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In vivo toxicity assessment of *Clinopodium vulgare* L. water extract characterized by UHPLC-HRMS

Dimitrina Zheleva-Dimitrova[a,*], Rumyana Simeonova[b], Reneta Gevrenova[a], Yonko Savov[c], Vessela Balabanova[a], Gazela Nasar-Eddin[b], Krum Bardarov[d], Nikolay Danchev[b]

[a] Department of Pharmacognosy, Faculty of Pharmacy, Medical University-Sofia, Bulgaria
[b] Department of Pharmacology, Pharmacotherapy and Toxicology, Faculty of Pharmacy, Medical University-Sofia, Bulgaria
[c] Institute of Emergency Medicine "N. I Pirogov", Bul. Totleben 21, Sofia, 1000, Bulgaria
[d] Institute of Molecular Biology and Biotechnology, 105 Ruski Bul., 4000, Plovdiv, Bulgaria

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ABSTRACT

Clinopodium vulgare L. (Lamiaceae) was used in the traditional Bulgarian medicine for treatment of wounds, diabetes and gastric ulcers. In this study we aimed at safety assessment of *C. vulgare* lyophilized water extract (CVE) characterized by ultra high-performance liquid chromatography–Orbitrap high resolution mass spectrometry (UHPLC-HRMS). The acute and sub-acute toxicity of CVE was determined in two rodent species (mice and rats), and two routes of administration – intraperitoneal (i.p.) and oral (p.o.). $LD_{50\ (i.p.)}$, were found to be 675 mg/kg (mice) and 500 mg/kg (rats). An acute i. p. administration resulted in central nervous system toxic effects. LD_{50} (p.o.) was higher than 2000 mg/kg for both species. In sub-acute oral administration, CVE did not exert any toxic effect on hematology, blood and urine biochemistry, and histomorphology in pancreas, liver, spleen and kidney. In addition, based on accurate masses, MS/MS and comparison with standards, a variety of flavonoids, caffeic acid oligomers and saponins were tentatively elucidated in CVE. Rosmarinic acid was the major compound. In conclusion, CVE did not cause hematological, biochemical and histopathological changes after oral administration and it is safe for internal use. The obtained UHPLC-HRMS profile revealed CVE as a new rich source of water soluble caffeic acid oligomers.

1. Introduction

The genus *Clinopodium* L. (Lamiaceae) consist of flowering plants, widely distributed in southern and southeastern Europe, North America, Latin America and Asia (Saltos et al., 2014). *Clinopodium vulgare* L. (wild basil) is a perennial aromatic plant with diverse ethnopharmacological applications used for treatment of diabetes, gastric ulcers, mastitis, prostatitis, skin irritation and swelling (Batsalova et al., 2017). Previous investigations revealed a variety of beneficial effects of *C. vulgare* extracts i.e. antibacterial, anti-inflammatory, antioxidant and anticancer activities (Tepe et al., 2007; Burk et al., 2009; Stefanovic et al., 2011; Batsalova et al., 2017). Recently, *C. vulgare* extract and its active constituents (catechin, caffeic and chlorogenic acids) were found to modulate cyclooxygenase-2 expression in neutrophils (Armirova et al., 2019). Despite the numerous pharmacological effects described for *C. vulgare*, there is no detailed investigation on its possible toxic effects.

With respect to *C. vulgare* chemical composition, there are a few earlier studies on phenolic acids, flavonoids, phenylpropanoids, caffeic acid oligomers, and saponins (Miyase and Matsushima, 1997; Obreshkova et al., 2001; Murata et al., 2009; Aoshima et al., 2012). *C. vulgare* essential oil composition was also established revealing the abundant quantity of thymol, γ-terpinene and *p*-cymene (Kokdil, 1998; Tepe et al., 2007).

Phytochemical screening using high-performance liquid chromatography coupled to mass spectrometry (LC-MS) quickly provides structural information, leading in many cases to the identification of compounds. This allows researchers to distinguish the known compounds (dereplication) and new molecules directly from the crude plant extracts. In particular, a hybrid quadrupole Orbitrap has high resolution and accuracy in both MS profiling and tandem mass spectrometry (Hostettmann et al., 2001).

The present study focused on the evaluation of the acute and sub-acute toxicity of *C. vulgare* lyophilized extract (CVE) on two rodent species - mice and rats. In addition, an in-depth characterization of CVE using UHPLC-HRMS Orbitrap acquisition strategy was performed.

* Corresponding author. Department of Pharmacognosy, Faculty of Pharmacy, Medical University-Sofia, 2 Dunav Str., 1000, Sofia, Bulgaria.
E-mail address: dimizheleva@gmail.com (D. Zheleva-Dimitrova).

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2. Material and methods

2.1. Chemicals and reagents

Apigenin-7-*O*-glucoside (**1**), luteolin-7-*O*-glucoside (**2**), luteolin-8-*C*-glucoside (orientin) (**3**), quercetin-3-*O*-glucoside (isoquercitrin) (**7**) and caffeic acid (**9**) were obtained from Extrasynthese (Genay, France), while rosmarinic acid (**13**) was purchased from PhytoLab (Vestenbergsgreuth, Germany). All reagents were of analytical grade.

2.2. Plant material

C. vulgare L. aerial parts were collected in July 2017 from region of German village near Sofia, Bulgaria (voucher specimen SO 107606). Air-dried powdered aerial parts (50 g) were triplicate extracted with water (500 ml) by ultrasound assisted extraction (15 min each time). A lyophilized *C. vulgare* extract (CVE) (5g) was used for phytochemical and pharmacological assays.

2.3. Experimental animals

Twenty two ICR female mice (22–24 g; 6 weeks old) and 40 female Wistar rats (180–230 g; 6 weeks old) were obtained from the National Breeding Center, Sofia, Bulgaria. The mice were housed in plexiglass cages (6 per cage), while rats were placed 3 per cage, in a 12/12 light/dark cycle, under standard laboratory conditions (ambient temperature 20 °C ± 2 °C and 72% humidity ± 4%) with free access to water and standard pelleted food 53-3, produced according ISO 9001:2008. Seven days acclimatization was allowed before the commencement of the study and a veterinary physician monitored the health of the animals regularly. The vivarium (certificate of registration of farm № 15320139/01.08.2007) was inspected by the Bulgarian Drug Agency in order to check the husbandry conditions (№ A-16-0532/14.10.2016). All performed procedures were approved by the Bulgarian Food Safety Agency (BFSA) (permissions № 190 and № 103/13.11.2017) and the principles stated in the European convention for the protection of vertebrate animals used for experimental and other scientific purposes (ETS 123, 1991) and were strictly followed throughout the experiment. Efforts were done to minimize the suffering of the animals.

2.4. Acute toxicity in mice and rats

To test the chemicals for acute toxicity, usually females are chosen because they are more sensitive than males (OECD 425, 2008). In our experiment the acute toxicity of CVE was assessed in 21 female mice and 24 female Wistar rats using simplified method of Chinedu et al. (2013) with modifications. We used 3 animals per dose, at 7 fixed-dose intervals with 2000 mg/kg being the highest dose. In order to calculate the index of absorption, the extract was administered by two routes of administration, oral and intraperitoneal at dose volume 0.1 mL/10 g body weight (bw) for mice and 0.5 mL/100 g bw for rats in the following doses: 2000, 1500, 1000, 800, 600, 400 and 200 mg/kg bw. Extract was easily dissolved in physiological saline buffer (PSB).

2.5. Sub-acute toxicity in rats

Based on the oral LD_{50} values, which was 2000 mg/kg for both species, two doses 50 mg/kg and 100 mg/kg (1/40 and 1/20 of LD_{50}) were selected for subacute toxicity test. Subsequent toxicity experiments were conducted with female Wistar rats in which CVE was administered daily for 14 days orally with a gastric tube at about the same time daily, 11.00 AM. The experiment also includes a control group of animals that were only saline-gavaged. CVE was dissolved in PSB and given in a volume of 0.5 mL/100 g bw. Animals were observed daily for behavioral changes and signs of toxicity.

2.6. Design of the in vivo subacute experiment

To assess the effect of the CVE 18 female rats were divided into three groups with six rats in each group (n = 6).

Group 1 – control animals;
Group 2 – animals treated with CVE 50 mg/kg, oral gavage (1/40 of LD_{50} = 2000 mg/kg p. o.)
Group 3 – animals treated with CVE 100 mg/kg, oral gavage (1/20 of LD_{50} = 2000 mg/kg p. o.)

