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CHARACTERIZATION OF URINARY BIOACTIVE PHENOLIC METABOLITES EXCRETED AFTER CONSUMPTION OF A CUP OF MOUNTAIN TEA (*Sideritis scardica*) USING LIQUID CHROMATOGRAPHY – TANDEM MASS SPECTROMETRY

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A nutrition experiment was performed for studying the bioavailability of polyphenols from *Sideritis scardica* with healthy human subjects, who consumed a standardized *S. scardica* decoction after which urine was collected and analyzed. Thirty five polyphenolic compounds in the ingested decoction and sixty three of their metabolites in urine collected after ingestion were identified using HPLC/MS^n. It was shown that phenolic compounds present in the *S. scardica* decoction are extensively conjugated to glucuronides, sulfates and also transformed to methylated forms after oral administration. Thirty one different metabolites of hypolaetin, methylhypolaetin, isoscutellarein, methylisoscutellarein and apigenin, and 32 phenolic acids metabolites were detected in the analyzed urine samples, whereas hypolaetin and isoscutellarein metabolites were the most abundant. This enabled polyphenols metabolites patterns to be obtained, which is a crucial step towards revealing the bioavailability and metabolism of phenolic compounds from *S. scardica* in human. The identification and structure elucidation of these metabolites provided essential data for further studies of *S. scardica* polyphenols bioavailability.

Key words: *Sideritis scardica*; mountain tea; flavonoids; polyphenols; metabolites; bioavailability

КАРАКТЕРИЗАЦИЈА НА БИОАКТИВНИ ФЕНОЛНИ МЕТАБОЛИТИ ВО УРИНА ПО КОНСУМИРАЊЕ НА ПЛАНИНСКИ ЧАЈ (*Sideritis scardica*) СО ПРИМЕНА НА ТЕЧНА ХРОМАТОГРАФИЈА СО ТАНДЕМ МАСЕНА СПЕКТРОМЕТРИЈА

За проучување на биорасположливоста на полифенолите од *Sideritis scardica* изведен е експеримент со здрави доброволци кои консумираа стандардизирана чајна напивка (декокт) од *S. scardica*, по што беше собирана и анализирана нивната урина. Со употреба на HPLC/MS^n во уринатa собрана по внесувањето на декоктот во организмот се идентификувани триесет и пет полифенолни соединенија и шеесет и три нивни метаболити. Покажано е дека фенолните соединенија присутни во декоктот од *S. scardica* по ингестијата во голема мера се конјугирани и се наоѓаат во облик на глукурониди и сулфати, а исто така се трансформирани и во метилирани форми. Во анализираните примероци урина е утврдено присуството на триесет и еден различен метаболит на хиполетин, метилхиполетин, изоскутелареин, метилизоскутелареин и апигенин, од кои најзастапени се метаболитите на хиполетин и изоскутелареин. Ова овозможува да се добие профилот на полифенолните метаболити, што е клучниот чекор во откривањето на биорасположливоста и метаболизмот на фенолните соединенија од *S. scardica* во човечкиот организам. Со идентификацијата и утврдувањето на структурата на овие метаболити се добиваат неопходни податоци за понатамошните студии на биорасположливоста на полифенолите од *S. scardica*.

Клучни зборови: *Sideritis scardica*; планински чај; флавоноиди; полифеноли; метаболити; биорасположливост

1. INTRODUCTION

In the Balkan countries, the dried inflorescences of number of species of genus *Sideritis* L. (*Lamiaceae*) are used to prepare traditional beverage so-called mountain tea. In R. Macedonia these species are called "sharplaninski chaj" or "planinski chaj" and are widely used as a refreshing herbal tea as well in the folk medicine for treatment of cough, bronchitis, asthma and curing gastrointestinal disorders. This type of drink is usually obtained for domestic use and consumption.

Pharmacological studies indicate that *Sideritis* samples have various bioactivities as anti-ulcer, anti-inflammatory and antioxidant activity [1]. Regarding the chemical constituents of *Sideiritis* species, more than 50 compounds have been isolated and identified up to now, such as flavone aglycones and glycosides [2–7], diterpenes [8] and phenylethanoid glycosides [6, 7, 9].

Although many investigations have been conducted in the fields of pharmacology and phytochemistry of *Sideritis*, the fate of its effective constituents after oral administration of the decoction in the human body is still not elucidated. The limited knowledge about the metabolism of *Sideritis* decoction restricts the thorougher pharmacological and clinical studies of the bioactive constituents of *Sideritis*.

There are a number of reports on the bioavailability of flavonoids in humans as well as on the biological activity of flavonoids in human clinical and intervention studies [10]. It is now clear that flavonoids are absorbed into the plasma and mostly are present in the blood and urine as conjugates, but the extent of absorption, bioavailability and biological activity is dependent on their nature. The use of *in vitro* studies and animal models has provided useful information on the bioavailability and has implied the small intestine as the site for their absorption. Since flavonoids exert biological effects *in vivo* in human intervention studies, one hypothesis to explain their efficacy and bioavailability is that flavonoid conjugates, i.e. metabolites retain some of the biologically active properties.

In our previous study, chemical characterization of *Sideritis scardica* and *Sideritis raeseri* decoction has been successfully performed using HPLC/DAD/ESI/MS^n and 35 major polyphenolic constituents were identified [6, 7, 11, 12]. In the present study, identification of these compounds and their conjugated forms in human urine after ingestion of *S. scardica* decoction was carried out. Urine samples were collected over a 24 h period post-ingestion of the decoction and analyzed, without prior hydrolysis, by HPLC/DAD/MS^n. This enabled polyphenols metabolites patterns to be obtained consisting of the individual flavonoid glucuronide and sulfate metabolites together with phenolic acids metabolites that appeared in urine after supplementation, which is a crucial step towards revealing the bioavailability and metabolism of phenolic compounds from *S. scardica* in human.

2. EXPERIMENTAL

2.1. *Reagents and standards*

Formic acid, methanol and water, all of analyFormic acid, methanol and water, all of analytical grade were purchased from Merck KGaA (Darmstadt, Germany). Authentic samples of verbascoside, forsythoside B, leucoseptoside A, hypolaetin 7-*O*-[6‴-*O*-acetyl]-allosyl(1→2)-glucoside, isoscutellarein 7-O-[6‴-*O*-acetyl]-allosyl(1→2)glucoside, hypolaetin 7-*O*-[6‴-*O*-acetyl]-allosyl(1→2)glucoside, 3'-*O*-methylhypolaetin 7-*O*-[6‴-*O*-acetyl]-allosyl-(1→2)glucoside, 4'-*O*-methylhypolaetin 7-*O*-[6‴-*O*-acetyl]-allosyl(1→2)glucoside, apigenin 7-(6"-*p*-coumaroylglucoside), isoscutellarein 7-*O*-[6‴-*O*-acetyl]-allosyl-(1→2)-[6″-*O*-acetyl]-glucoside, 4'-*O*-methylhypolaetin 7-*O*-[6‴-*O*-acetyl]-allosyl-(1→2)-[6″-*O*-acetyl]-glucoside and 4'-*O*-methyl isoscutellarein 7-*O*-[6‴-*O*-acetyl]-allosyl-(1→2)-[6″-*O*-acetyl]-glucoside were previously isolated from

Sideritis species and identified in the laboratory of the Institute of Organic Chemistry, Bulgarian Academy of Sciences, Sofia, Bulgaria, using NMR techniques [13].

