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***In vitro* metabolism of pyranocoumarin isomers decursin and decursinol angelate by liver microsomes from human and rodents**

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Abstract

The aim of this study is to investigate and compare the metabolic rate and profiles of pyranocoumarin isomers decursin and decursinol angelate (DA) using liver microsomes from humans and rodents, and to characterize the major metabolites of decursin and DA in human liver microsomal (HLM) incubations using LC-MS/MS. First, we conducted liver microsomal incubations of decursin and DA in the presence or absence of NADPH. We found that in the absence of NADPH, decursin was efficiently hydrolyzed to decursinol (DOH) by hepatic esterase(s), but DA was not. In contrast, formation of DOH from DA was mediated mainly by cytochrome P450(s). Second, we measured the metabolic rate of decursin and DA with liver S9 fractions from mice and humans. We found that human liver S9 fractions metabolized both decursin and DA more slowly than that of the mouse. Third, we characterized the major metabolites of decursin and DA from HLM incubations using HPLC-UV and LC-MS/MS methods and assessed the *in vivo* metabolites in mouse plasma from a one-dose PK study. Decursin and DA have different metabolite profiles. Nine metabolites of decursin and nine metabolites of DA were identified in HLM incubations besides DOH using a hybrid triple quadrupole linear ion trap LC-MS/MS system, and many of which were later verified to be also present in plasma samples from rodent PK studies.

Keywords

decursin; decursinol angelate; decursinol; *Angelica gigas* Nakai; Apiaceae; LC-MS/MS; *in vitro* liver microsomal incubations

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Introduction

Angelica gigas Nakai (AGN) is a traditional herb widely used in Asian countries [1]. AGN products are currently marketed as dietary supplements for pain, Alzheimer disease and women's health care in the United States and some Asian countries. Pyranocoumarin compounds decursin and its structural isomer decursinol angelate (DA) are the major chemical components in the ethanol extract of the root of AGN (Fig. 1) [2]. In the past decade, the *in vivo* anti-cancer, anti-nociceptive and other activities of decursin and/or DA have been increasingly reported [1,3–7].

Recently we and others have shown that both decursin and DA were rapidly converted to decursinol (DOH, Fig. 1) in rodents after administration either by oral gavage or intraperitoneal injection, in the form of pure chemicals or AGN extract [3,8–10]. However, we observed that plasma retained DA more than decursin when decursin/DA mixture was administered to mice [8] and rats [10]. These data suggested that decursin and DA might be metabolized differently in terms of metabolic rate and profiles, which might translate into different *in vivo* activities. Studies by Park *et al* and us indicated that liver might be the major organ site where decursin and DA are metabolized to DOH [9,10]. Since interspecies difference in oral bioavailability typically arises from differences in hepatic first pass metabolism [11], it will be important to investigate the *in vitro* metabolism of decursin and DA with human and rodent liver microsomes and S9 fractions to predict their metabolic fates in humans. In addition, DOH, the major *in vivo* major metabolite after decursin and/or DA administration in rodents, has been reported to lack direct *in vitro* anti-cancer cytotoxic activities [12,13]. One reasonable explanation is that further metabolites of DOH and/or metabolites of decursin/DA other than DOH account for the *in vivo* activities seen in decursin/DA. However, up to now, little is known about additional metabolites of decursin and DA. In this study, we investigated the *in vitro* metabolism of decursin and DA using liver microsomal and S9 fraction systems. We also profiled the *in vitro* metabolites in liver microsomal incubations of both compounds by LC-MS/MS, and further verified that most of *in vitro* metabolites were also present *in vivo*.

Results

Incubation of decursin or DA with human, rat or mouse liver microsomes (HLM, RLM or MLM) in the presence or absence of cofactor NADPH generated interesting metabolite patterns. As shown in Fig. 2A, when incubated with MLM (CD-1 background) in the absence of NADPH, the majority of decursin was rapidly hydrolyzed to DOH, with only 4% of decursin remaining after 5 min incubation. In sharp contrast with decursin, there was no detectable hydrolysis of DA to DOH in MLM incubations in the absence of NADPH (Fig. 2B). The RLM and HLM incubations produced similar contrasting patterns after 10 min or 60 min incubation for RLM and HLM, respectively (data not shown).

