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Article

Chemical Profile Analysis and Comparison of Two Versions of the Classic TCM Formula Danggui Buxue Tang by HPLC-DAD-ESI-IT-TOF-MS^n

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Abstract: Danggui Buxue Tang (DBT) is a Traditional Chinese Medicine (TCM) formula primarily used to treat symptoms associated with menopause in women. Usually, DBT is composed of one portion of Radix Angelicae Sinensis (RAS) and five portions of Radix Astragali (RA). Clinically, Radix Hedysari (RH) is sometimes used by TCM physicians to replace RA in DBT. In order to verity whether the chemical constituents of the DBT1 (RA:RAS = 5:1, w/w) and DBT2 (RH:RAS = 5:1, w/w) share similarities the chemical profiles of the two DBTs crude extracts and urine samples were analyzed and compared with the aid of HPLC-DAD-ESI-IT-TOF-MS^n, which determines the total ion chromatogram (TIC) and multi-stage mass spectra (MS^n). Then, the DBT1 and DBT2 were identified and compared on the basis of the TIC and the MS^n. In the first experiment (with crude extracts), 69 compounds (**C1**–**C69**) were identified from the DBT1; 46 compounds (**c1**–**c46**) were identified from the DBT2. In the second experiment(with urine samples), 44 compounds (**M1**–**M44**) were identified from the urine samples of rats that had been administered DBT1, and 34 compounds (**m1**–**m34**) were identified from the urine samples of rats that had been administered DBT2. Identification and comparison of the chemical compositions were carried out between the DBT1 and DBT2 of the crude extracts and urine samples respectively. Our results showed that the two crude extracts of the DBTs have quite

different chemical profiles. The reasons for their differences were that the special astragalosides in DBT1 and the isoflavonoid glycosides formed the malonic acid esters undergo single esterification and acetyl esters undergo acetylation in DBT1. In contrast, the urine from DBT1-treated rats strongly resembled that of DBT2-treated rats. These metabolites originate mainly from formononetin, calycosin and their related glycosides, and they were formed mainly by the metabolic process of reduction, deglycosylation, demethylation, hydrogenation and sulfation. The HPLC-DAD-ESI-IT-TOF-MS^n method was successfully applied for the rapid chemical profiles evaluation of two DBTs and their related urine samples.

Keywords: Radix Hedysari; Radix Astragali; Danggui Buxue Tang; HPLC-DAD-ESI-IT-TOF-MS^n; isoflavonoid; astragaloside

1. Introduction

Danggui Buxue Tang (DBT) is a Traditional Chinese Medicine (TCM) formula primarily used to treat symptoms associated with menopause in women. It is believed to invigorate 'Qi' (vital energy) and nourish the 'Blood' (body circulation) [1]. Nowadays, it is commonly used in China as an efficacious medicinal prescription and a healthy food supplement. Pharmacological studies have found that DBT promotes hematopoietic function [2,3], regulates blood lipid and anti-inflammatory activities in diabetic atherosclerosis [4,5], anti-fibrosis effects [6], prevents osteoporosis [7,8], and increases anti-oxidation activity as well as immune response [9]. According to its original formula, DBT comprises Radix Astragali (RA) and Radix Angelicae Sinensis (RAS) (5:1, w/w). More recently, Radix Hedysari (RH) has been used to replace RA. Thus, in current clinical applications, DBT is prescribed in two forms: RA:RAS (5:1) (called DBT1), and RH:RAS (5:1) (called DBT2) [1,10].

The plants RA and RH belong to the same botanical family but different genus, and have long been widely used as the same crude herb in DBT [1]. This is always a question of whether RH can replace RA in the DBT decoction. Chemically, RA-containing DBT showed higher amounts of calycosin-7-*O*-*β*-D-glucoside, ferulic acid, ononin, calycosin, astragaloside IV, astragaloside III, and *Z*-ligustilide. Only formononetin was higher in RH-containing DBT. In parallel, the estrogenic, osteogenic and erythropoetic effects of RA-containing DBT1 showed better activities than that of RA-containing DBT2 [1]. So far, the chemical differences between DBT1 and DBT2 has not been investigated. Therefore, we designed a systematic comparison of the chemical ingredients of DBT1 and DBT2.

Two experiments were designed, including thorough elucidation of the chemical profiles of DBT1 and DBT2 crude extracts and illumination of the metabolites of DBT1 and DBT2 after being administrated to rats. The chemical profiles of the two DBTs were compared by determining the total ion chromatogram(TIC) and the multistage mass spectra (MS^n) from HPLC-DAD-ESI-IT-TOF-MS^n. Subsequently, DBT1 and DBT2 were identified and compared on the basis of the TIC and the MS^n [11]. The results will be provide a solid evidence to understand the chemical profiles of the two different versions of DBT.

2. Results and Discussion

2.1. Optimization the Conditions of HPLC and Mass Spectrometry

In order to obtain desirable HPLC and mass spectrometry chromatograms, the procedures for preparation of the urine samples and crude extracted samples of the two DBTs were optimized in terms of the extraction solvents and extraction times. Methanol and acetonitrile were initially selected as the extraction solvents, but methanol is less poisonous and produced almost the same chromatograms as acetonitrile, so it was applied as the final extraction solvent. For comparison, different columns (Phenomenex RP C_{18}, Agilent RP C_{18}) were tested for sample separation, and Phenomenex RP C_{18} gave the best chromatographic resolution. The column was eluted with a gradient mobile phase that consisted of water-formic acid (100:0.1, v/v) (A), acetonitrile (B) and at a flow rate of 1.0000 mL/min, in addition, 0.1% (v/v) formic acid was added to improve the mass spectrometry ionization efficiency and enable symmetric peak shapes [12]. Both the positive ion (PI) and negative ion(NI) modes were tested for the experiment. Since MS and MS^n fragmentations gave more information about the isoflavones in PI mode but about saponins in NI mode, the analysis was simultaneously conducted in both PI and NI mode.

2.2. The Identification and Analysis of 19 Reference Compounds

Nineteen reference compounds which might represent the major structural types of the DBTs were analyzed. The characteristic fragment ions of 19 references are very useful for determining the structural skeleton and the substitution patterns of those related compounds in two DBTs. Their high resolution mass spectra (HRMS) data are summarized (see Table 1 and Supplementary: pages S2–S8). The base peak chromatograms (BPCs) detected in NI and PI mode were recorded (see Figure 1a). Furthermore, the structure of 19 reference compounds were shown (Figure 1b) [11,13].

According to our preliminary research, there three main types of isoflavonoids are found in Radix Astragali which were named as **a**: isoflavone (T1, T2, T5, T6, and T8), **b**: isoflavan (T4, T7, and T10) and **c**: pterocarpan (T3, and T9) [11,13–15]. To facilitate the structural identification of the isoflavonoids in the DBTs, the fragmentation behaviors of the three types of isoflavonoids were analyzed, which might represent the major structural types. We first studied the MS^n fragmentation behaviors in PI and NI mode, and found that the fragmentation behaviors in PI mode could give more information about the structure than in NI modes. Then we elucidated the structure of the three types of isoflavonoids mostly from the PI mass spectra.

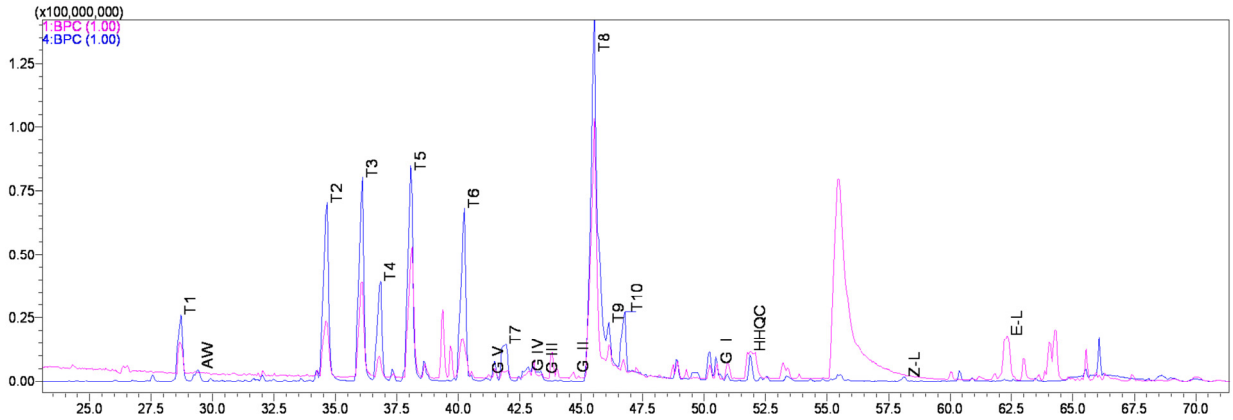
Isoflavone had the characteristic fragment ions $^{5}B^{+}$-2H, $^{0,3}B^{+}$-2H, $^{5}A^{+}$-2H, $^{1,3}A^{+}$-2H, $^{3,4}A^{+}$-2H, *etc.*, isoflavan had the characteristic fragment ions $^{5}B^{+}$-2H, $^{5}A^{+}$-2H, $^{1,3}A^{+}$-2H, *etc.*, and pterocarpan had the characteristic fragment ions $^{6,7}B^{+}$-2H, $^{1,4}B^{+}$-2H, $^{3,4}A^{+}$-2H, $^{5,6}A^{+}$-2H, *etc.*, based on MS^2 and MS^3 spectra by HPLC-DAD-ESI-IT-TOF-MS^n (see Figure 2).

For example, we identified the characteristic malonate-glucose-, acetyl-glucose- and glucose-binding ingredients with a neutral loss of 248 Da, 204 Da, and 162 Da, otherwise, glucuronide metabolites with a neutral loss of (−176 Da) and sulfated metabolites with a neutral loss of (−80 Da) from the molecular ion peaka in the MS^2 spectra [13,16,17].

