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Original Research Article

Metabolic determination of decursinol using human liver microsome

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Abstract

Purpose: *To determine new metabolites of the main components of Angelica gigas known to give anti-inflammation and pain relief*
Methods: *Decursinol and blank sample were metabolized in human liver microsomes. The metabolized samples were centrifuged and deproteinated by adding 3 mL acetonitrile. The acetonitrile layer was concentrated and reconstituted in methanol. Finally, the prepared sample was injected into the LC-Q-TOF-MS.*
Results: *Four new metabolites of decursinol with m/z ranging from 263.0912 ~ 263.0920 were identified as hydroxylated forms of decursinol, and the hydroxylated position of each metabolite was characterized using TOF mass spectrum. Their error values of detected m/z were 0.38 ~ 2.29 ppm, which indicates high accuracy of analysis.*
Conclusion: *Previously unreported decursinol metabolites have been identified in this study. The findings provide athe basis for further pharmaceutical studies and functional food development using decursinol.*

Keywords: *Angelica gigas, Decursinol, Metabolites, LC-Q-TOF-MS, Human liver microsome*

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INTRODUCTION

Angelica, a plant belonging to the genus *Angelica* and the family *Apiaceae*, has been widely used in Asia as a medicinal herb for many years because of its calming, analgesic, antitumor, and brain cell protection properties. In addition, it is effective in the treatment of women's diseases such as menstrual irregularities, amenorrhea, menopausal syndrome, and postpartum abdominal pain. Thus, it is sold as a functional food for women's health care in Europe and USA [1-3]. Korean Angelica (*Angelica gigas*), Japanese Angelica (*Angelica acutiloba*), and Chinese Angelica (*Angelica sinensis*) are named based on their places of origin and are known to be different in appearance, major components, and pharmacological effects [4]. *Angelica gigas* Nakai, a herb which grows for two to three years, exhibits activities such as immune enhancement, antioxidant, antidiabetic, anti-dementia, skin

whitening, and ultraviolet protection [5-9]. The anti-inflammatory properties of *Angelica gigas* Nakai assessed using an atopic dermatitis model as well as against curing dry eye syndrome have been recently reported [10,11].

The main components of *Angelica gigas* Nakai are decursinol, decursin, and decursinol angelate [12]. Decursin and decursinol angelate are structurally isomeric and their properties are very similar because their secondary alcohol groups are esterified to the 3,3-dimethyl acryloyl group and the angeloyl group, respectively [13]. Decursin and decursinol angelate are effective in the treatment of breast, prostate, and lung cancers, whereas decursinol does not directly kill cancer cells [14-16]. However, decursinol was effective in preventing tumor cell proliferation in an *in vitro* study and is being marketed as "Decursinol-50" owing to its anti-inflammatory and pain-relieving effects [14-18].

In recent studies, metabolic analyses of decursin and decursinol angelate were conducted using the human liver microsome and rodent plasma, which showed that decursinol is produced as a major metabolite of decursin and decursinol angelate [19]. In another study, oxidation and glucuronidation of decursinol were attempted in rats *in vitro* and *in vivo*, but no significant metabolites were found [21]. Owing to its remarkable pharmaceutical activity, decursinol has been the subject of significant research in multiple fields, including pharmacokinetic studies; however, metabolic studies of decursinol have not produced any tangible results. In this present study, the metabolism of decursinol *in vitro* was evaluated in human liver microsomes, and their structures were determined using LC-Q-TOF-MS.

EXPERIMENTAL

Reagents and chemicals

Decursinol standard was purchased from Chem Faces (Wuhan, China). β-Nicotinamide adenine dinucleotide phosphate sodium salt hydrate (≥98%), glucose-6-phosphate dehydrogenase from baker's yeast (*S. cerevisiae*), D-glucose 6-phosphate sodium salt, potassium phosphate monobasic, potassium phosphate dibasic, and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Mixed-gender pooled 150-donor human liver microsomes were supplied from BD Gentest (MA, USA). HPLC (High performance liquid chromatography) Grade methanol (99.9%) used for sample preparation was purchased from Duksan Reagent (Korea). HPLC Grade acetonitrile (99.9%) used for chromatographic mobile phase and sample deproteination was obtained from J.T. Baker (Phillipsburg, NJ, USA). Distilled water was purified using an ultrapure water preparation apparatus (Millipore Elix 3 and Milli-Q Academic Gradient A-10 Bedford, MA, USA).