2.2. *Preparation of Sideritis scardica decoction*

The flowering plant of *Sideritis scardica* Griseb. (wild growing) was collected from Kozjak Mountain, R. Macedonia, in July 2011. 8 g of dried *S. scardica* sample was extracted with 400 ml of boiling water during 10 min of boiling to the final volume of 300 ml. The phenolic composition of the sample was determined by LC–DAD-MS/MS.

Identification and peak assignment of all phenolic compounds was based on comparison of their retention times and mass spectral data with those of standards and published data.

2.3. *Study design*

Three male and seven female volunteers (19 – 26 years of age; mean body index = 24.2, range = 18.4–29.7) participated in the study divided in two stages. In stage 1, they followed a polyphenols restricted diet (without fruits and vegetables, high-fiber products, and beverages: tea, coffee, fruit juices and wine) for 2 days before the study. After the wash period of 2 days, the volunteers consumed 300 ml of the *S. scardica* decoction every morning in the next five days. Urine was collected over 24 h after ingestion of the drink. Urine samples were stored at –80 ºC.

In stage 2, the volunteers did not follow the flavonoids restricted diet, but continued with drinking *S. scardica* decoction, and collecting urine 24 h after ingestion of the drink.

2.4. *Urine extraction procedure for phenolic metabolites*

Phenolic metabolites in urine were extracted by solid-phase extraction (SPE). Prior to SPE, urine samples were centrifuged at 15000 g for 10 min at 4 ºC. After 5 min of vortex-mixing, 10 ml urine sample was applied to the preconditioned C18 Sep-Peak cartridge (Waters, USA) with 5 ml of methanol, 5 ml of water and 5 ml air. Sample clean up was performed with 3 ml of water, followed by drying with 3 ml air. Elution was performed with 3 ml of methanol. The eluted fraction was concentrated to dryness and residues dissolved in 500 μl of methanol.

2.5. *LC/DAD/ESI-MS^n analysis*

The native phenolic compounds present in *S. scardica* samples and the phenolic metabolites in urine samples were identified by LC–MS/MS analysis. Liquid chromatography separations were performed on Agilent Technologies 1100 LC system (Waldbronn, Germany) equipped with diode array and mass detector in series. It consisted of a G1312A binary pump, a G1329A auto-sampler, a G1379B degasser and G1315D photo-diode array detector, controlled by ChemStation software (Agilent, v.08.03).

Spectral data from all peaks were accumulated in range 190–600 nm and chromatograms were recorded at 280, 300 and 330 nm.

For both analyses of plant and urine sample extracts XDB-Eclipse (Agilent, USA) C18 (150 mm × 4.6 mm, 5 μm) column was used at 38 °C, with the sample injection volume of 20 μl.

Gradient elution was performed with a binary system consisting of (A) 1% aqueous formic acid and (B) methanol. A linear gradient starting with 20% B was installed to reach 80% B at 50 min and 100% B at 60 min. The flow rate was 0.4 ml min^{-1}.

The mass detector was a G2449A Ion-Trap Mass Spectrometer equipped with an electrospray ionization (ESI) system and controlled by LCMSD software (Agilent, v.6.1.). Nitrogen was used as nebulizing gas at pressure of 65 psi and the flow was adjusted to 12 l min^{-1}. The heated capillary and the voltage were maintained at 350 °C and 4 kV, respectively. MS data were acquired in the negative ionization

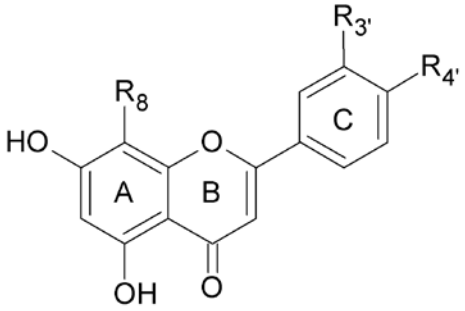
mode. The full scan covered the mass range at *m/z* 100–1200. Collision–induced fragmentation experiments were performed in the ion trap using helium as collision gas, with voltage ramping cycle from 0.3 up to 2 V. Maximum accumulation time of the ion trap and the number of MS repetitions to obtain the MS average spectra were set at 300 ms and 5, respectively.

3. RESULTS AND DISCUSSION

Relevant phenolic compounds in the *S. scardica* extracts were identified by their UV spectra, the deprotonated molecular ions and their corresponding ion fragments using LC-DAD-MS/MS (Table 1). Identification of phenolics was in accordance with previously reported work [6, 7]. Structures of the flavone aglycones from *S. scardica* decoction are given in Figure 1.

In *S. scardica* samples flavonoids are present in different chemical forms including aglycones, *β*-glycosides, *p*-coumaroyl and acetyl glycosides. Upon ingestion, the *β*-glycoside moiety is hydrolyzed by microbial *β*-glycosidase resulting in formation of the aglycones hypolaetin, metylhypolaetin, isoscutellarein, 4′-*O*-methylisoscutellarein and apigenin. These compounds are then absorbed in the gut and transported to the liver. During absorption processes, flavonoids may be conjugated with glucuronic or sulfonic acid in hepatic and/or epithelial cell membranes (Figure 2) and can be bound to plasma proteins such as albumin [14]. Due to their hydrophilic characteristics compared to parent aglycones, the metabolites are more readily transported in the plasma and excreted into urine.

The bioavailability of flavonoids is influenced by multiple factors including: composition of intestinal microflora, gender, age, diet, duration of exposure and chemical and physical nature of the flavonoids and the food matrix. The extensive metabolism of flavonoids has been attributed to the action of intestinal microflora [15]. Hydrolytic enzymes of intestinal microflora can convert certain flavonoid glycosides (e.g. rutin) to their corresponding aglycones (e.g. quercetin), which could be further metabolized by intestinal microflora to various single-ring aromatic compounds [15, 16]. Recently, however, intestinal hydrolysis of flavone monoglycosides was observed, suggesting that enterocytes might also play an important role in the hydrolysis of glycosides to aglycones [17].