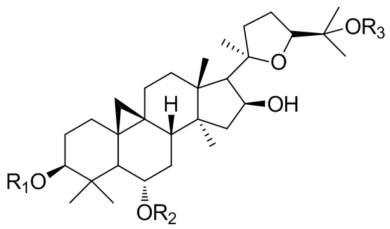
Table 1. The fragment ions of 19 kinds of reference compounds by HPLC-DAD-ESI-IT-TOF-MS^n.

NO.	T_R (min)	$[M+H]^+$	$[M-H]^-$	Predicted Formula	Fragment Ions Da	Error (ppm)	The Name of the Reference Compounds
1(T1)	28.595	447.1290		$C_{22}H_{22}O_{10}$	447,285,270,225	0.89	Calycosin-7-*O*-*β*-D-glucopyranoside
2(AW)	29.162		193.0501	$C_{10}H_{10}O_4$	193,178,134	−2.59	Ferulic acid
3(T2)	34.545		431.1326	$C_{22}H_{22}O_9$	431,269,253,237,213,197,163,134,107	−2.55	Ononin
4(T3)	36.038	485.1400	$[M+Na]^+$	$C_{23}H_{26}O_{10}$	485,463,323,301	−3.71	Astrapterocarpan-7-*O*-*β*-D-glucopyranoside
5(T4)	36.785	487.1574	$[M+Na]^+$	$C_{23}H_{28}O_{10}$	487,303,167	−0.21	Astraisoflavan-7-*O*-*β*-D-glucopyranoside
6(T5)	38.055	285.0754		$C_{16}H_{12}O_5$	285,270,253,225,197,137	−1.40	Calycosin
7(T6)	40.123	473.1432		$C_{24}H_{24}O_{10}$	473,269	−2.11	6"-*O*-acetyl-ononin
8(T7)	41.942	529.1691	$[M+Na]^+$	$C_{25}H_{30}O_{11}$	529,507,303	2.08	6"-*O*-acetyl-astraisoflavan-7-*O*-*β*-D-glucopyranoside
9(T8)	45.410	269.0795		$C_{16}H_{12}O_4$	269,270,237	−4.83	Formononetin
10(T9)	46.062	301.1076		$C_{17}H_{16}O_5$	301,271,251,167,151,134	1.66	(6aR,11aR)-3-hydroxy-9,10-dimethoxypterocarpan; Astrapterocarpan
11(T10)	46.655	303.1213		$C_{17}H_{18}O_5$	303,181,167,149,123	−4.62	(3R)-7,2'-dihydroxy-3',4'-dimethoxyisoflavan; Astraisoflavan
12(GⅤ)	41.110	$[M+HCOO]^-$	991.5156	$C_{47}H_{78}O_{19}$	991,783,397	3.73	Astragaloside Ⅴ
13(GⅣ)	42.775	$[M+HCOO]^-$	829.4615	$C_{41}H_{68}O_{14}$	829,783,621,489,383	2.89	Astragaloside Ⅳ
14(GⅢ)	43.410	$[M+HCOO]^-$	829.4585	$C_{41}H_{68}O_{14}$	829,783,651,489	−0.72	Astragaloside Ⅲ
15(GⅡ)	44.628	$[M+HCOO]^-$	871.4693	$C_{43}H_{70}O_{15}$	871,765,717	−0.46	Astragaloside Ⅱ
16(GⅠ)	50.525	$[M+HCOO]^-$	913.4802	$C_{45}H_{72}O_{16}$	913,867,807	0.00	Astragaloside Ⅰ
17(HHQC)	51.855	513.3550	$[M+Na]^+$	$C_{30}H_{50}O_5$	513,515,405,229	0.00	Cycloastragenol(HHQC)
18(ZL)	55.477	191.1056		$C_{12}H_{14}O_2$	191,173,117	−5.76	*Z*-ligustilide
19(EL)	58.102	191.1066		$C_{12}H_{14}O_2$	191,173	−0.52	*E*-ligustilide

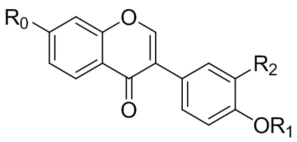
Figure 1. (**a**) LC-MS chromatogram of 19 reference compounds in PI (**1BPC**) and NI (**4BPC**) mode. (**b**) The chemical structures of 19 reference compounds.



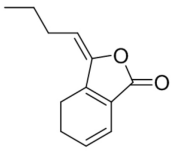
(**a**)



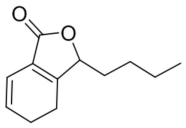
	R_1	R_2	R_3
G I	2',3'-*O*-Ac-*β*-D-xyl	*β*-D-glc	H
G II	2'-*O*-Ac-*β*-D-xyl	*β*-D-glc	H
G III	*β*-D-glc(1→2)-*β*-D-xyl	H	H
G IV	*β*-D-xyl	*β*-D-glc	H
G V	*β*-D-glc(1→2)-*β*-D-xyl	H	*β*-D-glc
HHQC	H	H	H



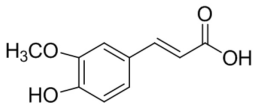
	R_0	R_1	R_2
T8	H	H	H
T2	*β*-D-glc	H	H
T5	H	H	OH
T1	*β*-D-glc	H	OH
T6	6''-*O*-Ac-*β*-D-glc	H	H



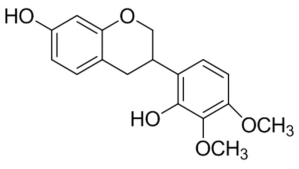
EL



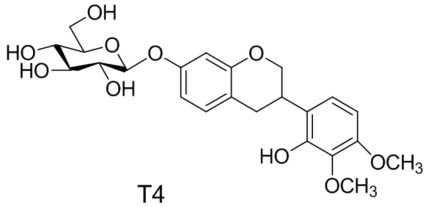
ZL



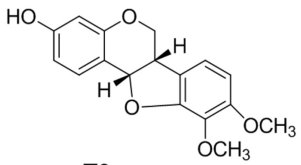
AW



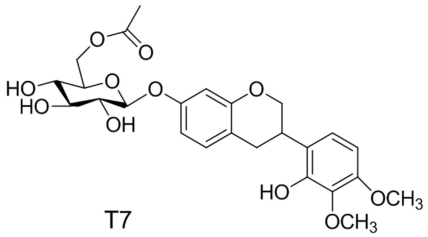
T10



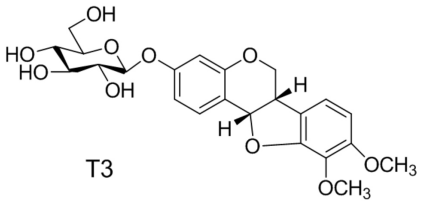
T4



T9



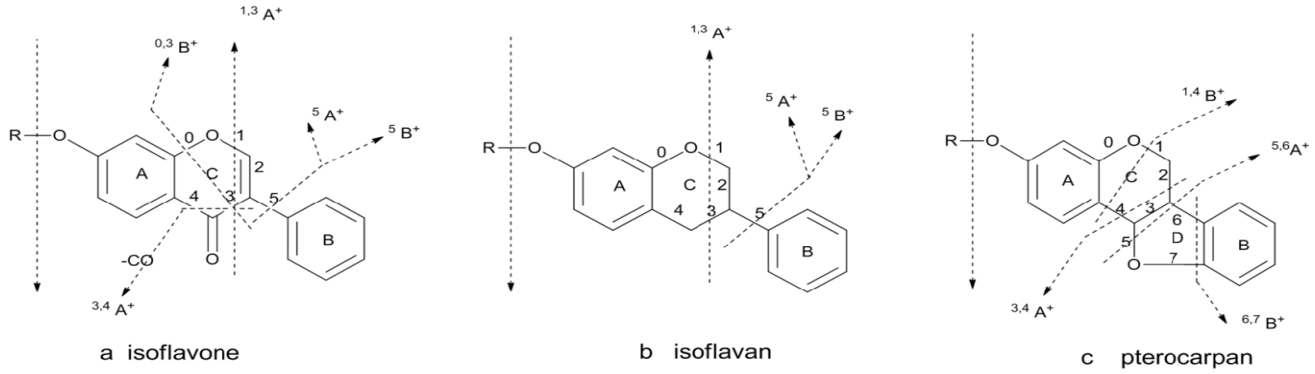
T7



T3

(**b**)

Figure 2. The bond cleavage pathways of the three types of isoflavonoids in RA.



2.3. Profiling and Identifying Chemical Compounds of the Two Crude Extracts (DBT1, and DBT2) by HPLC-DAD-ESI-IT-TOF-MS^n

2.3.1. Identification of the Chemical Profiles of DBT1 by HPLC-DAD-ESI-IT-TOF-MS^n

The HRMS data of these identified compounds are summarized (see Table 2 and Supplementary: pages S9–S35). The BPCs detected in PI (**1BPC**) and NI (**4BPC**) mode were also recorded (Figure 3a) [17–19].

By comparing the fragment ions and retention times and based on the high resolution mass spectra software predicted formulas with the reference compounds from the MS and MS^n, the compounds **C23**, **C33**, **C36**, **C37**, **C38**, **C44**, **C46**, **C56**, **C58**, **C59**, **C49**, **C53**, **C60**, **C64**, and **C68** were identified as the reference compounds [11,13].