It is well known that liver microsomes contain enzymes other than CYP p450s such as carboxylesterases. We deduced that liver carboxylesterase(s) hydrolyzed decursin to its alcohol moiety which is DOH. Somehow, DA was resistant to liver carboxylesterases catalyzed hydrolysis. Decursin and DA are structural isomers; the only difference is the

position of a methyl group on the butenyl side chain being on 3' position for decursin and 2' position for DA (Fig. 1). Based on the structural characteristics of decursin and DA, we proposed that steric shield may be the mechanism to account for the resistance of DA to liver carboxylesterase. The methyl group on the 2' position of DA may block the access of the carboxylesterase(s), leading to resistance of DA to carboxylesterase(s).

When incubated with MLM, RLM and HLM in the presence of NADPH, both decursin and DA were metabolized extensively to a variety of metabolites with DOH being the major one as shown in Fig. 2A and 2B. This suggested that CYP p450 enzymes compete with carboxylesterase(s) for metabolizing decursin and can efficiently metabolize DA. Overall, *in vitro* liver microsomal incubation data suggested that decursin was metabolized by both NADPH-independent esterase(s) and NADPH-dependent p450s, but DA was metabolized predominantly by NADPH-dependent p450s. Work is in progress to identify the enzymes responsible for the metabolism of decursin and DA and the mechanism involved.

We measured the metabolic rate of decursin and DA with liver S9 fractions from mouse and human. As shown in Fig. 3, mouse S9 fractions metabolized both decursin and DA much faster than human S9 fractions. The metabolic half-life of decursin and DA was approximately 3.5 and 12 min, respectively, in mouse S9 fraction, and approximately 85 and 100 min, respectively, in human S9 fraction. The data indicated that DA was metabolized slower than decursin by mouse S9 which is in agreement with our previous findings that DA was retained more than decursin in plasma samples from our previous PK studies in mice and rats [8,10].

We next compared the metabolite profiles of decursin and DA in MLM vs. HLM incubations in the presence of NADPH. HLM incubations produced different metabolite profiles in both decursin (Supplemental Fig. 1A) and DA (Supplemental Fig. 1B) compared to MLM, while MLM and RLM incubations showed similar metabolite profiles in both decursin and DA (data not shown). In addition, decursin displayed different metabolite profiles comparing to DA in MLM, RLM and HLM across the board. Since the activity of the commercially obtained liver microsomes could vary from batch to batch, and from vendor to vendor [14], we further confirmed our major findings by using human and rat liver S9 fractions obtained from another vendor, Invitrogen (Grand Island, NY, USA. data not shown).

Fig. 4A & 4B showed the MS/MS and MS³ spectra of decursin and its known metabolite DOH. The fragment ion m/z 83 was detected in MS/MS spectrum of 329 but not in the MS³ spectrum of 247, and was assigned to the butenyl moiety of decursin. The major fragment ions m/z 247, 229, 211, 213, 214 and 175 were assigned to the pyranocoumarin moiety of decursin as illustrated in Fig. 4C. DA displayed an identical MS/MS spectrum as of decursin (data not shown).

We then identified and tentatively elucidated the structure of the major metabolites of decursin and DA from 60min HLM incubation extracts using LC-MS/MS. As described in Method section, we used pMRM and PI/NL scan methods to screen for potential metabolites of decursin and DA. Supplemental Fig. 2 and 3 showed chromatograms of LC-MS/MS by pMRM scan (A) and PI/NL scan (B), and also by HPLC-UV (C) as well for decursin and

DA, respectively. Besides DOH, nine metabolites of decursin and nine metabolites of DA were identified in the HLM incubation extracts in positive mode by pMRM and PI/NL scans. HPLC-UV traces indicated that DOH was the most abundant metabolite for both decursin and DA in HLM incubation extracts. Metabolite M6 of decursin was the secondary abundant metabolite for decursin in HLM incubation extract (Supplemental Fig. 2), and metabolites N2 and N8 of DA were the other two most abundant metabolites for DA in HLM incubation extract (Supplemental Fig. 3).