Compounds:	R_8	$R_{3'}$	$R_{4'}$	MW
luteolin	H	OH	OH	286
hypolaetin	OH	OH	OH	302
4'-*O*-methylhypolaetin	OH	OH	OCH_3	316
apigenin	H	H	OH	270
isoscutellarein	OH	H	OH	286
4'-*O*-methylisoscutellarein	OH	H	OCH_3	300

Fig. 1. Structures of flavone aglycones present in *Sideritis scardica* samples

T a b l e 1

Identification of phenolic compounds in Sideritis scardica extract by LC-DAD-MS/MS data

	Compounds	t_R (min)	UV (nm)	$[M–H]^-$ (*m/z*)	$–MS^2[M–H]^-$ (*m/z*)
	Hydroxycinnamic derivatives				
1	3-caffeoylquinic acid	7.2	242, 296sh, 324	353	**191*,** 179
2	5-caffeoylquinic acid	12.0	242, 294sh, 326	353	**191,** 179
3	*p*-coumaric acid 4-*O*-glucoside	14.3	232, 312	325	**307**, 265, 235, 217, 187
4	feruloylquinic acid	16.6	295, 322	367	**191**, 173
	Phenylethanoid glycosides				
5	echinacoside	19.9	232, 246sh, 290, 332	785	**623**, 461
6	forsythoside B	20.2	232, 246, 290, 332	755	623, **593**, 461
7	verbascoside	20.6	232, 244, 290, 302sh, 332	623	487, 477, **461**, 443, 315, 297
8	forsythoside A	21.1	246, 286, 304, 334	623	578, 463, 461, 445, 316, **301**
10	samioside	22.4	234, 246, 288, 330	755	623, **593**, 461
11	isoverbascoside	22.7	232, 274, 298sh, 358	623	**461**, 315, 161
12	isoleucoseptoside	23.1	234, 288, 330	637	491, **461**, 443, 325
15	allysonoside	25.2	234, 290, 330	769	637, **593**, 575, 491, 475, 461
16	leucoseptoside A	25.7	234, 288, 330	637	491, **461**, 443, 325
	Luteolin derivatives				
17	luteolin 7-*O*-allosyl-(1→2)-[6″-*O*-acetyl]-glucoside	26.3	256, 350	651	609, 591, 447, 429, **285**
18	luteolin 7-*O*-[6‴-*O*-acetyl]-allosyl(1→2) glucoside	26.8	256, 350	651	**609**, 591, 447, 429, 285
21	luteolin 7-*O*-[6‴-*O*-acetyl]-allosyl-(1→2)-[6″-*O*-acetyl]-glucoside	28.4	256, 352	693	651, 633, 609, 489, 471, 429, **285**
	Hypolaetin derivatives				
9	hypolaetin 7-*O*-allosyl(1→2)glucoside	21.8	234, 256sh, 278, 300, 340	625	463, 445, **301**
14	hypolaetin 7-*O*-[6‴-*O*-acetyl]-allosyl(1→2) glucoside	24.8	230, 254, 276, 300, 344	667	**625**, 607, 505, 463, 445, 301
20	3′-*O*-methylhypolaetin 7-*O*-allosyl-(1→2)-[6″-*O*-acetyl]-glucoside	28.2	232, 256, 276, 302, 340	681	639, 621, 477, 459, **315**, 301
23	4′-*O*-methylhypolaetin 7-*O*-[6‴-*O*-acetyl]-allosyl(1→2)glucoside	30.3	226, 254, 276, 302, 340	681	**639**, 621, 477, 459, 315, 301
24	hypolaetin 7-*O*-[6‴-*O*-acetyl]-allosyl-(1→2)-[6″-*O*-acetyl]-glucoside	30.9	228, 254, 276, 302, 346	709	667, 649, 607, 505, 487, 427, **301**
29	3′-*O*-methylhypolaetin 7-*O*-[6‴-*O*-acetyl]-allosyl-(1→2)-[6″-*O*-acetyl]-glucoside	33.6	228, 256, 276, 302, 340	723	**681**, 663, 639, 621, 477, 315, 301
	Apigenin derivatives				
26	apigenin 7-(6″-*p*-coumaroylglucoside)	32.9	232, 268, 318	577	431, 413, 307, **269**
27	apigenin 7-(4″-*p*-coumaroylglucoside)	33.2	232, 270, 318	577	431, 413, 307, **269**
	Isoscutellarein derivatives				
13	isoscutellarein 7-*O*-allosyl(1→2)glucoside	23.7	230, 274, 306, 328	609	447, 429, **285**
19	isoscutellarein 7-*O*-[6‴-*O*-acetyl]-allosyl(1→2)glucoside	27.1	230, 276, 306, 330	651	**609**, 591, 447, 429, 285
22	4′-*O*-methylisoscutellarein 7-*O*-allosyl(1→2)glucoside	29.1	230, 292, 314	623	461, 443, **299**
25	4′-*O*-methylisoscutellarein 7-*O*-allosyl-(1→2)-[6″-*O*-acetyl]-glucoside	32.2	230, 276, 306, 328	665	623, 503, 461, 443, **299**, 285
28	isoscutellarein 7-*O*-[6‴-*O*-acetyl]-allosyl-(1→2)-[6″-*O*-acetyl]-glucoside	33.2	230, 276, 308, 328	693	651, 633, 609, 489, 471, 429, **285**
30	4′-*O*-methylisoscutellarein 7-*O*-[6‴-O-acetyl]-allosyl(1→2)glucoside	34.4	230, 276, 306, 328	665	**623**, 503, 461, 443, 299, 284
31	4′-*O*-methylisoscutellarein 7-*O*-[6‴-*O*-acetyl]-allosyl-(1→2)-[6″-*O*-acetyl]-glucoside	36.9	230, 280, 306, 330	707	665, 647, 605, 545, 503, **299**, 284