C27 has a RT at 30.773 min, $[M+HCOO]^-$ at *m/z* 671.2155 in MS (predicted formula: $C_{29}H_{38}O_{15}$: ppm error: −5.66), and characteristic fragment ions at *m/z* 625.2079 (−46 Da) $[M-H]^-$, *m/z* 463.1589 [M-162-H], and *m/z* 301.1108 [M-162-162-H] in MS^2. The neutral loss is mass 46 Da (CH_2O_2; identified as HCOOH), 162 Da*2 ($C_6H_{10}O_5$; identified as glucopyranoside), and the fragment ion *m/z* 301.1108 predicted as $C_{17}H_{18}O_5$. Then **C27** was identified as astraisoflavan-di-7-*O*-*β*-D-glucoside or its isomer. The $[M+H]^+$ or $[M-H]^-$ of **C24**, **C29**, **C30**, and **C41** shows the same neutral loss of −162 Da ($C_6H_{10}O_5$; glucoside) in them MS^2, so both of them were identified as the glycosides [17–19]. The characteristic fragment ions of **C31**, **C35**, **C43**, **C42**, and **C51** have a neutral loss of −248 Da ($C_9H_{12}O_8$; identified as the 6"-*O*-malonate-glucoside) in them MS^2, so both of them were identified as glycosides of 6"-*O*-malonate-glucoside [13].The HRM software predicted $[M+H]^+$ or $[M-H]^-$ of **C32**, **C40**, **C34**, **C47,** and **C18,** whose formulas have the same characteristic fragment loss (−204 Da; $C_8H_{12}O_6$) which was identified as 6"-*O*-acetylglucosides. In addition, **C32**, **C40**, **C34**, **C47**, and **C18** were identified as glycosides of 6"-*O*-acetylglucoside [16].

For the predicted formulas of $[M+H]^+$ or $[M-H]^-$, we tentatively identified **C39**, **C50**, **C62**, **C30**, and **C41** as the isoflavonoid-related constituents by referring to the literature [18,19], and **C45**, **C55**, **C48**, **C52**, **C54**, **C61**, and **C65** were tentatively identified as being related to saponins [13,17–19].

Using the $[M+H]^+$, $[M-H]^-$ or $[M+Na]^+$ data of **C7**, **C8**, **C10**, **C12**, **C13**, **C14**, **C21**, **C25**, **C26**, and **C28**, we predicted their formulas, which indicates that they are the ingredients of the samples. However, at this point, their exact structures could not be identified.

Table 2. The identified proposed compounds of the crude extract samples from Danggui Buxue Tang 1 and Danggui Buxue Tang 2 by HPLC-DAD-ESI-IT-TOF-MS^n.

NO.	T_R (min)	$[M+H]^+$	$[M-H]^-$	Predicted Formula	Fragment Ions Da	Error ppm	Identification	DBT1	DBT2
1	2.395		173.1044	$C_6H_{14}N_4O_2$		0.00	Arginine	**C1**	**c1**
2	2.692		195.0502	$C_6H_{12}O_7$		−4.10	Gluconic acid	**C2**	**c2**
3	2.695		341.1074	$C_{12}H_{22}O_{11}$		−4.40	D(+)sucrose	**C3**	**c3**
4	3.643		191.0187	$C_6H_8O_7$	191,173	−5.24	Citric acid	**C4**	**c4**
5	4.477		328.0427	$C_{11}H_{11}N_3O_9$		1.22	--	**C5**	**c5**
6	6.480	346.0529		$C_{11}H_{11}N_3O_{10}$		3.47	--	**C6**	**c6**
7	9.063		433.1364	$C_{18}H_{26}O_{12}$	301,191	4.91	--	**C7**	**--**
8	9.120	443.1146	$[M+Na]^+$	$C_{17}H_{24}O_{12}$		−3.16	----	**--**	**c7**
9	9.345	267.1369		$C_{18}H_{18}O_2$	267,225	−4.12	Magnolol	**--**	**c8**
10	9.398		433.1364	$C_{18}H_{26}O_{12}$	433,351,301,223	2.77	--	**C8**	**--**
11	10.773	188.0688		$C_9H_{11}NO_2$	146	3.19	L-phenylalanine	**C9**	**c9**
12	11.125		431.1192	$C_{18}H_{24}O_{12}$	431,299	−0.70	--	**C10**	**--**
13	12.653	384.1127		$C_{13}H_{21}NO_{12}$		−2.60	--	**C11**	**c10**
14	13.840		461.1283	$C_{19}H_{26}O_{13}$	461,167	−3.90	--	**--**	**c11**
15	13.847	485.1224	$[M+Na]^+$	$C_{26}H_{22}O_8$	485,317	3.50	--	**C12**	**--**
16	17.807		205.0701	$C_8H_{14}O_6$		−8.29	--	**C13**	**--**
17	21.147		315.2004	$C_{20}H_{28}O_3$		12.06	--	**C14**	**--**
18	21.207		433.1129	$C_{21}H_{22}O_{10}$	433,285	−2.54	--	**--**	**c12**
19	21.322		433.1121	$C_{21}H_{22}O_{10}$	433,285,241	−4.39	--	**--**	**c13**
20	22.020		417.1017	$C_{17}H_{22}O_{12}$	417,285,152	-5.03	--	**C15**	**c14**
21	23.080	389.2325		$C_{23}H_{32}O_5$		0.51	--	**C16**	**c16**
22	23.018		401.1445	$C_{18}H_{26}O_{10}$	401,269,161	−1.99	--	**C17**	**c15**
23	23.590		503.1175	$C_{24}H_{24}O_{12}$	503,443,299	−3.98	6"-*O*-acetyl-pratensein-7-*O*-*β*-D-glucoside	**C18**	**--**
24	24.347		239.0568	$C_{11}H_{12}O_6$		2.93	--	**C19**	**--**

Table 2. *Cont.*

NO.	T_R (min)	$[M+H]^+$	$[M-H]^-$	Predicted Formula	Fragment Ions Da	Error ppm	Identification	DBT1	DBT2
25	25.662	331.2296		$C_{21}H_{30}O_3$	331,299	8.45	--	**C20**	**--**
26	26.743	470.1534		$C_{18}H_{23}N_5O_{10}$		3.40	--	**C21**	**--**
27	26.967	289.1747		$C_{13}H_{24}N_2O_5$	289,272,152	−3.80	--	**C22**	**c17**
28	30.653		479.1492	$C_{30}H_{24}O_6$	479,317	−1.67	--	**C26**	**--**
29	30.773	$[M+HCOO]^-$	671.2155	$C_{29}H_{38}O_{15}$	671,625,463,301	−5.66	Astraisoflavan-di-7-*O*-*β*-D-glucoside	**C27**	**--**
30	31.005		579.2062	$C_{23}H_{36}N_2O_{15}$	579,417,387	3.28	--	**C28**	**--**
31	31.348		445.1123	$C_{22}H_{22}O_{10}$	445,283	−3.82	Glycetein-4'-*O*-*β*-D-glucoside	**C29**	**--**
32	32.078	463.1203		$C_{22}H_{22}O_{11}$	463,301	−6.91	Kaempferide-7-*O*-*β*-D-glucoside	**C30**	**--**
33	32.662	533.1267		$C_{25}H_{24}O_{13}$	533,285	−4.31	6"-*O*-malonate-calycosin-7-*O*-*β*-D-glucoside	**C31**	**--**
34	34.207	489.1398		$C_{24}H_{24}O_{11}$	489,285	1.43	6"-*O*-acetyl-calycosin-7-*O*-*β*-D-glucoside	**C32**	**--**
35	34.517	431.1322		$C_{22}H_{22}O_9$	431,269,237,118	−3.48	Ononin	**C33**	**c18**
36	35.348		489.1340	$C_{24}H_{26}O_{11}$	489,285,271,159	−12.68	6"-*O*-acetyl-isosakuranetin-7-*O*-*β*-D-glucoside	**C34**	**--**
37	35.580	549.1174		$C_{25}H_{24}O_{14}$	549,301	−11.84	6"-*O*-malonate-kaempferide-7-*O*-*β*-D-glucoside	**C35**	**--**
38	36.027	463.1615		$C_{23}H_{26}O_{10}$	485(+Na^+),463,301	3.45	Astrapterocarpan-7-*O*-*β*-D-glucopyranoside	**C36**	**--**
39	36.773		463.1577	$C_{23}H_{28}O_{10}$	463,301,271	−7.13	Astraisoflavan-7-*O*-*β*-D-glucopyranoside	**C37**	**--**
40	37.480		255.0657	$C_{15}H_{12}O_4$	255,135	−2.35	Isoliquiritigenin	**--**	**c19**
41	38.035	285.0744		$C_{16}H_{12}O_5$	285,270,225,137	−4.91	Calycosin	**C38**	**c20**
42	39.917		269.0807	$C_{16}H_{14}O_4$	269,253,227	−4.46	Isomer of alpinetin	**--**	**c21**
43	39.977		255.0657	$C_{15}H_{12}O_4$	255,237	−2.35	Liquiritigenin	**--**	**c22**
44	38.653	315.0844		$C_{17}H_{14}O_6$		−6.03	4-methoxy-maackiain or the isomer	**C39**	**--**
45	38.653	473.1445		$C_{24}H_{24}O_{10}$	473,269	0.63	The isomer of 6"-*O*-acetyl-ononin	**C40**	**--**
46	38.773	447.1264		$C_{22}H_{22}O_{10}$	447,285	−4.92	Glycetein-7-*O*-*β*-D-glucoside	**C41**	**--**
47	38.997	549.1545		$C_{26}H_{28}O_{13}$	549,301	−10.56	6"-*O*-malonate-astrapterocarpan-glucoside	**C42**	**--**
48	39.452	517.1301		$C_{25}H_{24}O_{12}$	517,269	−7.73	6"-*O*-malonate-ononin	**C43**	**--**
49	39.683		505.1699	$C_{25}H_{30}O_{11}$	505,301	−3.17	6"-*O*-acetyl-astraisoflavan-7-*O*-*β*-D-glucoside	**C44**	**--**