Evaluation of *in vitro* metabolism using human liver microsome

A 50mM substrate stock solution was prepared by dissolving decursinol in DMSO. 1 M phosphate buffer (pH 7.4) was prepared by mixing 1 M potassium phosphate dibasic and 1 M potassium phosphate monobasic at a volume ratio of 8:2. The NGS (NADPH(β-Nicotinamide adenine dinucleotide phosphate sodium salt hydrate) generating system)was prepared by adding 500 µL of 0.1 M glucose 6-phosphate, 250 µL of 10 mg/mL $NADP^+$, and 1.12 µL of glucose 6-phosphatedehydrogenase (1,000 units) to a total volume of 751.12 µL. The microsomes were stored at -80 °C and incubated at 37 °C prior to use.

In *in vitro* experiments, samples for experiments were prepared in 100 µL of 1 M phosphate buffer (pH 7.4), 645 µL of distilled water, 5 µL of substrate stock solution, and 50 µL of human liver microsomes, and pre-incubated at 37 °C for 5 min. Thereafter, the sample was added to a tube containing 200 µL of NGS, and another preincubated sample was added to a tube containing 200 µL of DW instead of NGS for blank sample. The samples were incubated at 37 °C for 2 h in a shaking incubator.

Sample preparation

To confirm the retention time of decursinol, the decursinol standard was diluted to a concentration of 10 ppm in methanol and kept at 4 °C until LC-Q-TOF-MS analysis. The samples incubated *in vitro* were centrifuged at 12,000 *g* for 10 min. After centrifugation, the supernatant was transferred to a glass tube and deproteinated by adding 3 mL of acetonitrile. The samples were centrifuged at 12,000 ×*g* for 10 min, and the acetonitrile layer was placed in another glass tube and concentrated using nitrogen gas. The residue was reconstituted in 100 µL of methanol and filtered. The filtered sample was injected into the LC-Q-TOF-MS.

LC-Q-TOF-MS conditions

The LC-Q-TOF-MS system was composed of an Agilent 1260 Infinity HPLC system (Agilent, Waldbronn, Germany) combined with an Agilent

6530 Q-TOF mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) with electrospray ionization capability. The chromatographic separation column was 4.6 × 50 mm I.D. 1.8 µm Eclipse XDB-C18 (Agilent Technologies, Foster city, CA, USA), and the column temperature was maintained at 40 ℃. The mobile phases used were solvent A (0.1% formic acid in water) and solvent B (acetonitrile). The flow rate was set at 0.5 mL/min. The chromatographic separation was done in a gradient with solvent B % set to 15% at 0 to 0.1 min, 15 to 100% at 0.1 to 7 min, 100% at 7 to 10 min, 100 to 15 % at 10 to 11 min and 15 % at 11 to 20 min.

For the mass spectrometric parameters used for the mass analysis were as follows: drying gas temperature 200 ℃, dry gas flow rate 10 L/min, nebulizer of 45 psi, fragmentor voltage 150 V, capillary 3000 V, skimmer 65 V, Oct RFV 750 V. The mass range was set from 20-1,000 m/z and scan rate was 1.00 spectra/sec. Mass measurements for each peak were performed in accordance with an automated calibration delivery system using a calibration solution (calibration solution A, Agilent Technologies) with the internal reference mass at m / z 121.0509 and 922.0098.

Statistical analysis

The ESI probe was operated in the positive mode and data were analyzed using Mass Hunter software (Agilent Technologies). The error ppm was determined by calculating the mass error in parts per million (ppm) from the observed and theoretical m/z values. To ensure accuracy, error ppm was analyzed less than 10.