At the end of the experiment (14th day) animals were placed in metabolic cages (Ugo Basile) and urine was collected for 24 h. On the next day after fasting overnight they were sacrificed by decapitation. Blood and urine were collected for haematological and biochemical analysis. Livers, kidneys, pancreas, spleen and brains were taken for histological analysis. Oxidative stress markers malondialdehyde (MDA), reduced glutathione (GSH), antioxidant enzyme activity of catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) were also measured in liver, kidney and brain homogenate.

For all the following experiments the excised organs were perfused with saline solution (0.9% NaCl, 40 °C), blotted dry, weighed, and homogenized with corresponding buffers.

2.7. Markers of oxidative stress

The quantity of thiobarbituric acid reactive substances (TBARS) (expressed as MDA equivalents) was assessed by the method described by (Polizio and Peña, 2005) and Bump et al. (1983). The antioxidant enzymes activity was measured in the supernatant of 10% homogenates, prepared in 0.05 M phosphate buffer (pH = 7.4). The protein content of investigated organ's homogenate was measured by the method of Lowry et al., 1951. Catalase activity was determined by measuring the decrease in absorbance at 240 nm and expressed as U/mg protein, as described by Aebi (1974). Superoxide dismutase (SOD) activity was measured according to the method of Misura and Fridovich (1972). Glutathione peroxidase activity (GPx) was assessed by NADPH oxidation, using a coupled reaction system consisting of reduced glutathione (GSH), glutathione reductase (GR), and cumene hydroperoxide (Tappel, 1978).

2.8. Blood hematological, serum biochemical parameters, urine analysis and histological investigation

White blood cells number (WBC), red blood cells count (RBC, Er), platelets (PLT) number, hemoglobin (Hb), and hematocrit (Ht) were measured using commercial kits for semi-automated hematological analyzer (BC-2800 Vet, Mindray, China) following the instructions of the manufacturer. Blood glucose level (Glu), cholesterol (Chol), triglycerides (Tgs), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), urea (U), creatinine (Cr), total protein (TP) and albumin (Alb) were measured using commercial kits for automated biochemical analyzer (BS-120, Mindray, China) following the instructions of the manufacturer. pH, protein, bilirubin, urobilinogen, nitrites, blood, glucose and ketones were evaluated using Urinalysis Reagent Strips (Condor-Teco Medical Technology - China).

For histological examination, small parts of the pancreas, liver, kidney, spleen and brain from control animals (n = 6) and rats treated with the higher dose of CVE 100 mg/kg (n = 6), were fixed in 10% buffered formalin. Thin sections (4 nm) were stained with hematoxylin/eosin (Bancroft and Gamble, 2002), then examined and evaluated under a light microscope (DMR + 550, Leica, Wetzlar, Germany).

2.9. Statistical analysis

Statistical programme 'MEDCALC' was used for analysis of the in

vivo data. The results are expressed as mean ± SEM for six animas in each group. The significance of the data was assessed using the non-parametric Mann–Whitney *U* test. Values of $p \leq 0.05$ were considered statistically significant.

2.10. Ultra high-performance liquid chromatography – orbitrap high resolution mass spectrometry (UHPLC-HRMS)

A lyophilized hot water extract was analyzed by UHPLC-HRMS using a quaternary pump and a hybrid quadrupol-"Orbitrap" high resolution "Q-Exactive" mass spectrometer coupled with a HESI (heated electrospray ionization) probe. The chromatographic separation was performed on RP (reversed phase) "Poroshell" C18 3 × 150 mm 2.7 μm column using as eluents: (A) 0.08% formic acid in water and (B) 0.08% formic acid in acetonitrile at flow rate of 250 μl/min. The elution program commenced at 10% B followed by a linear gradient to 95% B for 50 min. HESI worked at 250 °C, spry voltage 3 kV, ion transfer tube at 300 °C, sheath gas pressure 45 Psi and mass tolerance of 5 ppm. The mass analyzer scanned over a mass range 100–1500 Da in Full MS-ddMS2 scan type and negative ion mode. The higher energy collision-induced dissociation was set at 25. The identification of the studied compounds was based on the accurate masses, MS/MS data and comparison with fragmentation fingerprints observed for the reference standards, and literature data. Data acquisition and processing were carried out with XCalibur® software package (ThermoScientific Co, Waltham, MA, USA). The proposed chemical structures were drawn using the software ChemBioDraw Ultra 12.0.

3. Results

3.1. Toxicological assessment

3.1.1. Acute toxicity after intraperitoneal administration (i.p.)

Effects of CVE after intraperitoneal administration to mice and rats are presented in Tables 1 and 2. Animal death occurred as a result of difficulty in breathing, which appeared within 15 min–24 h after administration of the substance. In animals treated with 600/400 mg/kg bw, rapid respiration occurred, and was resolved after about an hour. The highest doses at which no death occurred was 600 mg/kg bw for mice and 400 mg/kg bw for rats. The lowest doses that produced mortality was 800 mg/kg for mice and 600 mg/kg for rats. Based on the obtained results, LD_{50} i. p. was calculated according to the simplified formula of Chinedu et al. (2013) and it was found to be 700 mg/kg bw and 500 mg/kg for mice and rats, respectively.

3.1.2. Acute toxicity after oral administration of CVE

CVE was administered orally at an initial dose of 2000 mg/kg bw, at a dose volume of 0.1 mL/10 g bw to rats and 0.5 mL/100 g bw to 3 mice. Within 24 h, all animals survived acute oral treatment with CVE without apparent symptoms. Based on the obtained results, LD_{50} p. o. for CVE is above 2000 mg/kg for mice and rats, so it could be classified as less dangerous or non-toxic when administered orally. The index of resorption was calculated according to the equation:

$$IR_{mice} = LD_{50} \text{ i.p.}/LD_{50} \text{ p.o. x } 100\ (\%) = 700/2000 \text{ x } 100 = 35\%$$

$$IR_{rats} = LD_{50} \text{ i.p.}/LD_{50} \text{ p.o. x } 100\ (\%) = 500/2000 \text{ x } 100 = 25\%$$

3.1.3. Sub-acute toxicity in rats and mice

For multiple oral administration (14 days), two doses of CVE 50 mg/kg and 100 mg/kg were used, or 1/40 and 1/20 from oral LD_{50}, which was 2000 mg/kg per os for both species. Animals were daily gavaged with the tested extract at the dose volume of 0.5 mL/100 g bw with a gastric tube.

3.1.4. Urine analysis

Number of leucocytes, erythrocytes, the presence of proteins and nitrites, the concentrations of urobilinogen, bilirubin, ketones, and glucose, as well as pH of the urine of experimental animals were measured. Significant deviations from the control group were not observed (Table 3).

3.1.5. Hematological and serum biochemical parameters

The results of repeated 14-day administration of CVE on haematological and biochemical parameters are presented in Tables 4 and 5. Most of the haematological parameters (hemoglobin, hematocrit, erythrocytes, leukocytes, platelets) in treated rats did not differ significantly from the controls. Both doses administration of CVE did not produce any changes in serum biochemical parameters. No statistically significant differences in hepatic function parameters (ALT, AST, ALP) were observed. There were no significant changes in total protein and albumin, as well as in blood glucose and lipid profile (Table 5).

3.1.6. Markers of oxidative stress

Markers of oxidative stress MDA, GSH, CAT, SOD and GPx, measured in liver, kidney and brain are presented on Table 6. The results showed that the CVE in both doses did not cause an oxidative misbalance in investigated organs. Even more, in the kidneys, the higher dose of CVE produced a statistically significant decrease ($p < 0.05$) of MDA quantity by 22.4%, increase of GSH level by 22%, increase in CAT and SOD activity by 38%, and by 16% respectively, compared to the control group. In the liver, the higher dose of CVE increased significantly ($p < 0.05$) the GSH content by 20% and CAT activity by 14%, compared to the matched control group.

3.1.7. Histological evaluation

Histological changes in organ sections were observed under a light microscope and no significant changes were noted between control and higher dose CVE-treated group. The morphology of renal glomeruli was normal and no basal membrane thickening or infiltration of inflammatory cells were observed (Fig. 1d). The microscopic images of the liver, spleen, and pancreas showed normal borders (Fig. 1b, i and 1j). Mild perivascular and peri-cellular edema on the brain were noticed (Fig. 1i). These changes could be associated with the method of killing animals.

Table 1
Effects after intraperitoneal administration of CVE to mice.