* The *m/z* value for the base peak is in bold

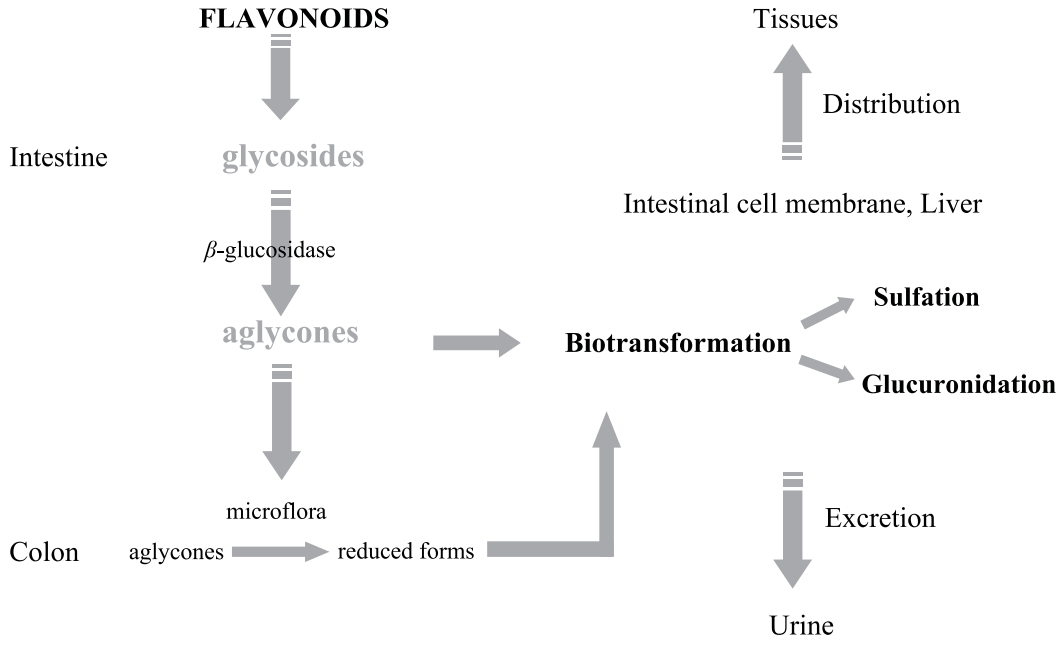


Fig. 2. General scheme for the absorption and metabolism of flavonoids

3.1. *Flavonoid metabolites*

HPLC/MS^2 and MS^3 was used to identify the metabolites of hydroxycinnamic acids, phenylethanoid glycosides and flavonoids from *S. scardica* excreted in urine collected 24 hours after ingestion. Phenolic metabolites identified in urine are listed in Table 2 and Table 3. Analysis of phenolic metabolites is more subtle than generally appreciated. This is due to the fact that, without reference compounds that can be separated by reversed phase HPLC, it is impossible to distinguish between 3′-*O*-methylhypolaetin and 4′-*O*-methylhypolaetin by mass spectrometry, as well as to locate the position of glucuronidation or sulfation. So, here we refer to metabolites as glucuronide, sulfate and glucuronide-sulfate without specifying the position of substitution.

A total of 31 different metabolites of hypolaetin, methylhypolaetin, isoscutellarein, 4′-*O*-methylisoscutellarein and apigenin were detected in the urine samples (Table 2).

Peaks **M3**, **M5**, **M6** and **M18** showed pseudomolecular ions at *m/z* 395, 395, 381 and 365, respectively, which yielded *m/z* 315, 315, 301 and 285 after loss of 80 amu (sulphate moiety) (Figure 3a). They were identified as methylhypolaetin sulfate (**M3**, **M5**), hypolaetin sulfate (**M6**) and isoscutellarein sulfate (**M18**).

Peaks **M16**, **M62** and **M63** were identified by their $[M–H]^-$ *m/z* at 429, 459 and 459, respectively. The initial MS^2 loss of 80 amu corresponds to one sulfate moiety and gave main product ion at *m/z* 349, 379 and 379 (Figure 3b). The fragments at *m/z* 269, 299 and 299 correspond to aglycones of apigenin and 4′-*O*-methylisoscutellarein. Therefore **M16** is presumably a disulfated apigenin, whereas **M62** and **M63** disulfated 4′-*O*-methylisoscutellarein.

Peaks **M7**, **M8**, **M10**, **M15**, **M17**, **M19**, **M23**, **M43**, **M46**, **M56** and **M58** produced $[M–H]^-$ *m/z* at 477 (**M8**, **M17**, **M19**), 491 (**M56**, **M58**), 461 (**M43**, **M46**), 475 (**M7**, **M10**) and 445 (**M15**, **M23**), respectively. After loss of 176 amu (a glucuronide unit) (Figure 3c) they gave ions at *m/z* 301, 315, 285, 299 and 269, respectively, which characterize these compounds as glucuronides of hypolaetin (**M8**, **M17**, **M19**), methylhypolaetin (**M56**, **M58**), isoscutellarein (**M43**, **M46**), 4’-*O*-methylisoscutellarein (**M7**, **M10**) and apigenin (**M15**, **M23**).

T a b l e 2

HPLC-MS² data for identification of flavonoid metabolites in urine collected 0-24 h after ingestion of mountain tea

Peak	t_R/ min	$[M–H]^-$ (*m/z*)	MS^2	Compound	
Hypolaetin derivatives					
M6	24.3	381	**301***	hypolaetin	sulfate
M8	24.4	477	459, **301**	hypolaetin	glucuronide
M17	31	477	459, **301**	hypolaetin	glucuronide
M19	31.8	477	459, **301**	hypolaetin	glucuronide
M40	37.3	653	**477**, 459, 301	hypolaetin	diglucuronide
M11	25.5	557	447, **301**	hypolaetin	glucuronide-sulfate
M3	21.8	395	**315**	methylhypolaetin	sulfate
M5	24.1	395	**315**	methylhypolaetin	sulfate
M56	43.6	491	473, 447, **315**	methylhypolaetin	glucuronide
M58	44.6	491	473, 447, **315**	methylhypolaetin	glucuronide
M44	38.5	623	447, **315**	methylhypolaetin	glucuronide + pentose
Isoscutellarein derivatives					
M18	31.7	365	**285**	isoscutellarein	sulfate
M43	38.3	461	443, **285**	isoscutellarein	glucuronide
M46	39.7	461	443, **285**	isoscutellarein	glucuronide
M24	32.7	541	**461**, 285	isoscutellarein	glucuronide-sulfate
M27	33.3	541	**461**, 285	isoscutellarein	glucuronide-sulfate
M29	34.5	541	461, **285**	isoscutellarein	glucuronide-sulfate
M32	35.1	541	461, **285**	isoscutellarein	glucuronide-sulfate
M62	49.2	459	**379**, 299	methylisoscutellarein	disulfate
M63	49.7	459	**379**, 299	methylisoscutellarein	disulfate
M7	24.3	475	457, **299**	methylisoscutellarein	glucuronide
M10	25.4	475	457, **299**	methylisoscutellarein	glucuronide
M4	23.3	651	475, 457, **299**	methylisoscutellarein	diglucuronide
M41	37.5	651	**475**, 299	methylisoscutellarein	diglucuronide
Apigenin derivatives					
M16	28.5	429	**349**, 269	apigenin	disulfate
M15	28.5	445	**269**	apigenin	glucuronide
M23	32.6	445	**269**	apigenin	glucuronide
M53	42.4	621	**445**, 427, 269	apigenin	diglucuronide
M28	34.0	525	**445**, 269	apigenin	glucuronide-sulfate
M36	36.5	525	445, **269**	apigenin	glucuronide-sulfate
M45	38.6	525	445, **269**	apigenin	glucuronide-sulfate

* The *m/z* value for the base peak is in bold

Peaks **M40** and **M41** showed deprotonated molecular ions $[M–H]^-$ at *m/z* 653 and 651 respectively. The MS^2 spectra of these ions yielded the base peak $[M–H–176]^-$ (*m/z* 477 and 475) corresponding to the loss of glucuronide residue (Figure 3d). In the MS^3 spectra of these compounds the base peak was derived from a second loss of 176 amu (*m/z* 301 and 299). Therefore, these compounds could be characterized as hypolaetin (**M40**) and 4′-*O*-methylisoscutellarein (**M41**) diglucuronides with two glucuronide units bonded to the aglycone in different positions supported by their consecutive loss evidenced in MS^2 and MS^3. In contrast, after MS^2 fragmentation of compound **M4**, the base peak was detected after the loss of 352 amu (2 glucuronide units) from the deprotonated ion (Figure 3e). The occurrence of $[M–H–176]^-$ ions (*m/z* 475 and 457) implied the presence of glucuronide residue. Thus, **M4** was also characterized as 4′-*O*-methylisoscutellarein diglucuronide, but with substitution in one position in the aglycone i.e. glucuronide moieties attached to each other.