By characterizing the MS/MS spectra of all the identified metabolites of decursin and DA, we found that the metabolites could be categorized into two groups. Group A displayed a fragmentation pattern with fragment ions m/z 247, 229, 211, 213, 214, 201, 187 and 175, which are exactly the same as the parent compounds. Group B displayed a fragmentation pattern with major fragment ions m/z 263, 245, 227, 217, 203 and 191, which are corresponding to the fragment ions of the parent compound plus 16. Representative MS/MS spectra for group A and B metabolites were shown in Fig. 5A & B. For the group A metabolites, a reasonable explanation is that the biotransformation had occurred on the butenyl side chain of decursin and DA only. On the other hand, for the group B metabolites, one oxygen atom had been added to the pyranocoumarin moiety of decursin and DA. We found that 7/9 of the decursin metabolites and 7/9 of the DA metabolites belonged to Group A, with the remaining two decursin metabolites and two DA metabolites belonged to Group B. This indicated that the butenyl moiety of decursin and DA is more susceptible to the metabolic biotransformation than the pyranocoumarin ring.

As summarized in Table 1 & 2, three decursin metabolites M2, M3 and M4 and three DA metabolites N3, N4 and N5 displayed a $[M+H]^+$ of 361, 32 Da greater than that of the parent, indicating a possible addition of two oxygen atoms. Three metabolites of decursin M6, M7, M8 and three DA metabolites N6, N8 and N9 had a $[M+H]^+$ of 345, 16 Da greater than that of the parent compound, suggesting an addition of one oxygen atom to decursin. Decursin metabolite M5 and DA metabolite N7 had a $[M+H]^+$ of 347, 18 Da greater than that of the parent indicating that $2H+O$ had been added. We proposed dehydration has occurred on the double bond of the butenyl moiety. Decursin metabolite M1 had a $[M+H]^+$ of 359, 30 Da greater than that of parent indicating that $2O-2H$ had been the possible biotransformation. We deduced that the possible biotransformation of M1 is the carboxylation of the methyl group ($CH_3 \rightarrow COOH$), which was consistent with the relatively shorter retention time of M1 at 6.9min comparing to 12.1min for the parent compound. Another decursin metabolite M9 had a $[M+H]^+$ of 343, 14 Da greater than the parent compound, indicating an addition of $O-2H$. We interpreted M9 with a possible transformation of aldehyde of the methyl group ($CH_3 \rightarrow CHO$). DA metabolites N1 and N2 displayed a $[M+H]^+$ of 363, 34 Da greater than that of the parent, indicating that very likely $2O+2H$ had been added.

We next moved forward to identify the *in vivo* metabolites of decursin and DA in plasma samples of mice treated with a single dose of 240 mg/kg decursin/DA (mixture) by oral gavage. Although both decursin and DA generated different metabolic profiles in HLM vs. MLM incubation extracts, the differences were mostly related to the relative amount of each individual metabolite, not the types of metabolites (Supplemental Fig. 1A & B). Therefore,

here we tried to relate the *in vitro* metabolites from HLM incubations to *in vivo* metabolites from mouse plasma. We found that in mouse plasma, besides the most abundant metabolite DOH, there were many other metabolites present and the parent form of decursin and DA as well [8]. By combining the information of retention times and MS/MS spectra, we were able to tell whether the metabolites identified from *in vitro* liver microsomal system were detectable or not in the plasma sample. The data were summarized in Table 1&2. We also identified multiple metabolites which were detected *in vivo* but not *in vitro* as shown in Table 3.