Dose, mg/kg bw	Effects		
	Lethality	Time of occurrence	Clinical signs
2000	3/3 (100%)	15–30 min after administration	Difficulty breathing, ataxia
1500	3/3 (100%)		
	2/3	30–45 min after administration	Difficulty breathing, ataxia
	1/3	15–20 h after administration	Difficulty breathing, somnolence, ataxia
1000	2/3	6–8 h after administration	Difficulty breathing, ataxia
800	1/3	15–20 h after administration	Ataxia
600	0/3 (0%)	–	Accelerated breathing, observation up to 14 days. Lack of macroscopic changes in internal organs
400	0/3 (0%)		Observation up to 14 days. Lack of macroscopic changes in internal organs

Table 2
Effects after intraperitoneal administration of CVE to female Wistar rats.

Dose, mg/kg bw	Effects		
	Lethality	Time to occurrence	Clinical signs
2000	3/3 (100%)	10–20 min after administration	Difficulty breathing, ataxia
1500	3/3 (100%)		
	2/3	20–60 min after administration	Difficulty breathing, ataxia
	1/3	1–2 h after administration	Difficulty breathing, ataxia
1000	3/3 (100%)		
	1/3	30 min after administration	Difficulty breathing, ataxia
	2/3	20 h after administration	Difficulty breathing, ataxia
800	2/3	20 h after administration	Difficulty breathing, ataxia
600	1/3	24 h after administration	Difficulty breathing, ataxia
400	0/3 (0%)		Somnolence, suppressed motor activity
200	0/3 (0%)		Somnolence

Table 3
Changes in urine parameters.

Parameters	Controls	CVE 50 mg/kg	CVE 100 mg/kg
LEU leu/μL	5.00 ± 7.75	10.00 ± 7.75	10.00 ± 7.75
NIT	–	–	–
URO mmol/L	2.63 ± 1.75	1.75 ± 2.02	1.75 ± 2.02
PRO	–	–	–
pH	7.0 ± 0.41	6.88 ± 0.25	7.13 ± 0.25
BLO Er/μL	–	–	–
KET mmol/L	0.375 ± 0.25	0.125 ± 0.25	0.250 ± 0.29
BIL μmol/L	–	–	–
GLU mmol/L	–	–	–

Treatment: as described in the experimental design section.

Table 4
Changes in hematological parameters.

Hematological parameters	Controls	CVE 50 mg/kg	CVE 100 mg/kg
WBC (*10^9)/L	6.5 ± 1.90	4.2 ± 1.10	5.3 ± 2.1
RBC (*10^12)/L	8.6 ± 0.70	9.2 ± 0.10	8.12 ± 1.2
HGB g/L	140.7 ± 20.4	157 ± 5.20	142.6 ± 4.3
HCT %	44.4 ± 2.20	45.9 ± 1.10	42.3 ± 2.06
PLT (*10^9)/L	734.8 ± 84.4	647.3 ± 75.1	812.2 ± 36.2

Treatment: as described in the experimental design section.
Data are expressed as mean ± SEM of six rats ($n = 6$). For comparison between groups Mann—Whitney *U* test was performed.

Table 5
Changes in serum biochemical parameters.

Serum biochemical parameters	Controls	CVE 50 mg/kg	CVE 100 mg/kg
Urea mmol/L	10.05 ± 0.56	10.98 ± 0.65	9.3 ± 2.30
Creat μmol/L	65.6 ± 3.25	62.9 ± 1.66	63.6 ± 4.32
ALB g/L	50.3 ± 3.76	56.6 ± 5.45	55.8 ± 2.63
TP g/L	75.7 ± 1.37	72.23 ± 1.96	69.2 ± 4.55
AST U/L	20.8 ± 2.70	18.1 ± 5.6	18.8 ± 2.6
ALT U/L	121.9 ± 5.10	122.7 ± 3.16	131.3 ± 7.3
ALP U/L	137.1 ± 6.6	143.9 ± 12.3	148.8 ± 11.8
Cholesterol mmol/L	1.49 ± 0.21	1.36 ± 0.42	1.26 ± 0.86
Triglicerides mmol/L	1.46 ± 0.16	1.35 ± 0.12	1.47 ± 0.21
Blood glucose mmol/L	5.3 ± 0.70	4.9 ± 0.82	5.6 ± 0.63

Treatment: as described in the experimental design section.
Data are expressed as mean ± SEM of six rats ($n = 6$). For comparison between groups Mann—Whitney *U* test was performed.

3.2. UHPLC-HRMS profiling of CVE

The total ion chromatogram of CVE was given on Fig. 2. Based on the HRMS and tandem mass spectrometry (MS/MS) data, as well as comparison with reference standards and literature, fifty three metabolites (7 flavonoids, 41 caffeic acids derivatives and 5 saponins) were identified or tentatively assigned in CVE (Table 7, Fig. 3). The accurate and exact masses of the deprotonated molecules $[M-H]^-$ and characteristic fragment ions corresponding to each compound are depicted in Table S1.

Table 6
Markers of oxidative stress.

		Control	CVE 50 mg/kg	CVE 100 mg/kg
MDA nmol/g tissue	Liver	4.45 ± 0.29	4.18 ± 0.16	4.3 ± 0.29
	Kidney	4.46 ± 0.28	4.18 ± 0.18	3.46 ± 0.38[ab]
	Brain	5.67 ± 0.25	5.22 ± 0.2	5.54 ± 1.2
GSH nmol/g tissue	Liver	6.62 ± 0.38	7.28 ± 0.64	7.92 ± 0.28[a]
	Kidney	4.46 ± 0.29	5.14 ± 0.58	5.44 ± 0.23[a]
	Brain	1.69 ± 0.17	1.43 ± 0.34	2.03 ± 0.69
CAT U/mg protein	Liver	42.13 ± 1.14	44.98 ± 3.62	47.95 ± 2.57[a]
	Kidney	16.33 ± 1.02	19.48 ± 1.85	22.48 ± 0.5[a]
	Brain	26.75 ± 1.26	25.08 ± 2.19	27.53 ± 0.58
SOD nmol/mg/min	Liver	0.298 ± 0.037	0.265 ± 0.025	0.312 ± 0.015
	Kidney	0.225 ± 0.005	0.208 ± 0.017	0.26 ± 0.018[ab]
	Brain	0.318 ± 0.022	0.308 ± 0.009	0.345 ± 0.031
GPx nmol/mg/min	Liver	0.428 ± 0.014	0.409 ± 0.043	0.45 ± 0.026
	Kidney	0.237 ± 0.013	0.187 ± 0.012[a]	0.248 ± 0.041[b]
	Brain	0.332 ± 0.010	0.305 ± 0.024	0.345 ± 0.037

Treatment: as described in the experimental design section.
Data are expressed as mean ± SEM of six rats ($n = 6$). For comparison between groups Mann—Whitney *U* test was performed.
[a]$p < 0.05$ vs control group; [b]$p < 0.05$ vs CVE 50 mg/kg group.

3.2.1. Flavonoids

Compounds **1**, **2**, **3**, and **7** were identified based on comparison with reference standards. The fragmentation fingerprint of **4** ($[M-H]^-$ at *m/z* 447.094) showed a base peak at *m/z* 271.061, resulting from the loss of hexuronic acid [M-H-176]$^-$. Fragment ions at *m/z* 151.002 ($^{1,3}A^-$), 119.049 ($^{1,3}B^-$) and 107.0122 ($^{0,4}A^-$) were attributed to the Retro-Diels Alder (RDA) cleavages of the flavane skeleton suggesting only one hydroxyl group in ring B and absence of both a double bond and a substituent at the C-3. Consequently, **4** was tentatively identified as naringenin-*O*-hexouronide. Two isobaric flavonoids **5** and **6** shared the same $[M-H]^-$ at *m/z* 593.151 (exact mass). **5** yielded low abundant ions at *m/z* 447.092 [M-H-dHex]$^-$ and a base peak at *m/z* 285.040 [M-H-Hex-dHex]$^-$, indicating the presence of hexose and deoxyhexose moieties. The inner hexose loss evidenced *O*-glycosilation at *C*-7 of the flavon skeleton. The assignment of the disaccharidic chain was based on the principles of Cuyckens and Claeys (2004). The absence of the interglycosidic linkage breakdown is favoured for 7-neohesperidoside. Fragment ions at *m/z* 133.028 ($^{1,3}B^-$), 151.003 ($^{1,3}A^-$) and 107.012 ($^{0,4}A^-$) were attributed to the RDA cleavages of luteolin (de Rijke et al., 2006). Thus **5**, was tentatively identified as luteolin-*O*-neohesperidoside. In the same manner, **6** yielded fragment ions at *m/z* 431.1347 [M-H-Hex]$^-$ and *m/z* 285.077 [M-H-Hex-dHex]$^-$together with *m/z* 533.168