Peaks **M11**, **M29**, **M32**, **M36** and **M45** had $[M–H]^-$ ions at *m/z* 557 (**M11**), 541 (**M29**, **M32**), 525 (**M36**, **M45**) and MS^2 produced sequential losses of 80, 176 and 256 amu indicating the presence of a glucuronide/sulfate conjugate (Figure 3f). The aglycone ions at *m/z* 301 (**M11**), 285 (**M29**, **M32**) and 269 (**M36**, **M45**) were hypolaetin, isoscutellarein and apigenin. These three metabolities are therefore hypolaetin-*O*-glucuronide-*O*-sulfate (**M11**), isoscutellarein-*O*-glucuronide-*O*-sulfate (**M29**, **M32**) and apigenin-*O*-glucuronide-*O*-sulfate (**M36**, **M45**).

Peaks **M24**, **M27** and **M28** produced deprotonated molecular ions $[M–H]^-$ at *m/z* 541 (**M24**, **M27**) and 525 (**M28**). The MS^2 spectra of these ions yielded the base peak $[M–H–80]^-$ corresponding to the loss of sulfate moiety. In their MS^3 spectra, the base peak was derived from a loss of 176 amu (glucuronide residue), typical for *O*-conjugated compounds in two positions on the flavonoid skeleton (Figure 3g).

These compounds could be characterized as isoscutellarein-*O*-glucuronide-*O*-sulfate (**M24**, **M27**) and apigenin-*O*-glucuronide-*O*-sulfate (**M28**) but the glucuronide and sulfate moities are linked in different positions on the aglycone.

Peak **M44** had an $[M–H]^-$ ion at *m/z* 623 and MS^2 fragmentation resulted in a 176 amu loss of a glucuronyl group followed by loss of pentose unit (Figure 3h). This metabolite can be identified as methylhypolaetin pentoside glucuronide.

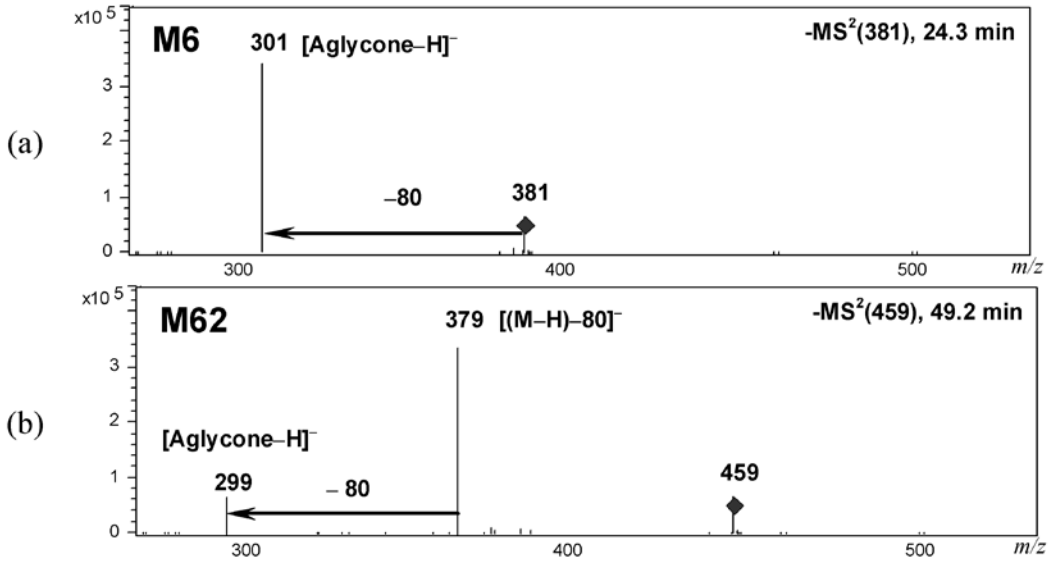


Fig. 3. MS^2 spectra of some characteristic metabolites of flavonoids present in urine (a) hypolaetin sulfate; (b) 4′-*O*-methylisoscutellarein disulfate

