Miller, S.B. Hawthorne, Method for determining the solubilities of hydrophobic organics in subcritical water, *Analytical Chemistry* 70 (1998) 1618–1621.
- [22] D.J. Miller, S.B. Hawthorne, A.M. Gizir, A.A. Clifford, Solubility of polycyclic aromatics in subcritical water, *Journal of Chemical & Engineering Data* 43 (1998) 1043–1047.
- [23] C. Noguera, B. Fritz, A. Clement, A. Baronnet, Nucleation, growth and ageing scenarios in closed systems II: dynamics of a new phase formation, *Journal of Crystal Growth* 297 (2006) 187–198.