RESULTS

To identify the metabolites of decursinol, sample chromatograms with and without NGS were compared and specific peaks in the NGS treated sample chromatogram were identified. Before metabolic identification, the retention time of decursinol in the sample was confirmed by comparing it with that of the decursinol standard. Decursinol and its metabolites were identified mainly in the form of $[M + H]^+$ using the Q-TOF-MS spectrum. The extracted ion chromatograms of decursinol and its metabolites analyzed *in vitro* are shown at Figure 1. All peaks were confirmed using the measured mass. The structures of decursinol metabolites were identified using LC-MS and the value of error ppm for determination accuracy was evaluated.

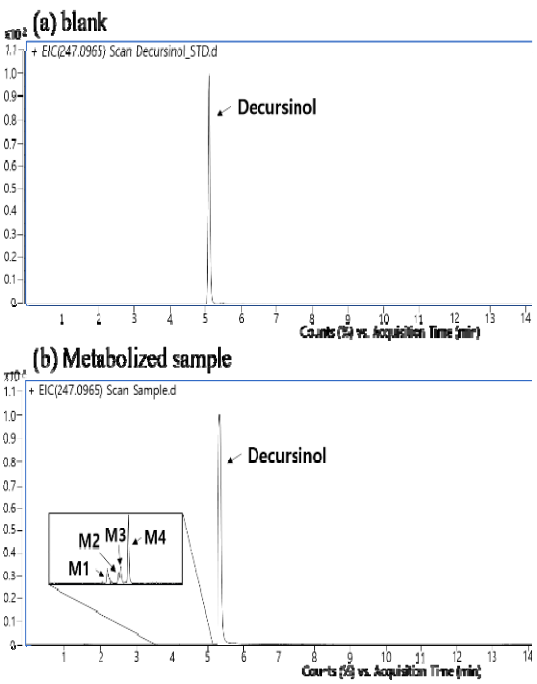

Figure 1: Extracted ion chromatogram of decursinol and its metabolites; (a) blank (b) metabolized sample

The decursinol peak in the samples appeared at the same retention time (5.467 min) as that of the decursinol standard. The mass of decursinol and sodium plus ion form identified with LC-Q-TOF-MS were m/z 247.0984 ($C_{14}H_{15}O_4^+$) and m/z 269.0779 ($C_{14}H_{14}O_4Na^+$) and the error ppm were 7.78 ppm and 4.01 ppm respectively, which indicates high detection accuracy by LC-Q-TOF-MS. The fragments m/z of decursinol were m/z 229.0878 ($C_{14}H_{13}O_3^+$), m/z 214.0617 ($C_{13}H_9O_3^+$), and m/z 201.0904 ($C_{13}H_{12}O_2^+$) (Figure 2(a)), respectively, confirming the generation of the same decursinol fragments, as previously observed [19].

The specific peaks detected only in NGS treated samples were regarded as metabolites of decursinol and their MS fragments were interpreted. As a result, a total of four metabolites were identified in the NGS treated samples. These four peaks were hydroxylated structures of decursinol at m/z 263.0915 ($C_{14}H_{15}O_5^+$) with the same molecular weights, and the retention times for M1-M4 were 4.224, 4.704, 4.787, and 5.102, respectively. All the metabolites eluted faster than decursinol. In addition, the molecular weights for M1–M4 were approximately m/z 263.0912–263.0920, and the error values of detected m/z were 0.38 ~ 2.29 ppm, with low factors suggestive of a high accuracy of mass analysis.

The common fragments of the four metabolites had an m/z 245.0814 ($C_{14}H_{13}O_4^+$), with the elimination of OH group to form a C-C double bond. The other fragments used to characterize the structure of the metabolites were identified as m/z 217.0861 ($C_{13}H_{13}O_3^+$), m/z 191.0342 ($C_{14}H_{15}O_5^+$), m/z 175.0393 ($C_{10}H_7O_3^+$), m/z 163.0393 ($C_9H_7O_3^+$), and m/z 147.0444 ($C_9H_7O_2^+$). Some of them overlapped with the fragments of decursinol, indicating that the fragments were not metabolized.