Discussion

Single dose PK studies in rodents with decursin or DA alone, decursin/DA mixture or AGN extract all showed that decursin and DA were extensively metabolized after oral or i.p. administration [3,8–10]. Hydrolyzed product, DOH, is the primary *in vivo* metabolite. Plasma chromatographic profiles also indicated that there were other metabolites present in plasma besides DOH. The structural isomers decursin and DA have been reported to have similar activities such as anti-cancer effect [4,13], neuro-protective activities [15] and CYP2A6 inactivation [16], primarily based on *in vitro* experiments. However, subtle structural difference could lead to different metabolic fate and different *in vivo* activities. Kim *et al* reported that bacterial enzyme was able to convert only decursin but not DA to DOH [17]. But it was unclear which enzyme was responsible for the hydrolysis of decursin to DOH in the bacteria and it was also unclear whether human enzymes behave the same as bacterial enzymes or not. Our previous PK studies in rodents indicated that DA was relatively more available than decursin *in vivo* in its original form [8,10]. To gain more knowledge on the metabolic fate of decursin and DA especially in humans, we evaluated the metabolic rate and profiles of decursin and DA using *in vitro* liver microsomal systems. In the absence of NADPH, *in vitro* liver microsomal incubations of decursin generated the hydrolytic metabolite DOH and DOH only, but incubations of DA did not. In the presence of NADPH, liver microsomal incubations of both decursin and DA produced a variety of metabolites including DOH and several other oxidative metabolites. This result suggested that formation of DOH from decursin was catalyzed by hepatic esterase(s) and the formation of DOH from DA was primarily mediated by p450. Structural difference between decursin and DA revealed that the methyl group on the 2' position of DA might produce steric effect which blocks the access of the esterase(s) resulting in resistance of DA to hepatic esterase hydrolysis. We previously reported that neither plasma nor intestine efficiently hydrolyzed decursin or DA to DOH [10]. We also found that decursin and DA were quite stable in the cell culture system. In a cellular uptake experiment using human prostate cancer cell lines, PC3 and LNCaP, we found that both of these two cell lines were able to take in decursin and DA in their original forms and no DOH was detected either inside the cells or in the culture media [18]. Therefore, future study should focus on hepatic esterases and p450s to evaluate the enzymes and mechanisms involved in ester cleavage of decursin and DA. HLM incubations also produced several other oxidative metabolites for both decursin and DA. Majority of the metabolites occurred on the butenyl side chain. Many of these metabolites were detected in plasma samples from a single dose PK study as shown in table 1 & 2. It would be interesting to evaluate whether these metabolites exhibit anti-cancer or other

biological activities seen in the parent compound decursin and DA or not. Considering the different metabolic rate and profiles between decursin and DA, in the future, it will be important to evaluate which compounds, decursin or DA, have better potential for each given indications such as cancer and Alzheimer's disease.

Another interesting finding is that both decursin and DA were metabolized much slower by HLM than by MLM and RLM. In general, rodents have a higher metabolic rate than humans, presumably due to greater hepatic P450 content (1.5–3 folds, rat/human) [19]. However, our comparison was based on equal amount of microsomes or S9 protein, so the bioavailability of decursin and/or DA might be even better in human. Therefore there is a need to perform an exploratory study in human subjects to provide important information on pharmacokinetics of decursin and DA. If their pharmacokinetic behaviors are similar to rodents, then mechanism studies should focus on DOH or other metabolites and their molecular targets rather than on the parent compounds, which might only serve as pro-drugs. If decursin and/or DA were more available in their parent form in humans, then many of the reported *in vitro* mechanistic studies would have relevance to human application. In this case, novel pharmacological/genetic approaches intend to decrease the conversion of decursin and/or DA to DOH would be useful to assess efficacy and toxicity to support Investigational New Drug (IND) application and future clinical trials in humans.

Materials and methods

Purification of decursin and decursinol angelate

AGN extract and decursin/DA were prepared as described before [8]. The decursin/DA mixture was further separated by HPLC [10], separate fractions of decursin and DA from multiple HPLC runs were collected, pooled and dried under reduced pressure and the resultant residues were dissolved in methanol. The purity of the above compounds was verified by HPLC to be higher than 99%.

Reagents and Chemicals

Decursinol was prepared by hydrolysis of decursin/DA mixture [8]. Liver S9 fractions and microsomes (20mg/mL) of human (pooled from 20 donors), male Sprague-Dawley rats, male B6C3F1 and female CD-1 mice were purchased from BD Biosciences. All S9 and microsomes were free of phenylmethylsulfonyl fluoride and other protease/esterase inhibitors as affirmed by the vendor. NADPH Regenerating Systems (solution A and B) were also from BD Biosciences. Ethyl acetate, methanol, ethanol and acetonitrile were from Fisher Scientific. All reagents used were of HPLC grade or mass spectrometry grade.

In vitro metabolism of decursin/DA and sample preparation

Decursin or DA (22.8 μ M) was incubated with HLM, RLM or MLM at 37 °C in the absence or presence of NADPH generating system (1mM) in potassium phosphate buffer (100mM, pH 7.4). The final concentrations of microsomes and methanol were 1mg/mL (protein) and 0.5% (v/v), respectively. At indicated time points, the reactions were terminated by adding 800 μ L of ice-cold ethyl acetate. The samples were then extracted twice with totally 1.3mL of ethyl acetate. Combined ethyl acetate fractions were dried under reduced pressure at

ambient temperature. The resultant pellet was reconstituted in 50 μ L of methanol, vortexed for 2 min and then centrifuged at 16000 g for 10 min. The supernatant was analyzed by HPLC-UV and LC-MS/MS [10].