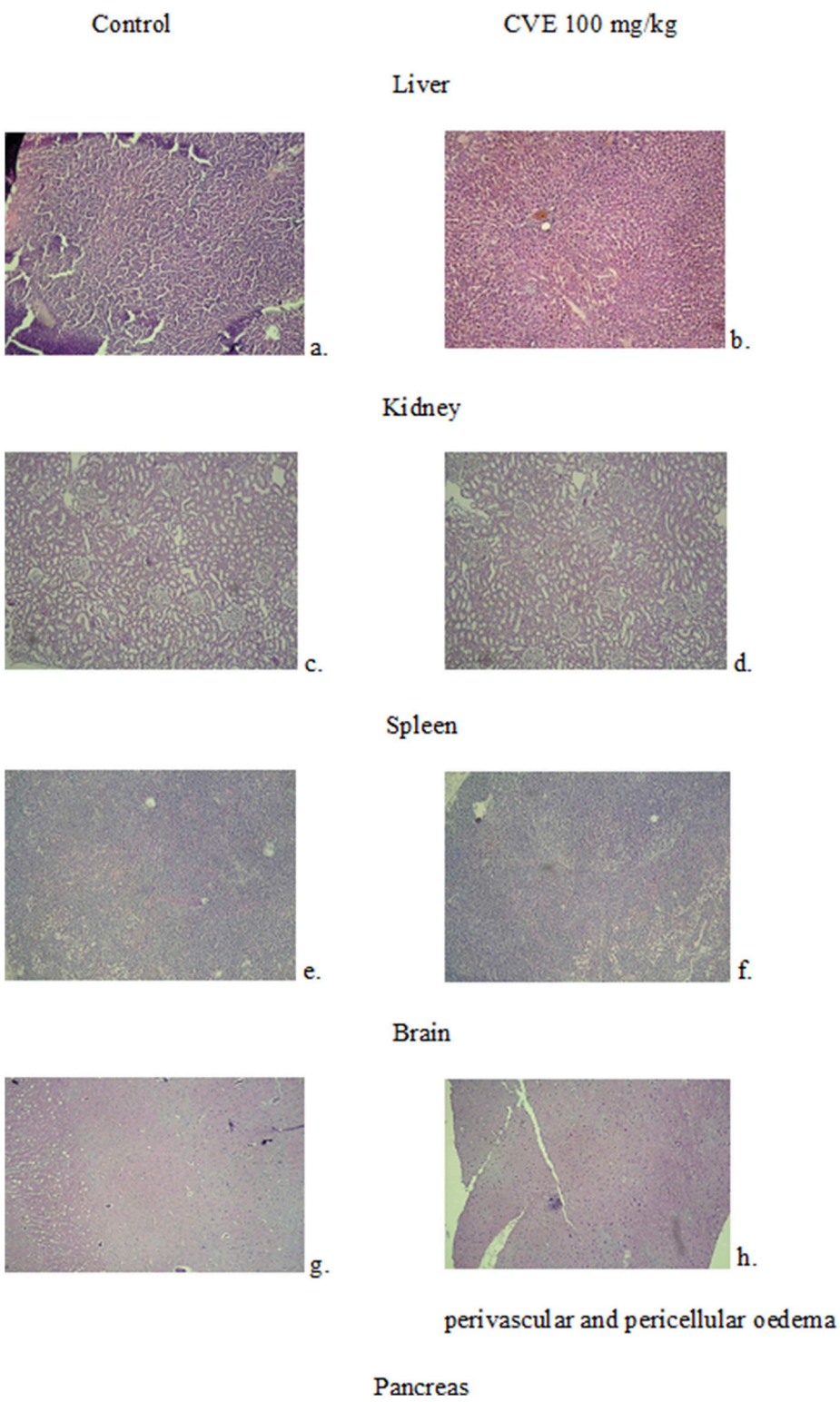

Fig. 1. Histology of liver, kidney, spleen, pancreas and brain of control rats and rats treated with 100 mg/kg CVE.

([M-H-60]$^-$) (Table S1). Regarding the aglycone, the loss of methyl group at *m/z* 179.034 and RDA fragmentation at *m/z* 119.049 ($^{1,3}B^-$) and *m/z* 179.034. ($^{0,4}A^-$) suggested the presence of methoxy group in ring B (de Rijke et al., 2006). Based on these data, **6** was tentatively elucidated as isosakuranetin-7-*O*-neohesperidoside.

3.2.2. Caffeic acids monomers

According to the number of aromatic rings, caffeic acids derivatives in CVE could be classified into monomers and oligomers (dimers, trimmers and tetramers). Oligomers consist of ester-bonded monomers such as danshensu, caffeic acid and others (Liu et al., 2007) (Fig. 3). Based on the, accurate masses, MS/MS data, and literature data, 2 monomers, 7 dimers, 23 trimers and 9 tetramers were tentatively identified. Fragmentation patters and retention times of caffeic (**7**) and rosmarinic acid (**13**) were compared with reference standards. The used nomenclature was adapted to Liu et al. (2007).

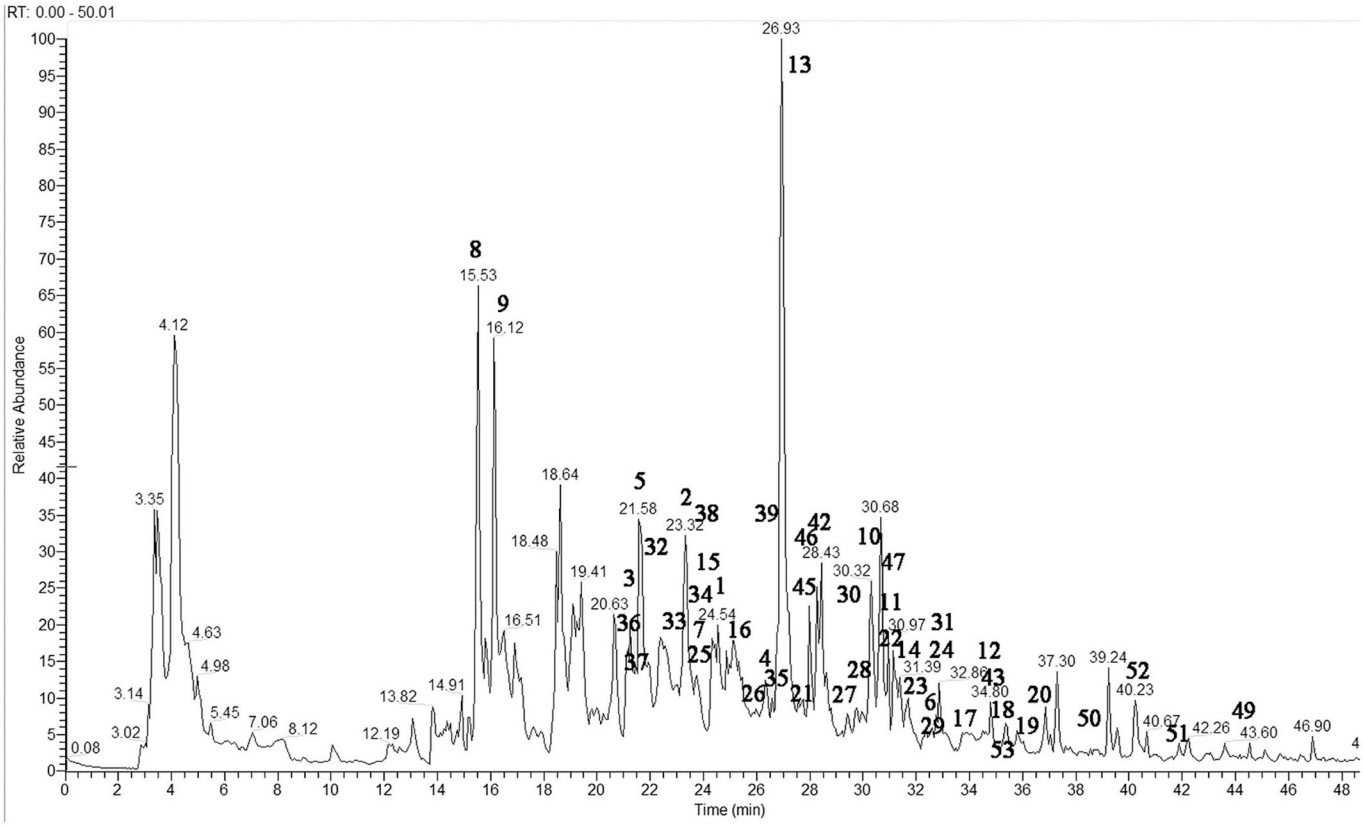

Fig. 2. Total ion chromatogram of CVE in negative ion mode. For compounds number see Table 7.