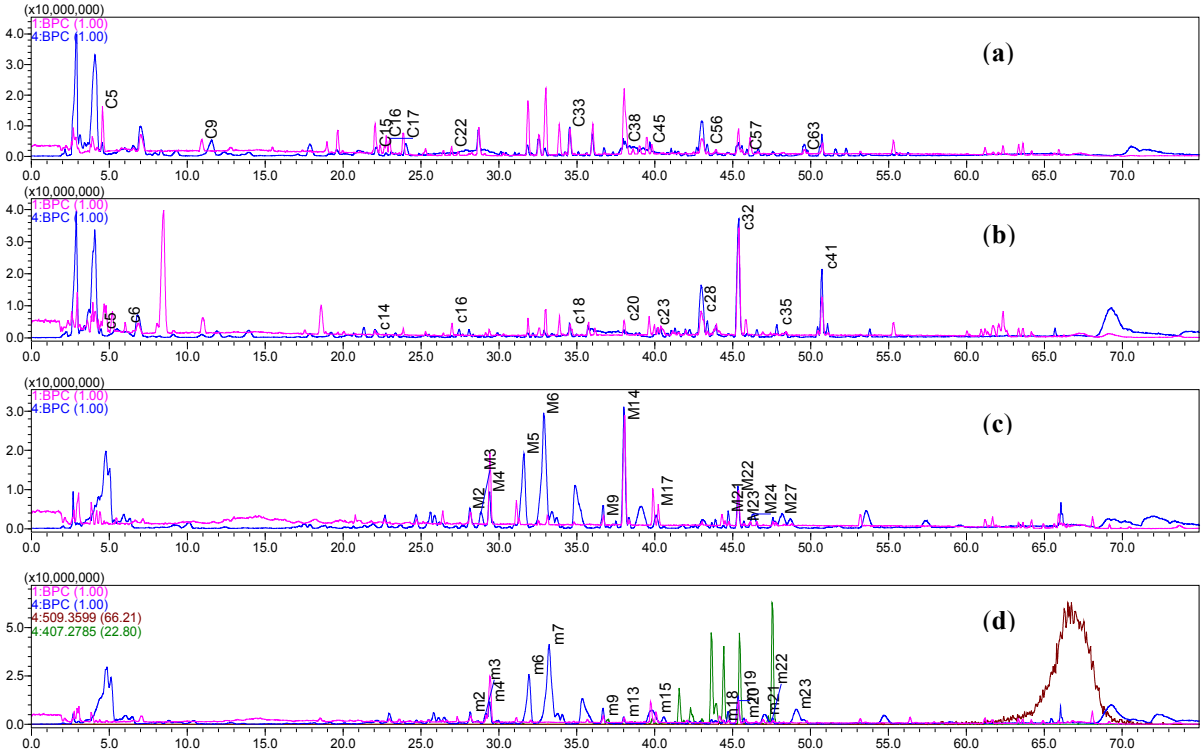
Table 2. *Cont.*

NO.	T_R (min)	$[M+H]^+$	$[M-H]^-$	Predicted Formula	Fragment Ions Da	Error ppm	Identification	DBT1	DBT2
50	39.745		957.5030	$C_{48}H_{78}O_{19}$	957,541,453	−3.66	Soyasaponin Ba	**C45**	**c23**
51	40.138	473.1424		$C_{24}H_{24}O_{10}$	473,269	−3.80	6"-*O*-acetyl-ononin	**C46**	**--**
52	40.903		927.4915	$C_{47}H_{76}O_{18}$		−4.74	Akebia saponin D	**--**	**c24**
53	40.430		503.1154	$C_{24}H_{24}O_{12}$	503,299	−8.15	6"-*O*-acetyl-kaempferide-7-*O*-*β*-D-glucoside	**C47**	**--**
54	40.825		785.4629	$C_{41}H_{70}O_{14}$		−8.15	Cyclocanthoside E	**C48**	**--**
55	41.108		991.5086	$C_{48}H_{80}O_{21}$		−3.33	Astragaloside V	**C49**	**--**
56	41.273		285.0751	$C_{16}H_{14}O_5$	285,194,109	−5.96	Isomer of isosakuranetin	**--**	**c25**
57	41.772		269.0456	$C_{15}H_{10}O_5$	269,237	0.37	Genistein	**--**	**c26**
58	41.778		315.0868	$C_{17}H_{16}O_6$	315,253	−1.90	Astragaluquinone or isomer	**C50**	**--**
59	41.950	533.1243		$C_{25}H_{24}O_{13}$	533,285	−8.82	6"-*O*-malonate-glycetein-7-*O*-*β*-D-glucoside	**C51**	**--**
60	42.062		867.4635	$C_{45}H_{72}O_{16}$		−13.03	Isoastragaloside I	**C52**	**--**
61	42.243		283.0602	$C_{16}H_{12}O_5$	283,268,224	−3.53	Glycetein	**--**	**c27**
62	42.612	$[M+HCOO]^-$	829.4572	$C_{41}H_{68}O_{14}$		−2.29	Astragaloside IV	**C53**	**--**
63	42.560		825.4532	$C_{43}H_{70}O_{15}$	871,825	−13.33	Isoastragaloside II	**C54**	**--**
64	42.965		329.2319	$C_{18}H_{34}O_5$		−4.25	--	**--**	**c28**
65	43.197		955.4857	$C_{48}H_{76}O_{19}$		−5.34	--	**--**	**c29**
66	44.690		287.0577	$C_{15}H_{12}O_6$		5.57	Dihydro-kaempferol	**--**	**c30**
67	44.982		255.0649	$C_{15}H_{12}O_4$	256,135	−5.49	Isomer of Liquiritigenin	**--**	**c31**
68	45.315	269.0791		$C_{16}H_{12}O_4$	269,254,237,118	−6.32	Formononetin	**C56**	**c32**
69	44.190		941.5081	$C_{48}H_{78}O_{18}$	941,525,437	−3.61	Soyasaponin Bb	**C55**	**c35**
70	45.745	299.0911		$C_{17}H_{14}O_5$	299,284,166		Pterocarpin	**--**	**c33**
71	45.935		329.2299	$C_{18}H_{34}O_5$		−10.33	--	**C57**	**--**
72	46.080		283.0599	$C_{16}H_{12}O_5$	283,255,240	−4.59	The isomer of glycetein	**--**	**c34**
73	46.158	301.1052		$C_{17}H_{16}O_5$		−6.31	Astraoptercarpan	**C58**	**--**

Table 2. *Cont.*

NO.	T_R (min)	$[M+H]^+$	$[M-H]^-$	Predicted Formula	Fragment ions Da	Error ppm	Identification	DBT1	DBT2
74	46.708	303.1181		$C_{17}H_{18}O_5$		−15.18	Astraisoflavan	**C59**	**--**
75	46.768	$[M+HCOO]^-$	871.4656	$C_{43}H_{70}O_{15}$	871,825,603	−4.70	Astragaloside II	**C60**	**--**
76	47.172	$[M+HCOO]^-$	911.4668	$C_{45}H_{70}O_{16}$	955,911	2.41	--	**C61**	**--**
77	47.772		299.0552	$C_{16}H_{12}O_6$		−3.01	Kaempferide or isomer	**--**	**c36**
78	47.943		909.4836	$C_{47}H_{74}O_{17}$		−1.87	Acetylastragaloside I	**--**	**c37**
79	48.347	$[M+HCOO]^-$	911.5011	$C_{46}H_{74}O_{15}$		0.11	Castaraleside H	**--**	**c38**
80	49.432		285.0423	$C_{15}H_{10}O_6$	285,163	6.31	Kaempferol or isomer	**C62**	**--**
81	50.065		939.4925	$C_{48}H_{76}O_{18}$		−3.62	--	**--**	**c39**
82	50.435	285.0748		$C_{16}H_{12}O_5$	285,253,152	−3.51	Isomer of calycosin	**--**	**c40**
83	50.667	335.2180	$[M+Na]^+$	$C_{18}H_{32}O_4$		−3.88	--	**C63**	**c41**
84	50.713	$[M+HCOO]^-$	913.4777	$C_{45}H_{72}O_{16}$		−2.74	Astragaloside I	**C64**	**--**
85	51.560	193.1212		$C_{12}H_{16}O_2$		−5.70	Senkyunolide A	**--**	**c42**
86	51.607	$[M+HCOO]^-$	953.4637	$C_{47}H_{72}O_{17}$	953,909	−12.06	--	**C65**	**--**
87	52.363	437.3374	$[M+Na]^+$	$C_{28}H_{46}O_2$		−3.66	--	**C66**	**--**
88	55.212	191.1043		$C_{12}H_{14}O_2$		−12.56	n-butyl-phthalide	**--**	**c43**
89	59.250	213.0876	$[M+Na]^+$	$C_{12}H_{14}O_2$	403,213	−4.69	*Z*-ligustilide	**C67**	**c44**
90	61.708	403.1867	$[2M+Na]^+$	$C_{12}H_{14}O_2$	403,381,191	−3.22	*E*-ligustilide	**C68**	**c45**
91	69.093		283.0257	$C_{15}H_8O_6$	283,203,147	3.18	--	**C69**	**c46**

Figure 3. (**a**) The BPC in NI and PI mode of the crude extracts of Danggui Buxue Tang 1 (RA:RAS = 5:1). (**b**) The BPC in NI and PI mode of the crude extracts of Danggui Buxue Tang2 (RH:RAS = 5:1). (**c**) The BPC in NI and PI mode of the urine samples of rats that had been administrated the Danggui Buxue Tang 1 (RA:RAS = 5:1) and (**d**) The BPC in NI and PI mode of the urine samples of rats had been administrated the Danggui Buxue Tang 2 (RH:RAS = 5:1).