Mouse plasma and sample preparation

Mouse plasma samples were collected from a previous study in which male C57BL6 mice were given a single dose of 240 mg/kg decursin/DA (mixture) by oral gavage [8]. The decursin/DA mixture contains 60% decursin and 40% DA as shown by HPLC. The animal protocol was approved by the University of Minnesota IACUC (Institutional Animal Care and Use Committee). IACUC number for this study was 0812A55141 and approved in February 2009. Plasma samples were prepared and extracted as described before [8].

HPLC-UV and UHPLC-MS/MS analysis

HPLC-UV analysis was performed on an Agilent 1260 HPLC system. The detection wavelength used was 330 nm [10]. The chromatographic separation was carried on a Phenomenex column Kinetex C18 (100mm $\times$ 3mm, particle size 2.6 μ m) with water and acetonitrile as solvent. The initial mobile phase was set as 10% acetonitrile, and slowly gradient to 62% acetonitrile in 25min. After that, a fast gradient to 90% acetonitrile in 2min, and kept at 90% for 5min. The column oven was set as 40°C and the flow rate was 0.4mL/min. Metabolite identification was carried out on a hybrid triple quadrupole linear ion trap mass spectrometer QTRAP5500 (AB Sciex, Foster City, CA) combined with a Nexera UHPLC system (Shimadzu) [10]. The QTRAP5500 mass spectrometer was equipped with a TurboIonSpray interface operating in the positive ESI mode. Analyst 1.5.2 and LightSight 2.2.1 software was used for data acquisition and data analysis. The same HPLC conditions as in the HPLC-UV method were applied to LC-MS/MS analysis.

Two approaches were adopted to detect the metabolites of decursin and DA. One is precursor ion/neutral loss (PI/NL) scan and the other is predictive MRM (pMRM) scan method. In these two methods, the PI/NL scans or MRM scans were used as survey scans and enhanced product ion scan (EPI) was used to obtain MS/MS spectra for structural identification. Instrument parameters were optimized by directly infusing parent compound decursin in acetonitrile/water (50:50). Parameters for decursin were set as declustering potential (DP) 100, EP 10, Collision energy 30, CXP 12, curtain gas 30, Ion Spray voltage 5200 V, source temperature 575°C, gas1 50psi, gas2 60psi. These parameters were then applied to generate the method for metabolite identification. We selected the most abundant product ion of decursin m/z 229 Da, and with the help of LightSight software we generated the PI/NL and pMRM methods for screening of Phase I metabolites of decursin and DA. DOH is a known hydrolyzed product of decursin and DA, however, it was not on the pMRM detection list generated by LightSight software due to the nature on how this metabolite was formed. We added the MRM transition for detection of DOH to the pMRM method to have this known metabolite included.

We injected both solvent control and decursin or DA HLM incubation extracts into the LC-MS/MS and only the compounds present in the sample but absent in the control (solvent) were regarded as possible metabolites.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AGN	<i>Angelica gigas</i> Nakai
DA	decursinol angelate
DOH	decursinol
HLM	human liver microsomes
RLM	rat liver microsomes
MLM	mouse liver microsomes

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Decursin **Decursinol Angelate (DA)** **Decursinol (DOH)**

Fig. 1.
Structures of decursin, decursinol angelate (DA) and decursinol (DOH).

Fig. 2.

The HPLC-UV analysis profiles of MLM incubation extracts with decursin (A) or DA (B) in the presence or absence of NADPH generating system. (A). Decursin; Top: with NADPH and 0 min incubation time; middle: without NADPH and 6.5 min; bottom: with NADPH and 6.5min. (B). MLM incubation extracts of DA. Top: without NADPH and 0 min incubation time; middle: without NADPH and 10 min incubation time; bottom: with NADPH and 10 min incubation time.

Fig. 3. Metabolic rate of decursin (D) and decursinol angelate (DA) in MLM and HLM incubations in the presence of NADPH generating system. HU S9: human S9 fraction; Mouse S9: mouse S9 fraction.

Fig. 4.
 (A) MS/MS spectrum and MS3 spectrum (m/z 329→247) of protonated decursin; (B)
 MS/MS spectrum and MS3 spectrum (m/z 247→229) of protonated DOH; (C)
 Fragmentation of protonated decursin.