The [M-H]$^-$ at *m/z* 197.045 of the monomer salvianic acid (danshensu; **8**), produced prominent ions resulting from the loss of H_20 at *m/z* 179.033 and CO_2 at *m/z* 135.043. However, for the other oligomers, the predominant fragment ions derived from the cleavage of *a* and *b* ester bonds with loss of danshensu [M-H-198]$^-$ and caffeic acid [M-H-180]$^-$, respectively (Liu et al., 2007).

3.2.3. Caffeic acid dimers

Dimers included rosmarinic acid (**13**), salvianolic acids F (**10**), G (**12**) and D (**13**, **14**), and clinopodic acids A (**11**) and B (**14**). In MS/MS, rosmarinic acid produced low abundant ion at *m/z* 197.044 [M-H-162]$^-$, and a base peak at *m/z* 161.023 [M-H-198]$^-$, corresponding to the cleavage of *a* bond (Fig. 3, Table S1). Regarding peaks **10**, **12**, **15** and **16**, decarboxylation resulted in diagnostic fragment ions [M-H-CO_2]$^-$. Furthermore, **10** produced more intensive fragment ion corresponding to the *a* bond cleavage at *m/z* 133.028, [M-H-180]$^-$ (37.25%) compared to *b* bond cleavage at *m/z* 117.523 [M-H-198]$^-$ (0.05%). In addition, caffeoyl residue yielded abundant ions at *m/z* 179.033 and 135.043. Consequently, **10** was assigned as salvianolic acid F (Liu et al., 2007). Isobaric pair **15**/**16** produced a base peak at *m/z* 175.038 [M-H-198-44]$^-$, suggesting the presence of second carboxyl group. This fragmentation behaviour together with a low intensive fragment ion at 237.040 [M-H-180]$^-$ were consistent with previously described salvianolic acid D (Liu et al., 2007). It seems that –CH_2COOH residue restrains the *b* bond cleavage. Fragmentation pathway of **11** matched that of rosmarinic acid (**13**), except for the lack of one hydroxyl group evidenced by the ion at *m/z* 145.027 [M-H-198]$^-$. *p*-Coumaroyl residue was found and **11** was ascribed to clinopodic acid A. **14** ([M-H]$^-$ at *m/z* 373.093), afforded prominent fragment ion at *m/z* 211.060 [M-H-162]$^-$, 14 Da more than danshensu residue, indicating methoxylation in the later residue. Thus, compound **14** was tentatively elucidated as clinopodic acid B.

3.2.4. Caffeic acid trimers

Four isobaric oligomers **17**–**20** shared the same [M-H]$^-$ at *m/z* 491.097 (exact mass). Due to the presence of benzofuran ring, **17**/**19** yielded a base peak at *m/z* 293.045 [M-H-198]$^-$, corresponding to the cleavage of *a* bond. Regarding **19**, low abundant ions at *m/z* 265.050 [M-H-198-CO]$^-$ and 249.055 [M-H-198-CO_2]$^-$ supported by an ion at *m/z* 311.056, were consistent with fragmentation pathway of salvianolic acid C (Liu et al., 2007). Similar fragmentation pathway was observed for **17**, except for the abundant ion at *m/z* 311.056 resulting from the easier cleavage of *b* bond than in **19**. Consequently **17** was assigned as salvianolic acid C isomer. In contrast, isobaric pair **18**/**20** afforded a base peak at *m/z* 311.056, corresponding to the easier loss of danshensu, probably due to the presence of dibenzooxepin structure, which restrains the cleavage of *a* bond. Based on comparison with literature data, **18**/**20** were tentatively identified as isosalvianolic acid C and its isomer, respectively (Liu et al., 2007).

The fragmentation of **21** [M-H]$^-$ at *m/z* 493.112 yielded base peak at *m/z* 295.313 together with diagnostic fragment ion at *m/z* 383.077, [M-H-110]$^-$, corresponding to the loss 3,4-dihydroxyphenyl residue (Liu et al., 2007). The aforementioned fragment pointed to the presence of –CH_2=CH_2 instead of furan ring in **19.** Thus, **21** was related to salvianolic acid A. Its isobars **22**–**24** yielding a base peak at *m/z* 161.023 could be ascribed to salvianolic acid A isomers.

MS/MS spectra of five isobars **25**–**29** ([M-H]$^-$ at *m/z* 537.102) were acquired. In compound **29**, there was one more carboxyl group attached to the benzofuran ring in comparison with the structure of salvianolic acid C (**19**). In fragmentation pathway of **29,** diagnostic ion at *m/z* 493.114 [M-H-CO_2]$^-$ was supported by ions at *m/z* 331.082 [M-H-44-162]$^-$ (100%) and *m/z* 295.065 [M-H-44-198]$^-$ corresponding to the concomitant losses of CO_2, caffeoyl and danshensu residues, respectively. This fragmentation was indicative for carboxyl group attached to the furan ring and was consistent with lithospermic acid A (Liu et al., 2007). Concerning **27**–**28**, the base peak at *m/z* 339.059 [M-H-198]$^-$

Table 7
Metabolites detected in *C. vulgare* extract.

Peak №	Exact mass	Formula	Number of the separated isobaric isomers	t_R min	Tentative assignment	Reference standard/Reference
	Flavonoids					
1	431.0983	$C_{21}H_{20}O_{10}$	1	25.46	apigenin-7-*O*-glucoside	*
2	448.1000	$C_{21}H_{20}O_{11}$	3	21.09	luteolin-8-*C*-glucoside (orientin)	*
3				23.21	luteolin-7-*O*-glucoside	*
4				26.41	naringenin-*O*-hexouronide	
5	464.0949	$C_{21}H_{20}O_{12}$	1	24.45	quercetin-3-*O*-glucoside (isoquercitrine)	*
6	594.1579	$C_{27}H_{30}O_{15}$	2	21.31	luteolin-*O*-neohesperidoside	
7				32.71	isosakuranetin-7-*O*-neohesperidoside	
	Caffeic acids derivatives **Caffeic acid monomers**					
8	198.0446	$C_9H_{10}O_5$	1	15.48	salvianic acid A (danshensu)	Liu et al. (2007)
9	180.0417	$C_9H_8O_4$	1	16.64	caffeic acid	*
	Caffeic acid dimers					
10	314.0785	$C_{17}H_{14}O_6$	1	30.54	salvianolic acid F	Liu et al. (2007)
11	344.0891	$C_{18}H_{16}O_7$	1	30.85	clinopodic acid A	
12	340.0578	$C_{18}H_{12}O_7$	1	34.76	salvianolic acid G	Liu et al. (2007)
13	360.0840	$C_{18}H_{16}O_8$	1	26.99	rosmarinic acid	*
14	374.0996	$C_{19}H_{18}O_8$	1	31.76	clinopodic acid B	
15	418.0894	$C_{20}H_{18}O_{10}$	2	24.50	salvianolic acid D isomer	Liu et al. (2007)
16				24.94	salvianolic acid D	Liu et al. (2007)
	Caffeic acid trimers					
17	492.0993	$C_{26}H_{20}O_{10}$	4	33.87	salvianolic acid C isomer	Liu et al. (2007)
18				35.61	isosalvianolic acid C	Liu et al. (2007)
19				36.10	salvianolic acid C	Liu et al. (2007)
20				36.69	isosalvianolic acid C isomer	Liu et al. (2007)
21	494.1207	$C_{26}H_{22}O_{10}$	4	27.10	salvianolic acid A	Liu et al. (2007)
22				30.74	salvianolic acid A isomer	Trifan et al. (2018)
23				31.52	salvianolic acid A isomer	Trifan et al. (2018)
24				32.81	salvianolic acid A isomer	Trifan et al. (2018)
25	538.1106	$C_{27}H_{22}O_{12}$	5	23.87	salvianolic acid T/U	Li et al. (2014)
26				26.17	salvianolic acid J/clinopodic acid C/E	Liu et al. (2007)
27				28.80	salvianolic acid H/I	Liu et al. (2007)
28				29.78	salvianolic acid H/I	Liu et al. (2007)
29				32.51	lithospermic acid A/clinopodic acid J/N	Liu et al. (2007)
30	552.1262	$C_{28}H_{24}O_{12}$	2	30.32	methoxy-lithospermic acid/clinopodic acid D/F	Liu et al. (2007)
31				31.82	methoxy-salvianolic acid H/I	Liu et al. (2007) Liu et al. (2007)
32	556.1211	$C_{27}H_{24}O_{13}$	4	21.64	salvianolic acid K isomer	Lu and Foo (1999)
33				23.21	salvianolic acid K isomer	Lu and Foo (1999)
34				24.24	salvianolic acid K isomer	Lu and Foo (1999)
35				26.77	salvianolic acid K	Lu and Foo (1999)
36	572.1161	$C_{27}H_{24}O_{14}$	3	21.05	yunnaneic acid E isomer	Liu et al. (2007)
37				21.26	yunnaneic acid E	Liu et al. (2007)
38				23.39	yunnaneic acid E isomer	Liu et al. (2007)
39	700.1634	$C_{33}H_{32}O_{17}$	1	26.04	clinopodic acid Q	Aoshima et al. (2012)
	Caffeic acid tetramers					
40	718.1528	$C_{36}H_{30}O_{16}$	5	24.44	salvianolic acid E	Liu et al. (2007)
41				26.23	yunnaneic acid G	
42				28.33	salvianolic acid L	Chen et al. (2011)
43				34.80	isosalvianolic acid B/salvianolic acid B/lithospermic acid B/ clinopodic acid I	Chen et al. (2011)
44				35.30	isosalvianolic acid B/salvianolic acid B/lithospermic acid B/ clinopodic acid I	Liu et al. (2007); Chen et al. (2011); Murata et al. (2009)
45	720.1685	$C_{36}H_{32}O_{16}$	1	27.05	sagerinic acid	Lu and Foo (1999)
46	1076.2217	$C_{54}H_{44}O_{24}$	2	28.33	clinopodic acid K	Aoshima et al. (2012)
47				29.68	clinopodic acid O	Aoshima et al. (2012)
48	1078.2373	$C_{54}H_{46}O_{24}$		30.74	yunnaneic acid A	Tanaka et al. (1996)
Saponins						
49	780.4654	$C_{42}H_{68}O_{13}$	1	43.04	saikogenin F-3-*O*-hexosyl-*O*-deoxyhexoside (clinoposaponin XV)	Miyase and Matsushima (1997)
50	912.5077	$C_{47}H_{75}O_{17}$	1	38.99	saikogenin F-*O*-Hex, dHex, Pent	
51	940.5026	$C_{48}H_{76}O_{18}$	1	41.59	clinoposaponin XVIII	Miyase and Matsushima (1997)