2.3.2. Identification of the Chemical Profiles of DBT2 by HPLC-DAD-ESI-IT-TOF-MS^n

The HRMS data of these identified compounds are summarized (see Table 2 and Supplementary: pages S36–S51). The BPCs detected in NI and PI modes were recorded (see Figure 3b).

Using their fragment ions and retention times in MS data, **c18**, **c20**, **c32**, **c44**, and **c45** were identified as the reference compounds [11,13].

Based the MS data, **c12** shows RT at 21.207 min, $[M-H]^-$ at *m/z* 433.1129 in MS (predicted the formula: $C_{21}H_{22}O_{10}$: ppm error: −2.54), and characteristic fragment ions at *m/z* 285.0744 (−148 Da; $C_5H_8O_5$; identified as the ribonic acid) and predicted as $C_{16}H_{14}O$. Compound **c12** was identified as the isomer of isosakuranetin-ribonic acid. Moreover, **c13** shows a characteristic neutral loss at −148 Da ($C_5H_8O_5$) with the same as **c12** [16].

By the formulas predicted of $[M-H]^-$ or $[M+H]^+$ and referring to literature [13,17–19], **c23**, **c24**, **c29**, **c35**, **c38,** and **c39** were tentatively identified as saponin-related constituents, and **c7**, **c8**, **c11**, **c19**, **c21**, **c22**, **c25**, **c26**, **c27**, **c31**, **c33**, **c34**, **c36**, **c40**, and **c43** were tentatively identified (see in Table 2).

By using the HRMS data (RT, Predicted the formulas and characteristic fragment ions) compared with the BDT1 crude extract samples, **C1**, **c1**; **C2**, **c2**; **C3**, **c3**; **C4**, **c4**; and **C9**, **c9** were identified as the same constituents [17–19].

The groups of **C5**, **c5**; **C6**, **c6**; **C11**, **c10**; **C15**, **c14**; **C16**, **c16**; **C17**, **c15**; **C22**, **c17**; **C57**, **c28**; **C63**, **c41**; and **C69**, **c46** between DBT1 and DBT2 were tentatively identified as the same compounds with uncertain structures.

From the analysis based on the comparison of TIC and MS^n: 69 compounds (**C1**–**C69**) were identified from the crude extracts of DBT1, 46 compounds (**c1**–**c46**) were identified from the crude extracts of DBT2. The isoflavonoids glycosides had experienced acetylation (seven compounds, **C18**, **C32**, **C34**, **C40**, **C44**, **C46**, and **C47**), formed the malonate acid esters (five compounds, **C31**, **C35**, **C42**, **C43**, and **C51**) and with special astragalosides (six compounds, **C49**, **C52**, **C53**, **C54**, **C60**, and **C64**) in DBT1. Thus, the number of identified components in DBT1 was significantly more than in DBT2 (the chemical structural diversity of isoflavonoids which were detected in DBT1 more than in DBT2 are shown in Figure 4). Among these, the 24 common chemical constituents accounted for approximately 27% to the total 91 identified compounds. However, and the proportion of the total isoflavonoids and saponins to the total identified ingredients accounted for nearly 62% (see Table 3).

Figure 4. The chemical structures of the main proposed different isoflavonoids in Danggui Buxue Tang 1 more than Danggui Buxue Tang 2.

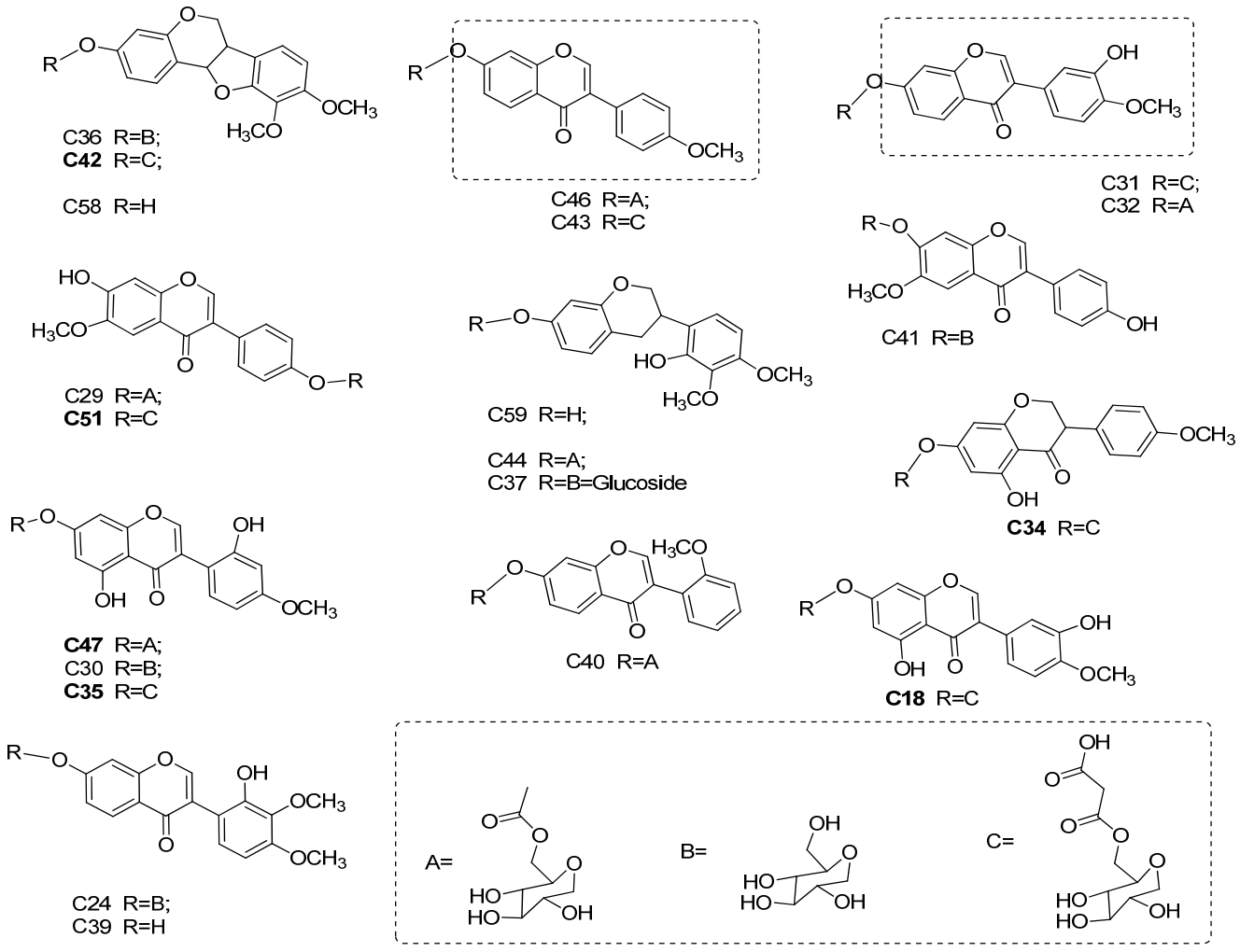


Table 3. The number comparison of the identified compounds between Danggui Buxue Tang 1 and Danggui Buxue Tang 2.

N.	ID.	S-ID.	T.ID.	S%	H + S	S%
DBT1	69	24	115−24 = 91	26.37	56	61.54
DBT2	46					
DBT1-U	44	19	78−19 = 59	32.20	48	81.35
DBT2-U	34					

ID. Total identified proposed compounds number; S-ID. Identified the common proposed compounds number between two Danggui Buxue Tangs; T.ID. Identified the unfamiliar proposed compounds number between two Danggui Buxue Tangs; H + S: The total isoflavones and the total saponins number; S% The ratio about the selective compounds in the total identified proposed compounds number.

2.4. Profiling and Identifying Chemical Profiles of the Urine Samples after Administration of the DBT1 and DBT2 Performed by HPLC-DAD-ESI-IT-TOF-MS^n

In the study of the existing literature, ingredients such as isoflavones, saponins in the two DBTs had no obvious differences in chemical profiles between the serum and bile samples collected from enterohepatic circulation. In addition, they had a lower concentration in serum samples, even when giving at dosages of 60–120 g/kg (w/w) several times to rats within 24 h [13,17,20]. Thus, this approach is not conducive to tracing these minor components. This study chose the normal usage of 10 g/kg (w/w) by comparing the urine samples of rats that were administrated two different DBTs, so as to improve the detection through enrichment of the treatments.

2.4.1. Identification of the Chemical Profiles of Urine Sample after Administration of the DBT1 by HPLC-DAD-ESI-IT-TOF-MS^n

The HRMS data of these identified metabolites are summarized (see Table 4 and Supplementary: pages S52–S66). The BPCs detected in NI mode were recorded (see Figure 3c). In addition, the main proposed structures of these metabolites identified from the urine samples of rats that had been administrated DBT1 were showed (see Figure 5).

Using MS data with the reference compounds, **M15** was identified as calycosin, and **M23** was identified as formononetin [11,13].

With the predictions of $[M-H]^-$ or $[M+HCOO]^-$, and the characteristic fragment ions, **M9**, **M7**, **M19**, **M8**, **M10**, **M11**, **M13**, **M14**, **M22**, **M16**, **M18**, **M20**, **M21**, **M24**, **M32**, and **M36** were tentatively identified as the metabolites of isoflavonoids [13,17–19,21].

M25 shows RT at 46.290 min, $[M+H]^+$ at *m/z* 335.0201 in MS (predicted formula: $C_{15}H_{10}O_7S$: ppm error: −5.67), and characteristic fragment ions at *m/z* 255.0637 (−80 Da; SO_3; identified as the sulfonyl hydroxide) and predicted as $C_{15}H_{10}O_4$. Then **M25** was identified as daidzein after sulfation. **M27**, **M26**, **M34**, **M28**, **M29**, **M30**, **M31**, **M33**, **M35**, **M37**, **M38**, **M43**, and **M44** have the same neutral loss of −80 Da, which was identified as the sulfonyl hydroxide (SO_3), so they were identified as the sulfated products [13,16].