The fragment ions of M1 were found at m/z 175.0384, indicating that the chromene of decursinol was not metabolized. Therefore, the position where OH can be bound is the para site or dimethyl of tetrahydropyran.

M2 and M4 showed the same fragment ions at m/z 245.0816 (M2), m/z 245.0809 (M4), m/z 191.0335 (M2), and m/z 191.0355 (M4). This indicates that a hydrogen of the 2H-chromen-2-one is metabolized to OH. However, the mass spectral information on the fragments was insufficient, and it was difficult to precisely identify the hydroxylated positions of M2 and M4.

M3 was observed at m/z 245.0794, m/z 191.0342, and m/z 147.0449. Among these ratios, m/z 147.0449 indicated that the chromen of decursinol was not metabolized, and m/z 191.0342 indicated that methyl-chromen of decursinol was hydroxylated. The hydroxylated site of M3 could be identified using the para position of tetrahydropyran. In addition, the OH position of M3 was identified, and it was concluded that a dimethyl group bound to tetrahydropyran is present at the OH position of M1. All MS data are summarized in Figure 2 and Table 1.

DISCUSSION

In previous metabolic study of decursin and decursinol angelate using the human liver microsomes and rodent plasma, several metabolites were identified [19]. Decursin was found to be metabolized to 10 metabolites *in vitro*, including decursinol. The previously identified metabolites of decursin were one carboxylated, one ester hydrolyzed, one hydrated, one methyl oxidated, three hydroxylated, and three dihydroxylated metabolites, indicating that the metabolism of decursin mainly involved a hydroxylation reaction. In the case of decursinol angelate, a total of 10 metabolites were identified *in vitro*, including decursinol. Three metabolites formed through hydroxylation and dehydroxylation, two metabolites formed through the epoxidation of hydroxylated forms, one formed through hydration, and one formed through ester hydrolysis. However, the metabolic pathways suggested that none of the compounds were expected to be the metabolites of decursinol. In another study, oxidative and glucuronic metabolism studies were carried out, but no significant metabolites of decursinol have been found [21].

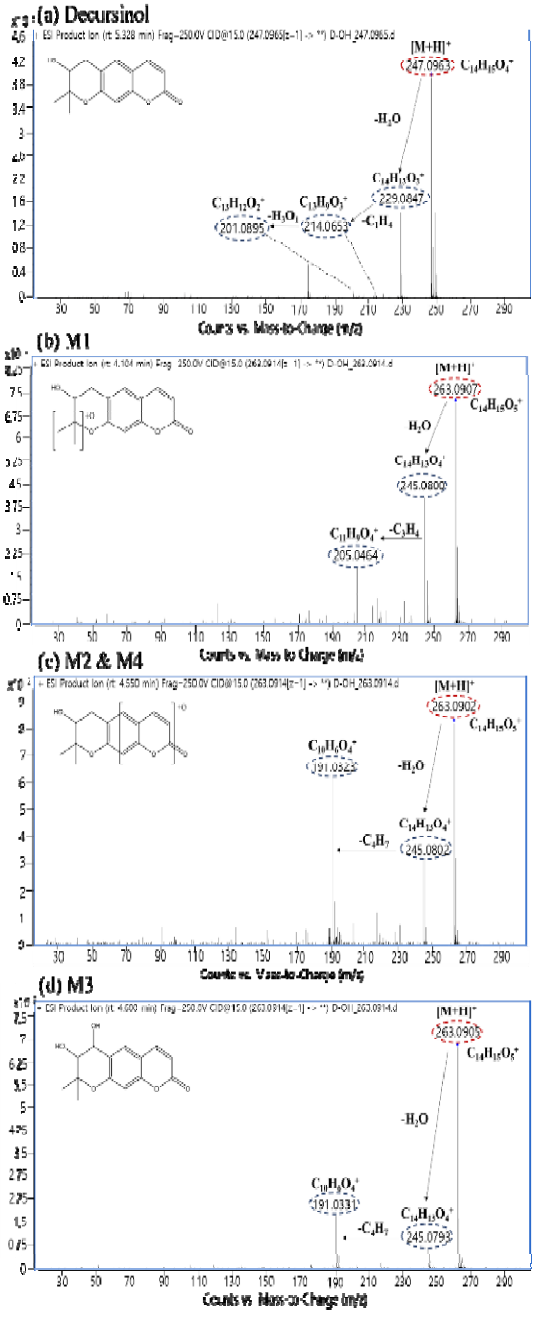

Figure 2: LC-Q-TOF- MS data of decursinol and its metabolites; (a) decursinol (b) M1, (c) M2, (c) M3, (d) M4