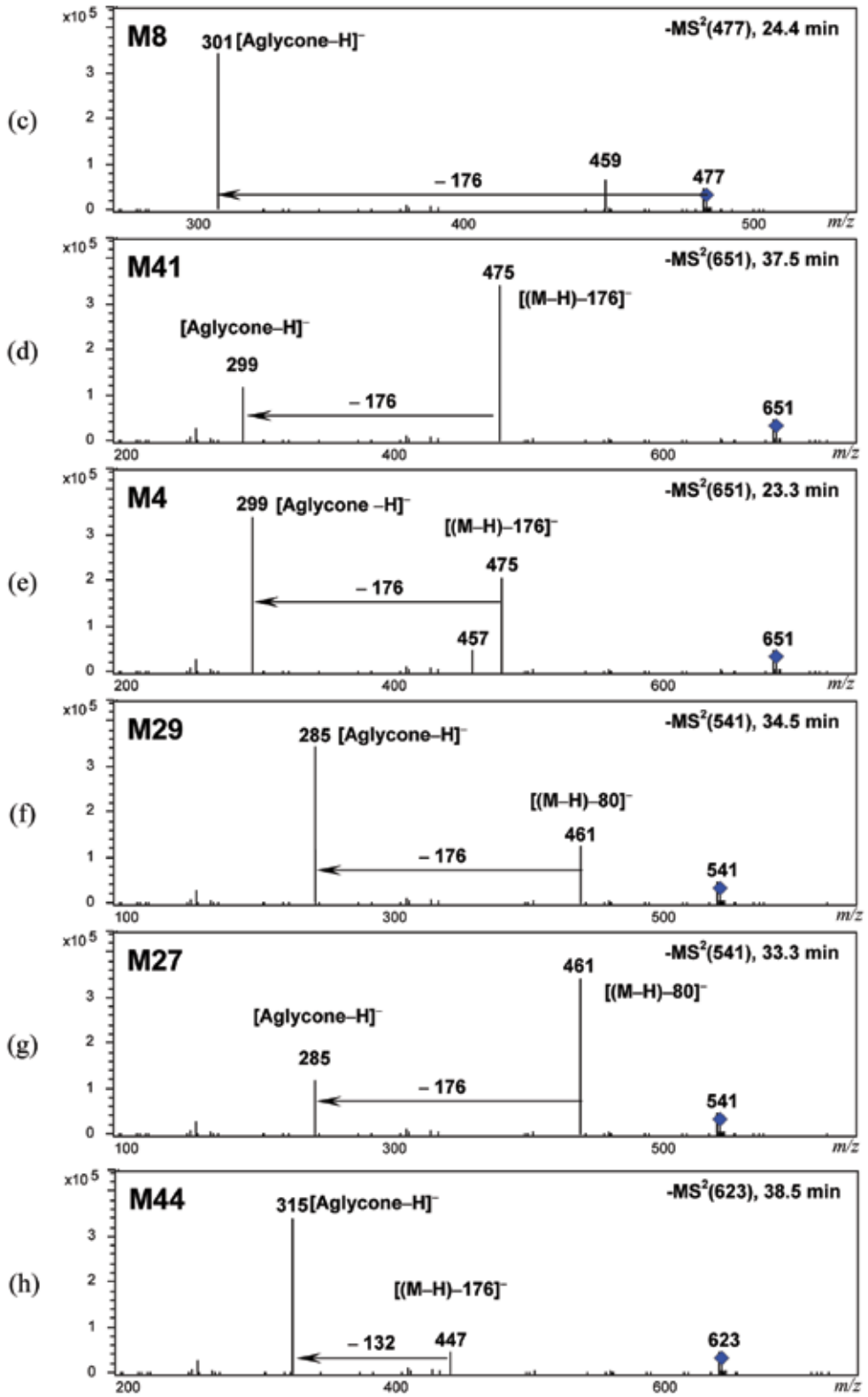


Fig. 3. MS^2 spectra of some characteristic metabolites of flavonoids present in urine
(c) hypolaetin glucuronide; (d, e) 4′-*O*- methylisoscutellarein diglucuronide; (f, g) isoscutellarein glucuronide-sulfate and (h) methylhypolaetin glucuronyl-pentoside

T a b l e 3

HPLC-MS^2 identification of phenolic acids metabolites in urine collected 0-24 h after ingestion of mountain tea

Peak	t_R/min	$[M–H]^-$ (*m/z*)	MS^2	Compound	
Quinic acid derivatives					
M22	32.4	431	**351***, 271, 191	quinic acid	trisulfate
M30	34.6	367	**191**	quinic acid	glucuronide
M61	48.6	367	**191**	quinic acid	glucuronide
M51	41.5	447	367, **191**	quinic acid	glucuronide-sulfate
M59	46.7	447	271, **191**	quinic acid	glucuronide-sulfate
Caffeic acid derivatives					
M2	15.6	179	**161**	caffeic acid	
M39	37.1	435	259, **179**	caffeic acid	glucuronide-sulfate
M12	27.7	383	**207**, 179	dimethylcaffeic acid	glucuronide
M14	28.1	383	**207**, 179	dimethylcaffeic acid	glucuronide
Chlorogenic acid derivatives					
M26	33.1	433	**353**, 179	chlorogenic acid	sulfate
M31	35.0	433	353, **179**	chlorogenic acid	sulfate
M54	42.5	513	**433,** 353	chlorogenic acid	disulfate
M55	42.9	513	433**, 353**	chlorogenic acid	disulfate
M47	39.7	529	353, **179**	chlorogenic acid	glucuronide
M48	40.2	529	**353**, 179	chlorogenic acid	glucuronide
M49	40.9	529	**353**, 179	chlorogenic acid	glucuronide
Ferulic acid derivatives					
M13	27.9	273	**193**	ferulic acid	sulfate
M20	31.9	369	**193**	ferulic acid	glucuronide
M33	35.2	369	**193**	ferulic acid	glucuronide
M38	36.9	369	**193**	ferulic acid	glucuronide
M42	37.5	369	**193**	ferulic acid	glucuronide
M25	32.8	449	**369**, 193	ferulic acid	glucuronide-sulfate
M50	41.3	449	369, **193**	ferulic acid	glucuronide-sulfate
M60	47.3	449	369, **193**	ferulic acid	glucuronide-sulfate
M9	24.4	301	**273**, 193	dimethylferulic acid	sulfate
M1	13.8	397	**221**, 193	dimethylferulic acid	glucuronide
M35	35.6	397	**221**, 193	dimethylferulic acid	glucuronide
M34	35.5	527	447, **367**	feryoil quinic acid	disulfate
M21	32.4	543	**367**	feryoil quinic acid	glucuronide
M37	36.5	571	395, **367**	dimethylferyoil quinic acid	glucuronide
Coumaric acid derivatives					
M52	41.9	513	**337**	coumaric acid	glucuronide
M57	43.7	513	**337**	coumaric acid	glucuronide

* The *m/z* value for the base peak is in bold

3.2. *Phenolic acids metabolites*

A total of 32 phenolic acid metabolites were identified in urine samples after 24 h excretion (Table 3). Peak **M2** had $[M–H]^-$ ions at *m/z* 179, which fragmented in MS^2 to *m/z* 161. This fragmentation pattern is characteristic for caffeic acid.

Peaks **M13**, **M26** and **M31** had $[M–H]^-$ ions at *m/z* 273 (**M13**) and 433 (**M26**, **M31**) which led to *m/z* 193 (**M13**) in MS^2, the molecular ion of ferrulic acid and *m/z* 353 and 179 (**M26**, **M31**) the molecular ions characteristic for chlorogenic acid (Figure 4a). These metabolites were identified as ferulic acid sulfate and chlorogenic acid sulfate.

Peak **M9** had $[M–H]^-$ ion and MS^2 spectrum for 28 amu higher than that of **M13**. The main MS^2 fragment (*m/z* 273) had an MS^3 spectrum characteristic of ferulic acid (ions at *m/z* 193, 179, 149 and 135), which identified this compound as dimethylferulic acid sulfate.

Peaks **M22**, **M34**, **M54** and **M55** had $[M–H]^-$ ions at *m/z* 431 (**M22**), 527 (**M34**) and 513 (**M54**, **M55**), which yielded MS^2 fragment ions at *m/z* 351, 271 and 191 (**M22**) obtained by sequential losses of 80, 160 and 240 amu indicating the presence of three sulfate groups (Figure 4c), and at *m/z* 447, 367 for **M34** and 433, 353 (**M54**, **M55**) – losses of 80 and 160 amu indicating the presence of two sulfate groups (Figure 4b). These MS spectra characterized these compounds as quinic acid trisulfate (**M22**), feruloylquinic acid disulfate (**M34**) and chlorogenic acid disulfate (**M54**, **M55**).