When predicting their formulas, **M39**, **M41**, and **M42** were identified as related metabolites of saponins [13,17].

Table 4. The identified proposed metabolites from the urine samples of rats that had been administrated Danggui Buxue Tang1 and Danggui Buxue Tang 2.

NO.	T_R (min)	$[M+H]^+$	$[M-H]^-$	Predicted Formula	Fragment Ions	Error ppm	Identification	DBT1	DBT2
1	4.132		287.0065	$C_{10}H_8O_{10}$		6.97	--	**M1**	**m1**
2	28.200		231.0768	$C_{12}H_{12}N_2O_3$	463,231	−3.03	--	**M2**	**m2**
3	29.298		233.0115	$C_{12}H_2N_4O_2$	233,169	4.29	--	**M3**	**m3**
4	29.298		337.1408	$C_{16}H_{22}N_2O_6$	337,253	0.89	--	**M4**	**m4**
5	31.083	268.1164	266.1021	$C_{13}H_{17}NO_5$		−4.89	--	**--**	**m5**
6	31.338		275.0209	$C_{13}H_8O_7$	275,195	4.36	--	**M5**	**m6**
7	32.927		273.0056	$C_{13}H_6O_7$	273,193	5.49	--	**M6**	**m7**
8	33.038	271.0585		$C_{15}H_{10}O_5$		−5.90	Hydroxydaidzein	**M7**	**--**
9	34.518	$[M+HCOO]^-$	475.1244	$C_{22}H_{22}O_9$	475,267	−0.42	Isomer of ononin	**--**	**m8**
10	36.155		303.0863	$C_{16}H_{16}O_6$	303,151	−3.63	Hydroxylcalycosin, direduction(C^2=C^3; C^4=O)	**M8**	**--**
11	36.705		253.0492	$C_{15}H_{10}O_4$		−5.53	Daidzein	**M9**	**m9**
12	36.808		477.1372	$C_{23}H_{26}O_{11}$	477,301	−6.29	Astraisoflavan, glucuronidation	**M10**	**--**
13	36.868		255.0662	$C_{15}H_{12}O_4$	255,149	−0.39	Daidzein, reduction(C^2=C^3)	**M11**	**m10**
14	37.255	385.1478		$C_{18}H_{24}O_9$		−3.89	Hydroligustilide, glucuronidation	**M12**	**--**
15	37.442		255.0655	$C_{15}H_{12}O_4$		−3.14	Daidzein, reduction(C^4=O)	**--**	**m11**
16	37.502		285.0751	$C_{16}H_{14}O_5$	285,269,149	−5.96	Calycosin, reduction(C^2=C^3)	**M13**	**m12**
17	37.195		257.0809	$C_{15}H_{14}O_4$		−3.89	Daidzein, direduction(C^2=C^3; C^4=O)	**M14**	**--**
18	38.033		283.0608	$C_{16}H_{12}O_5$	283,268	−1.41	Calycosin	**M15**	**m13**
19	38.362		285.0751	$C_{16}H_{14}O_5$	285,270	−5.96	Calycosin, reduction(C^4=O)	**M16**	**--**
20	39.047		273.0761	$C_{15}H_{14}O_5$	273,240,109	−2.56	Hydroxydaidzein, direduction (C^2=C^3; C^4=O)	**--**	**m14**
21	39.367		233.0098	$C_{12}H_2N_4O_2$		−3.00	--	**M17**	**m15**
22	40.260	$[M+HCOO]^-$	363.0748	$C_{16}H_{14}O_7$		7.16	Dihydroxycalycosin, reduction(C^2=C^3)	**M18**	**--**

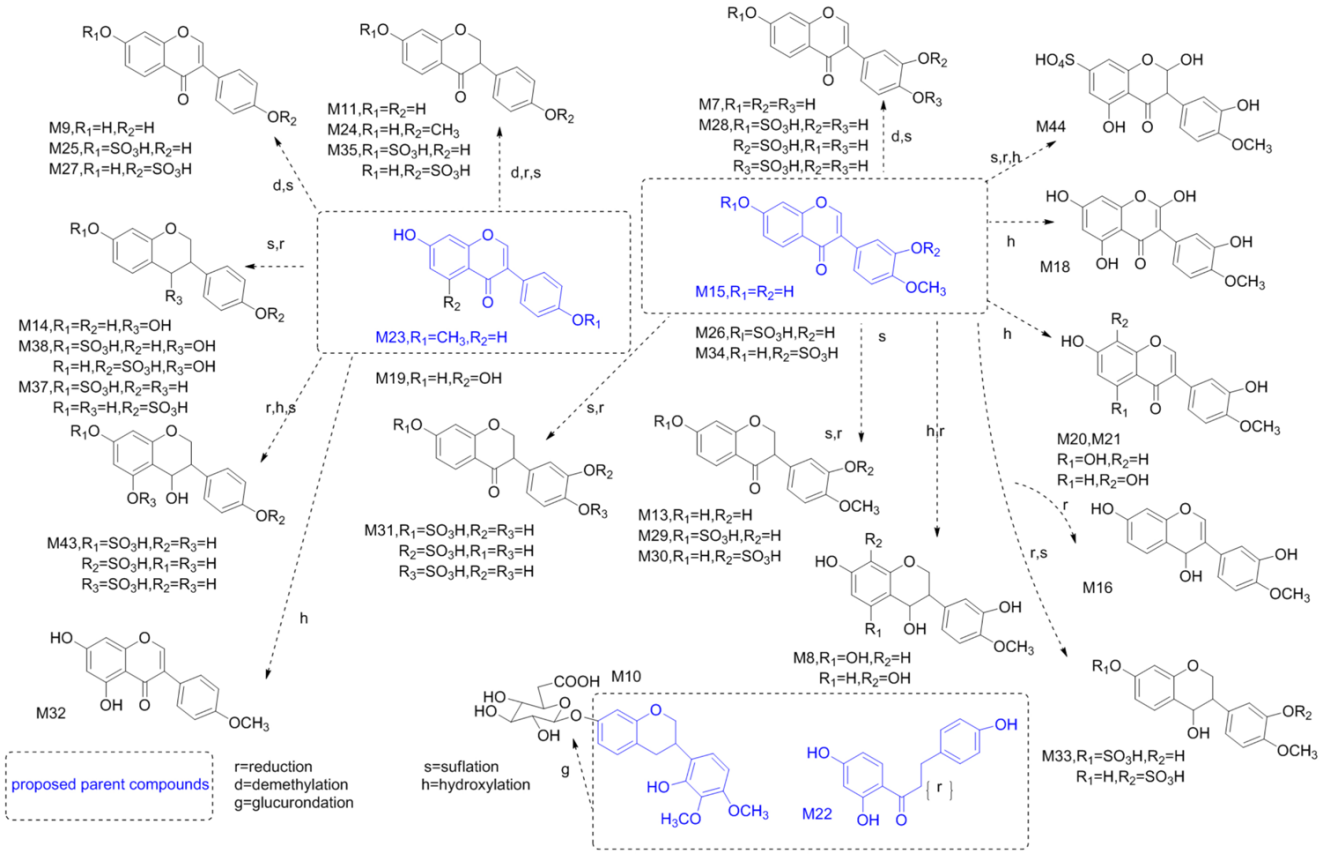
Table 4. *Cont.*

NO.	T_R (min)	$[M+H]^+$	$[M-H]^-$	Predicted Formula	Fragment Ions	Error ppm	Identification	DBT1	DBT2
23	41.630		283.0609	$C_{16}H_{12}O_5$	283,268,224	−1.06	Isomer of calycosin		**m16**
24	41.755		269.0441	$C_{15}H_{10}O_5$		−5.20	Hydroxydaidzein	**M19**	--
25	42.180		283.0603	$C_{16}H_{12}O_5$	283,268,224	−3.18	Isomer of calycosin	--	**m17**
26	42.365		299.0554	$C_{16}H_{12}O_6$	299,284	−2.34	Hydroxycalycosin, or isomer	**M20**	--
27	42.923		299.0556	$C_{16}H_{12}O_6$	299,284	−1.67	Hydroxycalycosin	**M21**	--
28	44.315		257.0819	$C_{15}H_{14}O_4$		0.00	Isoliquiritigenin, reduction(C=C)	**M20**	**m18**
29	45.322	269.0796		$C_{16}H_{12}O_4$	269,253,237	−4.46	Formononetin	**M23**	**m19**
30	45.733		269.0804	$C_{16}H_{14}O_4$	269,254,135	−5.57	Formononetin, reduction(C^2=C^3)	**M24**	**m20**
31	46.290	335.0201		$C_{15}H_{10}O_7S$	335,255	−5.67	Daidzein, sulfation	**M25**	**m21**
32	47.775		299.0556	$C_{16}H_{12}O_6$	299,256	−1.67	Hydroxycalycosin	--	**m22**
33	47.862		363.0174	$C_{16}H_{12}O_8S$	363,268	−1.65	Calycosin, sulfation	**M26**	--
34	47.922		333.0059	$C_{15}H_{10}O_7S$	333,253,225	−4.50	Daidzein, sulfation	**M27**	--
35	48.747		365.0347	$C_{16}H_{14}O_8S$	365,285	2.74	Calycosin, reduction(C^2=C^3), sulfation	**M29**	--
36	49.072		333.0056	$C_{15}H_{10}O_7S$	333,253,208	−5.41	Daidzein, sulfation	--	**m23**
37	49.543		333.0059	$C_{15}H_{10}O_7S$	333,253	−4.50	Isomer of daidzein, sulfation	--	**m24**
38	49.193		365.0360	$C_{16}H_{14}O_8$ S	365,285	6.30	Calycosin, reduction(C^2=C^3), sulfation	**M30**	--
39	49.810		349.0033	$C_{15}H_{10}O_8S$	349,269,225	2.58	Hydroxydaidzein, sulfation	**M28**	**m25**
40	49.623		351.0187	$C_{15}H_{12}O_8S$	351,271,149	1.99	Hydroxydaidzein, reduction(C^2=C^3), sulfation	**M31**	--
41	50.325		337.0395	$C_{15}H_{14}O_7S$	337,257	2.37	Daidzein,direduction(C^2=C^3; C^4=O), sulfation	--	**m27**
42	50.467		283.0595	$C_{16}H_{12}O_5$	283,268	−6.01	Isomer of calycosin	**M32**	--
43	50.973		367.0483	$C_{16}H_{16}O_8S$	367,272,150	−2.72	Calycosin, direduction(C^2=C^3; C^4=O), sulfation	**M33**	--
44	51.515		363.0180	$C_{16}H_{12}O_8S$	363,283	0.00	Calycosin, sulfation	**M34**	**m26**
45	52.563		335.0250	$C_{15}H_{12}O_7S$	335,255,135	5.67	Daidzein, reduction(C^2=C^3), sulfation	**M35**	--