(*continued on next page*)

Table 7 (*continued*)

Peak №	Exact mass	Formula	Number of the separated isobaric isomers	t_R min	Tentative assignment	Reference standard/Reference
	Flavonoids					
52	942.5182	$C_{48}H_{78}O_{18}$	1	40.23	saikogenin F-3-*O*-(hexosyl-[*O*-hexosyl]-deoxyhexoside (buddlejasaponin IV))	Liu et al. (1995)
53	1265.6189	$C_{60}H_{98}O_{28}$	1	35.62	saikogenin F-3-*O*-(hexosyl-[*O*-hexosyl-*O*-hexosyl-*O*-hexosyl]-deoxyhexoside (clinoposaponin V))	Yamamoto et al. (1993)

*Compared to reference standard.

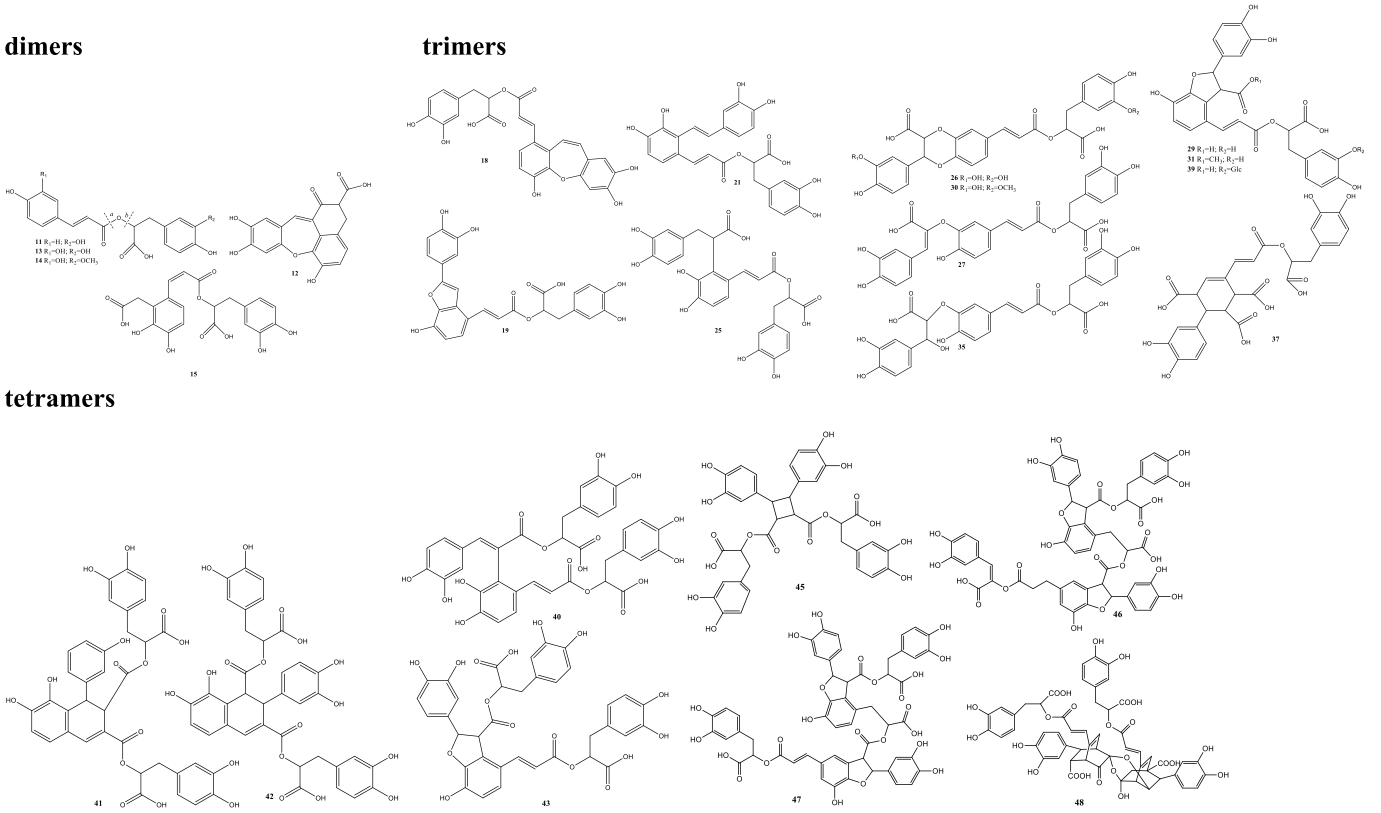

Fig. 3. Structures of the caffeic acid oligomers, identified in *C. vulgare* extract.

was registered indicating that danshensu is easier to loss than carboxyl in **29**. Moreover, $[M\text{-}H\text{-}CO_2]^-$ was not detected, assuming the lack of furan ring in both compounds. This allowed us to deduce a linear structure, and **27**/**28** were tentatively identified as salvianolic acid H/I (Liu et al., 2007). Regarding **25**, a prominent ion at *m/z* 357.062 $[M\text{-}H\text{-}180]^-$ and a base peak at *m/z* 297.076 $[M\text{-}H\text{-}180\text{-}CH_3COOH]^-$ are in good agreement with the structure of salvianolic acid T/U (Li et al., 2014). Compound **26** yielded a diagnostic ion at *m/z* 493.115 $[M\text{-}H\text{-}CO_2]^-$, indicative for carboxyl group attached to the heterocycle structure. An abundant ion at *m/z* 295.061 $[M\text{-}H\text{–}CO_2\text{–}198]^-$, resulted from the easily *b* bond cleavage. The fragmentation pathway of **26** and the base peak at *m/z* 197.0446 (loss of the danshensu residue) showed a stable *a* bond, and corresponded to salvianolic J/clinopodic acid C/E (Liu et al., 2007; Murata et al., 2009).

Peaks **30** and **31** ($[M\text{-}H]^-$ at *m/z* 551.117) differed from **27**–**29** with 14 Da and gave a fragment ion at 507.130 ($[M\text{-}H\text{-}44]^-$), suggesting their methoxylated derivatives. The MS/MS spectra of **30** yielded ion at *m/z* 463.140 $[M\text{-}H\text{-}44]^-$ indicating carboxyl group attached to the furan ring as was seen in **29**. Thus, **30** was tentatively identified as methoxy-lithospermic acid. In the same manner **31** was ascribed to methoxy-salvianolic acid H/I (Liu et al., 2007).