Peaks **M1**, **M12**, **M14**, **M20**, **M21**, **M30**, **M33**, **M35**, **M37**, **M38**, **M42**, **M47**, **M48**, **M49**, **M52**, **M57** and **M61** had neutral loss of 176 amu (Figure 4d and 4e). The fragments at *m/z* 191 (**M30**, **M61**), 193 (**M20**, **M33**, **M38**, **M42**), 207, 179 (**M12**, **M14**), 353, 179 (**M47**, **M48**, **M49**), 221, 193 (**M1**, **M35**), 367 (**M21**), 337 (**M52**, **M57**) and 395, 367 (**M37**) correspond to quinic acid (**M30**, **M61**), ferulic acid (**M20**, **M33**, **M38**, **M42**), dimethylcaffeic acid (**M12**, **M14**), chlorogenic acid (**M47**, **M48**, **M49**), dimethylferulic acid (**M1**, **M35**), feruloylquinic acid (**M21**), coumaric acid (**M52**, **M57**) and dimethylferuloylquinic acid (**M37**).

Peaks **M25**, **M39, M51**, **M50**, **M59** and **M60** had $[M–H]^-$ ions at *m/z* 435 (**M39**), 447 (**M51**, **M59**) and 449 (**M25**, **M50**, **M60**). The neutral losses of 80 and 176 indicated cleavage of both sulfate and glucuronide moiety. These compounds were identified as caffeic acid glucuronide-sulfate (**M39**), quinic acid glucuronide-sulfate (**M51**, **M59**) and ferulic acid glucuronide-sulfate (**M25**, **M50**, **M60**) (Figure 4f and 4g).

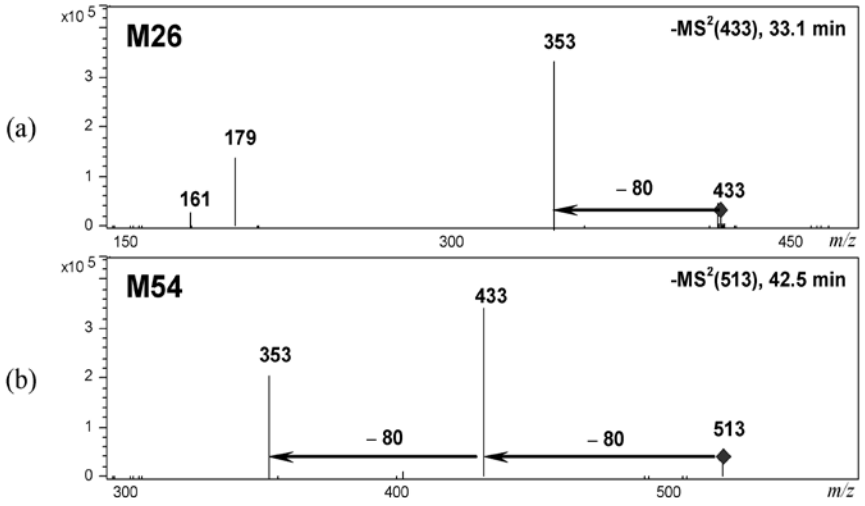


Fig.4. MS^2 spectra of some characteristic metabolites of phenolic acids present in urine (a) chlorogenic acid sulfate; (b) chlorogenic acid disulfate