Table 4. *Cont.*

NO.	T_R (min)	$[M+H]^+$	$[M-H]^-$	Predicted Formula	Fragment Ions	Error ppm	Identification	DBT1	DBT2
46	53.637		343.0835	$C_{18}H_{16}O_7$		3.50	--	**M36**	**--**
47	55.693		351.0172	$C_{15}H_{12}O_8S$	351,271	−2.28	Hydroxydaidzein, reduction(C^2=C^3), sufaltion	**--**	**m28**
48	55.165		321.0417	$C_{15}H_{14}O_6S$	321,241	−6.54	Equol, sulfation	**M37**	**m29**
49	59.468		337.0388	$C_{15}H_{14}O_7S$	337,257,243	0.30	Daidzein, direduction(C^2=C^3; C^4=O), sulfation	**M38**	**--**
50	59.728	619.3669	$[M+Na]^+$	$C_{36}H_{52}O_7$		10.33	Related to astragaloside	**--**	**m30**
51	61.838	683.4277		$C_{35}H_{64}O_{11}$		−9.57	Related to astragaloside	**M39**	**--**
52	62.002	639.4061		$C_{35}H_{58}O_{10}$		−6.57	Related to astragaloside	**M40**	**--**
53	62.113	595.3742		$C_{33}H_{54}O_9$		−16.63	Related to astragaloside	**M41**	**m31**
54	63.292		509.3599	$C_{33}H_{50}O_4$		−7.26	Related to astragaloside	**--**	**m32**
55	62.414	507.3296		$C_{29}H_{46}O_7$		−3.94	Related to astragaloside	**M42**	**--**
56	71.557		353.0324	$C_{15}H_{14}O_8S$	353,273	−3.68	Hydroxydaidzein,direduction(C^2=C^3;C^4=O) sulfation	**M43**	**--**
57	71.557		397.0250	$C_{16}H_{14}O_{10}S$	397,317	3.78	Dihydroxycalycosin, reduction(C^2=C^3), sulfation	**M44**	**--**
58	72.797		363.0197	$C_{16}H_{12}O_8S$	363,283	4.68	Calycosin, sulfation	**--**	**m33**
59	73.543		347.0213	$C_{16}H_{12}O_7S$	347,267	−5.19	Formononetin, sulfation	**--**	**m34**

Figure 5. The main proposed metabolites identified from the urine samples of rats that had been administrated Danggui Buxue Tang 1.



2.4.2. Identification of the Chemical Profiles of Urine Sample after Administration of the DBT2 by HPLC-DAD-ESI-IT-TOF-MS^n

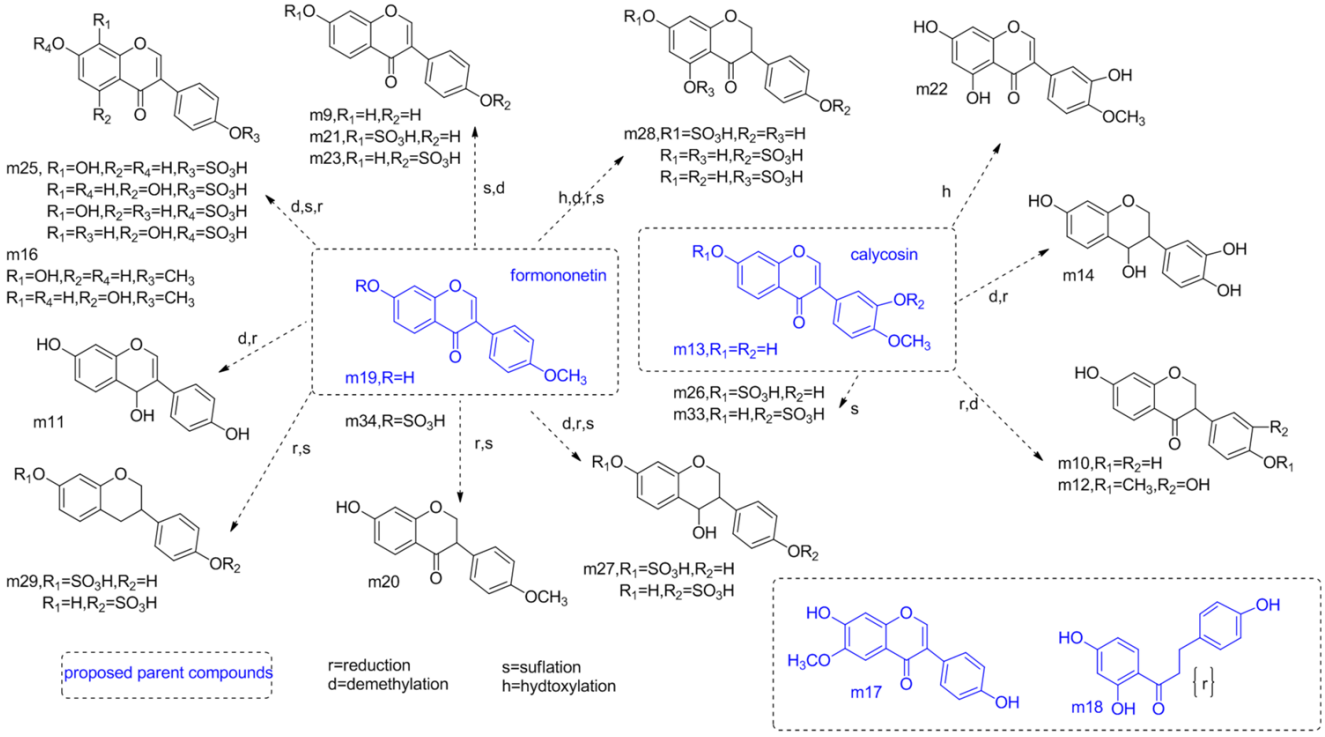
The HRMS data of these identified metabolites are summarized (see Table 4 and Supplementary: pages S67–S77). The BPCs detected in NI model were recorded (see in Figure 3d). The main proposed structures of metabolites identified from the urine samples of rats that had been administrated DBT2 were showed (see Figure 6).

The MS data of **m13**, and **m19** show that they are the reference compounds. In addition, **m13** was identified as calycosin, and **m19** was identified as formononetin [11,13].

Moreover, **m8** has a RT at 34.518 min, $[M+HCOO]^-$ at *m/z* 475.1244 in MS (predicted formula: $C_{22}H_{22}O_9$: ppm error: −0.42), and characteristic fragment ions at *m/z* 267.0656 that are predicted as $C_{16}H_{12}O_4$, the neutral loss is 46 Da (HCOOH) + 162 Da ($C_6H_{10}O_5$; identified as glucoside). Thus, **m8** was identified as the isomer of ononin [13].

In their MS and MS^2 Data, **m21**, **m23**, **m22**, **m24**, **m25**, **m26**, **m33**, **m27**, **m28**, **m29**, and **m34** have the same neutral loss of −80 Da, which was predicted as the sulfonyl hydroxide (SO_3), so they were identified as the sulfated products [13,16].

Figure 6. The main proposed metabolites identified from the urine samples of rats that had been administrated Danggui Buxue Tang 2.



By the MS data of $[M-H]^-$ or $[M+HCOO]^-$ and the characteristic fragment ions, **m9**, **m10**, **m11**, **m12**, **m14**, **m16**, **m17**, **m18**, and **m20** were identified as the metabolites of isoflavonoids [13–15,17,18,21]. Using their predictions of $[M+Na]^+$ or $[M+H]^+$, **m30**, **m31**, and **m32** were tentatively identified as saponin-related metabolites [13,17].

Between DBT1 and DBT2, the groups **M1**, **m1**; **M2**, **m2**; **M3**, **m3**; **M4**, **m4**; **M5**, **m6**; **M6**, **m7**; and **M15**, **m17** were identified as the same compounds, respectively.

In this part of the experiment, decoctions of DBT1 and DBT2 were administered to rats, and an analysis was conducted on the rats' urine for metabolites: 44 compounds (**M1**–**M44**) were identified from the urine samples after DBT1 was administrated, 34 compounds (**m1**–**m34**) were identified from the urine samples after DBT2 was administrated. The number of the chemical components in the urine samples from DBT1 was administrated to rats are slightly more than these of DBT2. The proportion of the 19 common constituents to the total 59 identified compounds accounted for approximately 33%. The proportion of 48 total isoflavonoids and saponins to the total identified compounds, however, reached approximately 82% (See Table 3).