In MS/MS spectra of **35** ($[M\text{-}H]^-$ at *m/z* 555.113), a base peak was observed at *m/z* 357.061 $[M\text{-}198]^-$, together with the prominent ion at *m/z* 359.114, corresponding to the loss of 3-(3,4-dihydroxyphenyl)-3-hydroxypropanoic acid (Lu and Foo, 1999). The isobars of **35** (**32**–**34**) demonstrated similar fragmentation pathway, but different base peaks were registered. Thus, **35** and **32**–**34** were tentatively elucidated as salvianolic acid K and its isomers, respectively (Lu and Foo, 1999).

The MS/MS spectrum of **37** ($[M\text{-}H]^-$ at *m/z* 571.108) included three consecutive losses of CO_2 and danshensu, and was consistent with yunnaneic acid E (Liu et al., 2007). Isobars **36** and **38** produced the same fragments, but with lower intensity and were identified as yunnaneic acid E isomers. Fragmentation pathway of **39** ($[M\text{-}H]^-$ at *m/z* 699.155) was similar to those of lithospermic acid (**29**) except for the presence of hexosyl moiety at *m/z* 493.115 $[M\text{-}H\text{–}CO_2\text{–}Hex]^-$. Consequently, **39** was assigned to clinopodic acid Q (Aoshima et al., 2012).

3.2.5. Caffeic acid tetramers

Five isobaric tetramers **40**–**44** shared the same $[M\text{-}H]^-$ at *m/z* 717.145 (exact mass). **40** gave abundant ions at *m/z* 519.094 $[M\text{-}H\text{-}198]^-$, 339.051 $[M\text{-}H\text{-}198\text{-}180]^-$ and a base peak at *m/z* 161.023 $[RA\text{-}198]^-$, corresponding to linear connection of two rosmarinic acids. In fragmentation pathway of **41** a base peak at *m/z* 475.103 $[M\text{-}H\text{-}198\text{-}44]^-$ was in line with the presence of condensed system in the structure.

Thus, **40** and **41** corresponded to salvianolic acid E and yunnaneic acid G, respectively (Liu et al., 2007). **42** yielded a base peak at *m/z* 321.040 $[M\text{-}2x198]^-$ showing an easier loss of two danshensu residues compared to **41**, probably due to the symmetrical position of catechol residues. In addition, the fragmentation pathway of **42** was in accordance with those of previously described for salvianolic acid L (Chen et al., 2011). The MS/MS spectra of **43** gave prominent ions at *m/z* 519.093 $[M\text{-}H\text{-}198]^-$ and 491.0992 $[M\text{-}H\text{-}198\text{-}CO]^-$, corresponding to the consecutive *α* bond cleavage and loss of carboxyl group attached to the furan ring. Thus, **43** was assigned as isosalvianolic acid B. **44**, yielded a base peak at m/z 357.061 [M-2x180] and could be related to salvianolic acid B/lithoospermic acid B/clinopodic acid I (Liu et al., 2007; Chen et al., 2011; Murata et al., 2009). Fragmentation pathway of **45** matched that of rosmarinic acid (**13**). Due to the absence of fragment ions $[M\text{-}H\text{-}180]^-$ and $[M\text{-}H\text{-}198]^-$, **45** could could be associated with sagerinic acid possessing a cyclobutan ring (Lu and Foo, 1999). Isobaric pair **46**/**47** gave base peaks at *m/z* 339.081 and 359.077, corresponding to salvianolic acid G and rosmarinic acid, respectively. Fragmentation pathway of **46** included prominent ions at *m/z* 877.157 $[M\text{-}H\text{-}198]^-$, 833.177 $[M\text{-}H\text{-}198\text{-}CO_2]^-$ and 679.114 $[M\text{-}H\text{-}2x198\text{-}CO_2]^-$ indicating consequently losses of two danshensu residues and carboxyl group. Moreover, diagnostic ions at *m/z* 519.093 $[M\text{-}H\text{-lythospermic acid-}H_2O]^-$ and 357.061 $[M\text{-}H\text{-lythospermic acid–}H_2O\text{–}162]$ suggested a terminal caffeoyl residue linked to lithospermic acid. In contrast to **46**, **47** yielded an abundant ion at *m/z* 661.099 $[M\text{-}H\text{-}180\text{-}198]^-$ corresponding to the presence of terminal salvianic acid A (danshensu). Thus **46** and **47** were tentatively identified as clinopodic acid K and clinopodic acid O, respectively (Aoshima et al., 2012). Based on the accurate mass, molecular formula and fragmentation pattern, **48** corresponded to yunnaneic acid A (Tanaka et al., 1996).

3.2.6. Saponins

Five oleanane-type triterpenoid saponins were tentatively elucidated in CVE (Table 7, Table S1, and Fig. 2). The fragmentation of **49** ($[M\text{-}H]^-$ at *m/z* 779.4576) afforded an abundant fragment ion at *m/z* 617.406 $[M\text{-}H\text{-}Hex]^-$ (26.41%) indicating terminal hexose. The concomitant loss of (Hex + dHex) was attributed to disaccharide (hexose and deoxyhexose). The fragmentation pathway gave prominent ion at *m/z* 471.346 [sapogenin-H]$^-$ supported by an ion at *m/z* 439.321 deriving from the loss of CH_3OH (32 Da). The abovementioned pattern is consistent with the presence of CH_2OH group in the sapogenin and **49** was assigned to saikogenin F-3-*O*-hexosyl-*O*-deoxyhexoside (Miyase and Matsushima, 1997). The structure of **50** was in accordance with that of **49** except for the appearance of one additional pentose unit (Table S1). Thus, 3-*O*-saccharidic chain could be attributed to trisaccharide (Hex, dHex, Pent) attached to saikogenin F. This compound was not described in the literature. Peak **51** ($[M\text{-}H]^-$ at *m/z* 939.497) yielded prominent ions at *m/z* 777.444 ($[M\text{-}H\text{-}Hex]^-$), 615.392 ($[M\text{-}H\text{-}2Hex]^-$), and 439.322 ($[M\text{-}H\text{-}2Hex\text{-}dHex\text{-}30$), indicating trisaccharidic chain. The characteristic fragment ions at *m/z* 747.435 $[M\text{-}H\text{-}Hex\text{-}CH_2O]^-$, 585.380 $[M\text{-}H\text{-}2Hex\text{-}CH_2O]^-$, and 567.369 $[M\text{-}H\text{-}2Hex\text{-}\text{-}CH_2O\text{–}H_2O]^-$ was consistent with clinoposaponin XVIII previously isolated from *C. vulgare* (Miyase and Matsushima, 1997).

The MS/MS fragmentation pattern of **52** ($[M\text{-}H]^-$ at *m/z* 941.513) revealed a subsequent losses of two hexoses at *m/z* 779.460 and 617.407, and deoxyhexose at *m/z* 471.353 [sapogenin-H]$^-$. Moreover, a fragment ion at *m/z* 581.386 $[M\text{-}H\text{-}2Hex\text{-}2H_2O]^-$ indicated a branched *C*-3 oligosaccharide chain (Hex, Hex, dHex) with terminal hexoses. Thus, **52** was tentatively identified as saikogenin F-3-*O*-(hexosyl-[*O*-hexosyl]-deoxyhexoside; this structure could be related to buddlejasaponin IV (Liu et al., 1995). Peak **53** showed [M-H]- at *m/z* 1265.618, 324 Da (2 hexoses) more than **52**. The prominent ions at *m/z* 941.515 $[M\text{-}H\text{-}2Hex]^-$ and 471.308 $[M\text{-}H\text{-}4Hex\text{-}dHex]^-$ allowed to deduce pentasaccharidic chain at *C*-3 of saikogenin F, related to clinoposaponin V (Yamamoto et al., 1993).

4. Discussion

In order to discover new natural bioactive compounds, the assessment of their toxic characteristics is the initial step. Despite the numerous pharmacological effects described for *C. vulgare*, there is no detailed investigation on its possible toxic impact. The present study focused on evaluation of the acute and subacute toxicity of CVE on two rodent species - mice and rats. In addition, the chemical composition of *C. vulgare* lyophilized extract was characterized.