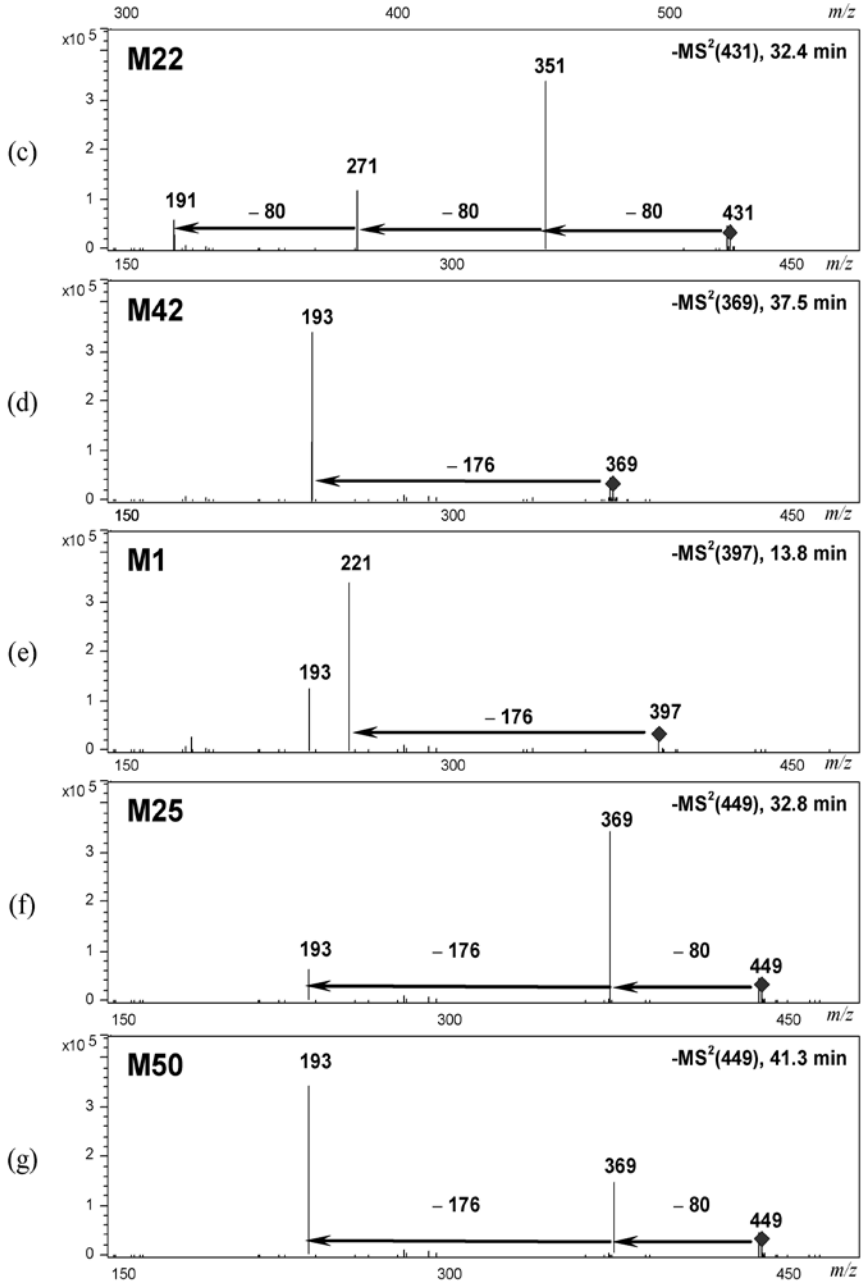


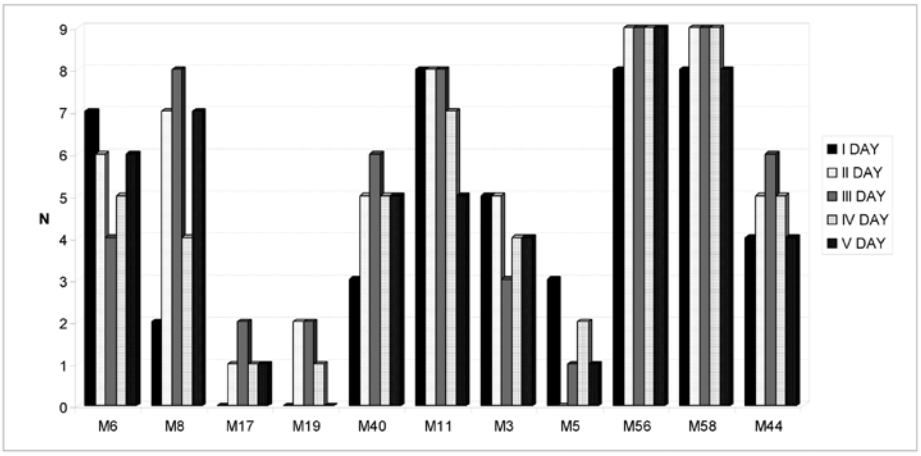
Fig.4. MS^2 spectra of some characteristic metabolites of phenolic acids present in urine (c) quinic acid trisulfate (d) ferulic acid glucuronide; (e) dimethylferulic acid glucuronide; (f, g) ferulic acid glucuronide-sulfate

3.3. *Polyphenols metabolites occurrence*

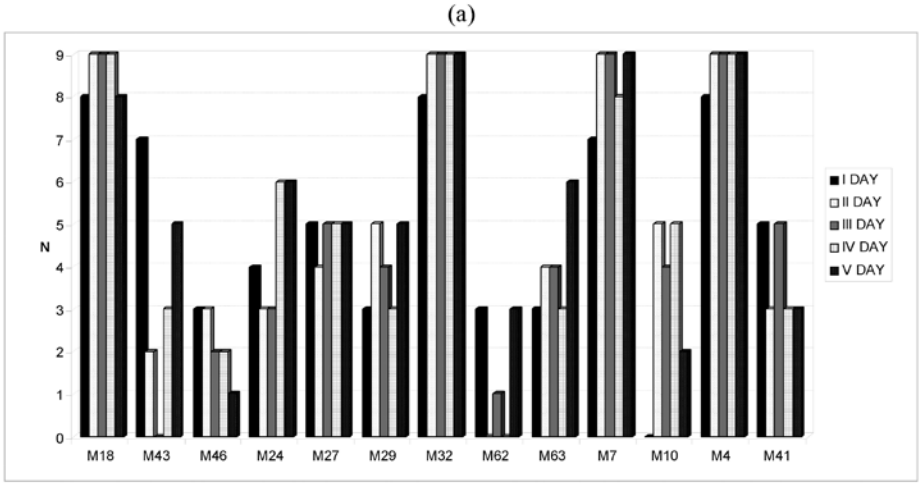
In the *S. scardica* decoction a total of 31 compounds were identified which were classified in six groups of polyphenols: hydroxycinnamic acid derivatives, phenylethanoid glycosides, luteolin, hypolaetin, apigenin and isoscutellaerin derivatives (Table 1). In contrast, in urine samples, a total of 63 different metabolites were detected implying that these compounds are subjected to different metabolic pathways. Following the prior grouping, the metabolites can be summarized in four groups as follows: hypolaetin, isoscutellaerin and apigenin derivatives, and phenolic acids derivatives (quinic acid, caffeic acid, chlorogenic acid, ferrulic acid and coumaric acid) as presented in Table 2 and Table 3.

By *in vivo* experiments performed in this work, it was shown that phenolic compounds present in *S. scardica* decoction are extensively conjugated to glucuronides, sulfates and also transformed to methylated forms after their oral administration. Upon ingestion, the *β*-glycosides are hydrolyzed by microbial *β*-glycosidase resulting in the formation of the aglycones hypolaetin, metylhypolaetin, isiscutellarein, 4′-*O*-methylisoscutellarein and apigenin, which are subsequently absorbed in the gut and transported to the liver. After drinking a standardized extract of mountain tea, 31 different metabolites of hypolaetin, methylhypolaetin, isoscutellarein, 4′-*O*-methylisoscutellarein and apigenin, and 32 phenolic acid metabolites were detected in the urine samples.

Hypolaetin and isoscutellarein were the dominant groups present in the urine with the highest number of different metabolites. These two types of flavones are quite specific for the genus *S. scardica* which are not so abundant in of the plant derived food in contrast to apigenin and luteolin, so there were not significant differences in the samples when the non-flavonoid diet was or was not followed. A total of eleven hypolaetin metabolites were present in urine; six of them were hypolaetin derivatives (**M6**, **M8**, **M17**, **M19**, **M40**, **M11**) and five methylhypolaetin derivatives (**M3**, **M5**, **M56**, **M58**, **M44**). In the *S. scardica* decoction, hypolaetin was present with nine derivatives: six hypolaetin and three methylhypolaetin derivatives. As can be seen in Figure 5a, compounds **M11** (hypolaetin glucuronide-sulfate), **M56**



(a)



(b)

Fig. 5. Distribution of (a) hypolaetin derivatives, and (b) isoscutellarein derivatives per day in urine samples of 9 volunteers (N)

and **M57** (methylhypolaetin glucuronide) were the most abundant metabolites detected in the urine samples of volunteers in the different days of the study.

Isoscutellarein derivatives were present as thirteen different metabolites; seven isoscutellarein derivatives (**M18**, **M43**, **M46**, **M24**, **M27**, **M29**, **M32**) and six methylisoscutellarein derivatives (**M62**, **M63**, **M7**, **M10**, **M4**, **M41**). In the *S. scardica* decoction, isoscutellarein was present with seven derivatives: three isoscutellarein and four methylisoscutellarein derivatives. Isoscutellarein derivatives **M18** (isoscutellarein sufate), **M32** (isoscutellarein glucuronide-sufate), **M7** (methylisoscutellarein glucuronide) and **M4** (methylisoscutellarein diglucuronide) were the dominant metabolites present in urine samples of all volunteers (Figure 5b).

It is noticeable that hypolaetin and isoscutellarein derivatives were mostly found (almost in all volunteers in all days) in the forms of sulfate (**M18**) or glucuronide-sulfate (**M11**, **M32**), whereas methylhypolaetin and methylisoscutellarein derivatives were mainly in the forms of glucuronides (**M56**, **M57**, **M7**, **M4**). This is an interesting finding that has to be further studied and compared with analogous flavonoids pairs.

As for the other most abundant group of polyphenols in the *S. scardica* decoction, phenylethanoid glycosides, it was found that they showed very low bioavailability after oral administration of the decoction rich with these compounds. This has also been pointed out previously and has been attributed to their poor absorption in the gastrointestinal tract [18]. In contrast, flavonoid glycosides have shown significant bioavailability, which has been previously also pointed out and attributed to the fact that hypolaetin and isoscutellarein posses hydroxyl groups at specific positions (3′ and 4′) [19].

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