Fig. 5. Representative MS/MS spectra of Group A and Group B metabolites. MS/MS spectra of decursin metabolites M6, M7 and M8 with molecular ion $[M+H]^+$ at 345. (A) MS/MS spectrum of metabolites M6 and M7; (B) MS/MS spectrum of metabolite M8.

Table 1.

Metabolites identified from HLM incubation of decursin

Decursin metabolites	nominal mass shift (Da)	Metabolic Reaction	[M+H] ⁺	RT (min)	MS/MS Fragment ions	One dose mouse PK plasma ^I
M1	+30	carboxylation of methyl	359	6.9	247, 229, 214, 213, 211, 201, 187	Y
DOH	-82	ester hydrolysis	247	7.9	229, 214, 213, 211, 201, 175	Y
M2	+32	dihydroxylation	361	8.34	247, 229, 214, 213, 211, 201, 187	Y
M3	+32	dihydroxylation	361	8.41	247, 229, 214, 213, 211, 201, 187	N.D.
M4	+32	dihydroxylation	361	8.59	263, 245, 227, 217, 191, 187	Y
M5	+18	hydration	347	9.38	247, 229, 214, 213, 211, 201, 187	Y
M6	+16	hydroxylation/epoxidation	345	9.63	247, 229, 214, 213, 211, 201, 187	Y
M7	+16	hydroxylation/epoxidation	345	10.5	247, 229, 214, 213, 211, 201, 187	Y
M8	+16	hydroxylation/epoxidation	345	10.88	263, 245, 227, 217, 203, 191, 189	Y
M9	+14	methyl oxidation to aldehyde	343	10.94	247, 229, 213, 211, 187	Y
decursin		parent	329	12.08	247, 229, 214, 213, 211, 201	Y

RT: retention time; Y: detected; N.D.: not detected.

Note:

^IOne dose PK studies were performed in mice given 240mg/kg decursin/DA mixture by oral gavage.

Table 2.

Metabolites identified from HLM incubation of decursinol angelate (DA)

DA metabolites	nominal mass shift (Da)	metabolic reaction	[M+H] ⁺	RT (min)	MS/MS Fragment ions	One dose mouse PK plasma ^I
DOH	-82	ester hydrolysis	247	7.91	229, 214, 213, 211, 201, 175	Y
N1	+34	epoxidation and hydration	363	8.28	247, 229, 214, 213, 211, 201, 187	Y
N2	+34	epoxidation and hydration	363	8.42	247, 229, 214, 213, 211, 201, 187	Y
N3	+32	dihydroxylation	361	8.66	263, 245, 229, 217, 203, 191, 189, 187	N.D.
N4	+32	dihydroxylation	361	8.83	247, 229, 214, 213, 211, 187	N.D.
N5	+32	dihydroxylation	361	9.31	247, 229, 214, 213, 211, 201, 187	N.D.
N6	+16	hydroxylation/epoxidation	345	9.48	247, 229, 214, 213, 211, 201, 187	Y
N7	+18	hydration	347	9.64	247, 229, 214, 213, 211, 201, 187	N.D.
N8	+16	hydroxylation/epoxidation	345	10.53	247, 229, 214, 213, 211, 201, 187	Y
N9	+16	hydroxylation/epoxidation	345	10.97	263, 245, 227, 217, 205, 191	N.D.
DA		parent	329	12.19	247, 229, 214, 213, 211, 201	Y

Y: detected; N.D.: not detected.

Note:

^I is as Table 1.

Table 3.

In Vivo metabolites of decursin/DA in mouse plasma

<i>In vivo</i> metabolites	nominal mass shift (Da)	Metabolic Reaction	[M+H] ⁺	RT (min)	MS/MS Fragment ions
V1 [*]	+30	carboxylation of methyl	359	6.47	341, 229, 214, 213, 211, 201, 187
V2	+48	Tri-oxidation	377	7.00	359, 341, 295, 245, 217, 203, 187
V3	+50	demethylation+oxidation (+4O)	379	7.35	361, 343, 321, 283, 261, 229, 187
V4	+50	demethylation+oxidation (+4O)	379	7.68	361, 343, 321, 261, 245, 203, 187
V5	+80	oxidation (+5O)	409	8.06	391, 345, 247, 229, 214, 201, 187

^{*} Metabolite V1 had the same [M+H]⁺ of 359 as metabolite M1 but with a different retention time.