The phase Ⅱ metabolites from the urine samples of DBT1 and DBT2 were dominantly sulfated products, with rare or no glucuronide metabolites. This result still requires further research as the literature reports the chief presence of mainly glucuronide metabolites [13–16]. Metabolites that originated from RAS are relatively rare or not detected. This is likely due to the low proportion of RAS in DBT and even the low dosage that was given to rats in this study, those chemical constituents of RAS being easy to volatilize, or a loss when sampling was conducted by concentrated processes [22,23]. At this normal dosage of DBT and due to poor absorption, the content levels of astragalosides are

much lower. In addition, saponins and their metabolites that originated from astragalosides are rarely detected [24–27].

The HPLC-DAD-ESI-IT-TOF-MS^n method adopted in our research was confirmed to be a powerful method to evaluate the chemical profiles of the crude extracts and the related urine samples. As we know, the chemical composition found in a Chinese herbal decoction is rather complicated. In this study, the chemical profile analysis of DBT1 and DBT2 was conducted, which provided a comprehensive understanding of that those isoflavonoids that play an important role in the main common chemical basis when they are used in clinical practice. Some identified metabolites are known to have many bioactivities, such as calycosin, formononetin, daidzein and equol (which are well-known phytoestrogens), and most of them displayed many beneficial effects to humans [28–30].

Through the comparison of chemical profiles of two DBTs at our used normal dosage, the similarity of urine samples is higher than that of the crude extract samples. This leads us to believe that the main chemical basis of the chemical constituents is almost the same. Whether the DBT2 of RH:RAS can replace the DBT1 of RA:RAS, we need to further investigate different ratios of RH:RAS with the RA:RAS (5:1) when using equivalent pharmacological research.

3. Experimental

3.1. Materials and Reagents

Radix Astragali was collected from Shanxi Province (Voucher No. 130401, Specimen No. 1167), Radix Hedysari was collected from Neimeng Province (Voucher No. 130401, Specimen No. 1168) and Radix Angelica sinensis was collected from Gansu Province (Voucher No. 130401, Specimen No. 1169). All of those medicinal materials were purchased from Guangzhou Zixing Herbal Company in Guangzhou at June in 2013 by Liu Jing and they are identified by Prof. Chen Hu-Biao. The following reference compounds: (6aR,11aR)-3-hydroxy-9,10-dimethoxypterocarpan (astrapterocarpan), ononin, formononetin, 6''-*O*-acetyl-ononin, calycosin, ferulic acid, (3R)-7,2'-dihydroxy-3',4'-dimethoxyisoflavan (astraisoflavan), calycosin-7-*O*-*β*-D-glucoside, astrapterocarpan-7-*O*-*β*-D-glucoside, *Z*-ligustilide, *E*-ligustilide, astragaloside I, astragaloside II, astragaloside III, astragaloside IV, astragaloside V, astraisoflavan-7-*O*-*β*-D-glucoside and 6''-*O*-acetyl-astraisoflavan-7-*O*-*β*-D-glucoside were prepared and identified in our preliminary work [10–14]. Acetonitrile (Merck Co., Darmstadt, Germany) and formic acid (Mreda Technology Inc., Beijing, China) were of HPLC grade. Ultra-pure water was prepared by a Milli-Q water purification system (Millipore, Billerica, MA, USA).

3.2. Sample Preparation

In clinical use, DBT is typically boiled with water twice, then the two decoctions are combined and applied [1]. Therefore, in our study, DBT1, consisting of RA 100 g and RAS 20 g was boiled in 1,000 mL of water (w:v) for 45 min, and then the decoction was filtered. The residue was again boiled in 700 mL of water (w:v) for 30 min. The two decoctions were evaporated to dryness under reduced pressure at 50 °C to 100 mL volume. Samples of DBT2, consisting of RH 100 g and RAS 20 g, were prepared in the same way.

3.3. Animals and Administration

12 male Sprague-Dawley (SD) rats (220–250 g) were provided by the Experimental Animal Center of Peking University Health Science Center (Beijing, China) and divided into two groups. They were housed in metabolic cages (Type: DXL-DL, Suzhou Fengshi Laboratory Animal Equipment Co. Ltd., Suzhou, China), and kept in an environmentally controlled breeding room for one-week acclimation. Throughout the experiments, rats had unrestricted access to laboratory chow and water. The DBT1 and DBT2 were administrated by oral at a dose of raw medicinal material 10 g/kg body weight once a day (at 17:30 pm) respectively. Totally for 2 days. All procedures used in the animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the US National Institute of Health. The experiments were reviewed by the Biomedical Ethical Committee of Peking University (Approval No. LA2013-193).

3.4. Urine Sample Collection and Pretreatment

Urine samples in each group (n = 6) were collected during the first 48 h after administration of the drugs began(Blank urine collected by self-control.); Finally, all urine samples from the same group were merged into one sample, then dried under vacuum at 50 °C using a Heidolph Laborota 4001 rotatory evaporator (Heidolph Instruments GmbH & Co., Schwabach, Germany), and then 1.00 g of the dried samples were reconstituted in 10 mL methanol, followed by 30 min ultrasonic extraction and 15 min centrifugation at 5,000 rpm. Afterward, the supernatant was collected for detection.

3.5. Instrumentations and Conditions

HPLC analysis was performed on a Shimadzu HPLC (Shimadzu, Kyoto, Japan) equipped with two LC-20AD pumps, aCTO-20A column oven, an SIL-20AC autosampler, an SPD-M20A PDA detector and a CBM-20A system controller. The chromatographic separation was carried out on a Phenomenex Gemini C_{18} column (250 × 4.6 mm, 5 μM) protected with a Phenomenex Security Guard column (4 × 3.0 mm, 5 μM) (Phenomenex, Torrance, CA, USA). For each sample, an aliquot of 20 μL was injected with needle wash. The thermostatted auto-sampler was maintained at 15 °C; column oven temperature was kept at 30 °C. The column was eluted with a gradient mobile phase consisted of water-formic acid (100:0.1, v/v) (A) and acetonitrile (B) at the flow rate of 1.0000 mL/min. Gradient program was adopted in the following manner: 5% B at 0–10 min, 5%–15% B at 10–20 min, 15%–40% B at 20–40 min, 40%–65% B at 40–55 min, 65%–100% B at 55–65 min, 100% B at 65–75 min, 5% B at 75–85 min.

High resolution mass spectra were recorded on an IT-TOF mass spectrometer (Shimadzu). The ESI source was operated both in negative and positive ion mode. The mass spectrometry was programmed to carry out full scan over *m/z* 100–1000 Da (MS^1), *m/z* 50–1000 Da (MS^2 and MS^3). A trifluoroacetic acid sodium solution (2.5 mM) was used to calibrate the mass range from 50 to 1000 Da. The other parameters were set as follows: flow rate, 0.20 mL/min (split from 1.00 mL/min HPLC effluent); heat block and curved desolvation line temperature, 200 °C; nebulizing nitrogen gas flow, 1.5 L/min; interface voltage: (+), 4.5 kV; (−), −3.5 kV; detector voltage, 1.70 kV; relative collision-induced dissociation energy (50%) [15].

3.6. Data Analysis

All data were recorded and processed by Shimadzu software LCMS solution version 3.60, Formula Predictor version 1.2 and Accurate Mass Calculator (Shimadzu).

4. Conclusions

A comparison was conducted on the similarities and differences of crude extracts and urine samples of DBT1 and DBT2. The chemical profiles of the crude extracts comprised a total of 115 proposed chemical components. There were 24 common ingredients, which was accounted for 27% in the total 91 identified components. There were a total of 56 isoflavonoids and saponins identified, which accounted for nearly 62% in the total identified components. Since isoflavonoid glycosides had acetylation (**C18**, **C32**, **C34**, **C40**, **C44**, **C46**, and **C47**), the formation of malonate acid esters (**C31**, **C35**, **C43**, **C42**, and **C51**) and special astragalosides (**C49**, **C52**, **C53**, **C54**, **C60**, and **C64**) in DBT1, the identified compounds from DBT1 were significantly greater than DBT2. Of these, **C18**, **C34**, **C35**, **C42**, **C47**, and **C51** were identified from DBT for the first time.

In total, 78 proposed chemical components in the urine samples of rats that had been administrated DBT1 and DBT2, respectively, were found. These included 19 common ingredients, which accounted for approximately 33% in the total identified constituents. In addition, 48 of total isoflavonoids and saponins were found, which accounted for nearly 82% of the total 59 identified components. The differences between those metabolites in the urine samples were revealed to be less than the crude extracts. These identified metabolites are mainly originated from formononetin, calycosin and their related glycosides, which are formed mainly through the metabolic processes of reduction, deglycosylation, demethylation, hydrogenation and sulfation. Through the comparison of chemical profiles of two DBTs at our used doses, the similarity of urine samples is higher than that of the crude extract samples, one can think the main chemical constituents are almost the same as when administrated to rats.

The HPLC-DAD-ESI-IT-TOF-MS^n method was successfully applied for the chemical profile comparison of two different DBTs and its related medicinal materials. The proposed assay provides an important reference and can be a suitable method for the rapid and accurate chemical basis evaluation of TCM or their related prescriptions.

Supplementary Materials

Supplementary materials can be accessed at: http://www.mdpi.com/1420-3049/19/5/5650/s1.

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Authors Contributions

Ya-Zhou Zhang, who presided the research work, finished the data analysis, and written this paper; Feng Xu, The main works by the writer are the data analysis, and participated in the writing of this paper; Tao Yi, who participated in the design of research and modified the paper; Jian-Ye Zhang, who participated the research design, and the data analysis; Jun Xu, who participated in the research design, and modified the paper; Yi-Na Tang, who participated in the research design and data analysis; Xi-Chen He, who participated in the data analysis of LC-MS; Jing Liu, who participated in the experiment work; Hu-Biao Chen, who is providing fund for this research work, the design of the work and modified the paper.

Conflict of Interest

The authors declare no conflict of interest.

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Sample Availability: Samples of the compounds are available from the authors.