Table 1: LC-Q-TOF- MS and metabolite analysis of decursinol and its metabolites (M1-M4)

Metabolite	Formula $[M+H]^+$	RT (min)	Theoretical Mass (Da)	Measured mass (Da)	Error (ppm)	Metabolic reaction	m/z of major fragment fragment ion (Da)
Decursinol	$C_{14}H_{15}O_4^+$	5.467	247.0965	247.0984	7.78	Parent	229.0878, 214.0617, 201.090447
M1	$C_{14}H_{15}O_5^+$	4.224	263.0914	263.0920	2.29	Hydroxylation	175.0384
M2	$C_{14}H_{15}O_5^+$	4.704	263.0914	263.0915	0.38	Hydroxylation	245.0816, 191.0335
M3	$C_{14}H_{15}O_5^+$	4.787	263.0914	263.0912	-0.76	Hydroxylation	245.0794, 191.0342, 147.0449
M4	$C_{14}H_{15}O_5^+$	5.102	263.0914	263.0917	1.14	Hydroxylation	245.0809, 191.0355

Time-of-flight mass spectrometry with high accuracy and high sensitivity made it possible to analyze the decursinol metabolites in this study, and their structures were identified. The four new metabolites detected in the present study were, previously, not included as the metabolites of decursin and decursinol angelate. However, they can be presumed to be metabolites of decursin and decursinol angelate, because decursinol is metabolized from decursin and decursinol angelate. The metabolic pathway of decursinol induced by decursin and decursinol angelate is shown in Figure 3.

These four new metabolites can be used as additional data for further research on decursinol and can be used as significant data for drug interaction analysis. In addition, the metabolism of decursinol and further interpretation was useful in the identification of decursin and decursinol angelate metabolites as well as form a basis for future research on new metabolites of decursin and decursinol angelate.

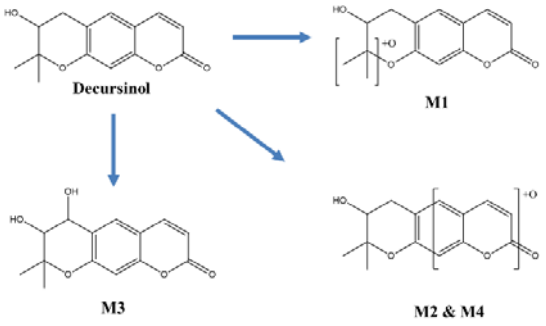

Figure 3: Proposed metabolic pathway of decursinol

CONCLUSION

Decursinol is one of the main bioactive components of *Angelica gigas*, whose metabolites have not been identified in previous studies. In this study, four new metabolites of decursinol have been identified for the first time in human liver microsomes using high-accuracy LC-Q-TOF-MS. These metabolic data may be useful for food development, as well as studies on *Angelica gigas* and decursinol, and metabolic enzymes of *Angelica gigas*. Furthermore, these data can be applied to metabolic and pharmacokinetic studies of decursin and decursinol angelate.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Jiye Gu and Jaesung Pyo designed the study and wrote the manuscript. Young Jin Park and Chaeyeong Ahn performed the experimental work. Dayoung Heo carried out a literature study and compiled the data. Yuseong Chung and Kang Min Kim performed a literature survey and analyzed the data. Jae Seon Kang compiled the data. † The first two-named authors contributed equally to this work. The research article was thoroughly read by all the authors before commination for the consideration of publication. These authors contributed equally to this work.

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