Intraperitoneal administration of CVE (not likely to be used in human) at dose of 600 mg/kg in rats produced adverse effects. Animals were somnolent with discoordination. At higher doses, they experienced difficulty of breathing and ataxia. It has been described that another *Clinopodium* species have central depressive effects (Estrada-Reyes et al., 2010). The authors noticed sedative, hypnotic, analgesic and antiepileptic effect of *C. mexicanum*. They hypothesize that the flavanone glycosides, the main compounds in the aqueous extract, are involved in GABAergic inhibitory mechanism. Toxicity signs as breathing difficulty and asthenia were also observed in p. o and i. p. administration of *Salvia przewalski* extract, containing water-soluble phenolic acids and lipophilic diterpenes (Li et al., 2010). Gao et al. (2009) also noticed asthenia, hypoactivity, and orthotonus after injection of danshensu, the chemical backbone of various salvianolic acids, when administrated i. v. at higher doses (> 2000 mg/kg bw) in mice. In other toxicity study of Danshen (*Salvia miltiorriza* aqueous extract), containing salvianolic A and B, danshensu and protocatechuic aldehyde, the sign of struggling was observed in some animals at the moment of intravenous administration. LD_{50} of Danshen injection was greater than 64 g/kg bw in rats (Wang et al., 2012). In light of this, it was interesting to examine the effects of CVE on the central nervous system. However, salvianolic acids are drawing increased interest from medical scientists; much of the research highlights their importance in the treatment of oxidative stress-linked disorders, fibrosis disease and cancer (Ma et al., 2019). In the present study, rosmarinic acid along with a great number of salvianolic acids was the dominant compounds of *C. vulgare* aqueous extract. We found that CVE is much more toxic than *C. mexicana* aqueous extract and salvianic acid A alone, where $LD_{50\ (i.p.)}$ for mice were found to be 2154 mg/kg and 2356 mg/kg, respectively (Estrada-Reyes et al., 2010; Gao et al., 2009). However, $LD_{50\ (i.p.)}$ for CVE was in the same order as *Salvia przewalski* ethanol extract ($LD_{50\ (i.p.)}$ = 780.8 mg/kg) (Li et al., 2010). Although, rosmarinic acid (the main compound in CVE) demonstrated very low i. v. toxicity with LD_{50} 561 mg/kg in mice (Parnham and Kesselring, 1985), it could be suggested that the abundance of caffeic acid trimers and tetramers have function for the somnolent effect and ataxia in the i. p. administration of CVE. Thus, the i. p. CVE treatment had marked impact on the central nervous system. In addition, clinoposaponins from CVE could contribute to toxicity by increasing the membrane permeability of the other accompanying bioactive compounds (Lorent et al., 2014).

According to Loomis and Hayes (1996) classification, chemical substances with a LD_{50} within the range of 1–5 g/kg is considered a practically low-toxic. The calculated LD_{50} being found in this range suggests that CVE should be regarded as practically low-toxic in acute oral administration for rodents we used. More recently, a toxicological assessment of essential oil from other *Clinopodium* species - *C. nepeta* (syn. *Calamintha nepeta*) revealed also a low toxicity with LD_{50} of 1500 mg/kg in acute oral administration (Arantes et al., 2019).

The acute dose study provides a guideline for selecting doses for the subacute and chronic low dose study, which may be more clinically relevant (Zbinden and Flury Roversi, 1981; OECD, 2001).

In the subacute toxicity test (upon repeated 14-day oral administration), both doses 50 mg/kg and 100 mg/kg did not produce adverse effects. There was no change in the consumption of water or food and no drastically change in body weight (data not shown). Therefore, CVE might be considered safe in the oral route of administration, consistent to the use in the traditional medicine.

A full blood count assay showed no significant changes in the treated animals, evidenced that the extract did not affect hematopoiesis. Macroscopic observation of the organs did not show any change in the color compared to the control group. Organ hypertrophy or other changes in the form and structure of the investigated organs were not observed. The heart, liver, spleen, kidneys and lungs were not adversely affected nor showed clinical signs of toxicity during the treatment confirming CVE safety for the analyzed organs. Serum hematological and biochemical assays were performed to assess possible changes in the liver and kidney functions. The lack of liver function abnormalities assessed by transaminase activity measurement correlated with the lack of morphological changes in histological examination. Normal levels of total protein and albumin in the serum confirmed that CVE, in both doses, did not impair hepatocellular or secretory liver functions. No changes in blood glucose, cholesterol and triglyceride levels were observed, indicating the absence of influence on glycemic and lipid status in experimental animals, as evidenced by the intact morphological characteristics of the pancreas. Similarly, normal urea and creatinine levels correlate with the normal histological characteristics of functional nephrons, also confirmed by the lack of changes in biochemical parameters in the urine. The CVE safety profile could be attributed to the high content of phenolic compounds with beneficial pharmacological effects and low toxicity. Consistent with the previously published data on Lamiaceae species, we found rosmarinic acid and a variety of salvianolic and clinopodic acids (Li et al., 2010; Domitrović et al., 2013; Ramalho et al., 2014).

Fourteen days oral administration of CVE in both doses did not cause an oxidative stress in animals. Moreover, the CVE higher dose produced a statistically significant antioxidant effect in kidney tissue discerned by a decrease of MDA quantity and increase of GSH level, and activity of the main antioxidant enzymes, catalase and SOD. These effects could be associated with the presence of salvianolic and clinopodic acids with antioxidant and radical scavenging activity (Zhao et al., 2008).

Histological examination of the organs did not show changes or adverse effects on the cell structure. Pathological abnormalities were not observed in the histological sections of the liver, spleen, kidney, and pancreas in the higher dose treated animals, compared to the control group. Minor changes in cerebral vessels were detected, probably due to the method of euthanasia.

A variety of compounds, including phenolic and acylquinic acids, flavonoids and triterpenoid saponins have been previously identified in *C. vulgare* extracts (Bardarov et al., 2016; Obreshkova et al., 2001; Miyase and Matsushima, 1997).

In the present study, a detailed UHPLC-HRMS profiling of CVE was performed. This approach allowed tentative identification of a total 52 compounds, including 7 flavonoids, 41 caffeic acids derivatives and 5 saponins. Based on the number of aromatic rings caffeic acid derivatives in CVE can be definitely classified into four groups: monomers, dimers, trimes and tetramers. Previous investigation revealed rosmarinic acid as a main compound in CVE (Nasar-Eddin et al., 2019), while caffeic acid, chlorogenic acid and catechin were found to be the most abundant in *C. vulgare* 50% aqueous methanol extract (Armirova et al., 2019). A large number of caffeic acid oligomers arise from ester bonding of caffeic acid and salvianic acid A (danshensu) in linear chain (clinopodic acid A, B; salvianolic acid A, D, E, F, G, H/I, K, T/U). Clinopodic acids A and B have been previously reported for *C. chinense* (Murata et al., 2009; Zeng et al., 2016), while closely related open chain salvianolic acids have been found largely in *Salvia* species (Liu et al., 2007; Lu and Foo, 1999; Chen et al., 2011; Li et al., 2014), and lately in *Symphytum officinale* (Trifan et al., 2018). The organ protective, anticancer, and antioxidant effects found in many studies are thought to be due to these substances. Other oligomers possess benzofuran ring (salvianolic acid C, J; clinopodic acid C, E, I, J, K, N, O; isosalvianolic acid B, lithospermic acid), cyclohexene ring (yunnaneic acid E), dihydronaphtalene structure (yunnaneic acid G, salvianolic acid L) benzoxepinyl structure (isosalvianolic acid C), or spiroketal linkage (yunnaneic acid A). Among all oligomers, only clinopodic acid Q is a glucoside. Clinopodic acids A-I have been previously isolated from *C. chinense* var. *parviflorum* (Murata et al., 2009), while clinopodic acids J-Q were found in *C. gracile* (Aoshima et al., 2012), salvianolic acids A-L in *Salvia miltiorrhiza* (Liu et al., 2007), and yunanneic acids E-H in *Salvia yunnanensis* (Tanaka et al., 1997). Caffeic acid oligomers are the most abundant water-soluble compounds in CVE as has been seen in *Salvia miltiorrhiza* (Liu et al., 2007). Based on accurate masses, fragmentation patterns and literature data, 5 saikosaponins were tentatively elucidated in CVE. The saponins involved the sapogenin saikogenin F and one oligosaccharide chain at C-3. These findings are in agreement with previously isolated clinoposaponin XV (**49**), XVIII (**51**) and V (**53**) from *C. vulgare*, *C. chinense* and *C. chinense* var. *parvifolium* (Miyase and Matsushima, 1997; Yamamoto et al., 1993), while buddlejasaponin IV (**52**) were found only in *C. chinense* (Liu et al., 1995) Moreover, this is the first report for saikogenin F-*O*-pentosyl-*O*-deoxyhexosyl-*O*-hexoside (**50**) in *C. vulgare*.

5. Conclusion

In the present study valuable data on the acute and subacute toxicity of CVE were provided. CVE was found to be practically low-toxic in acute and subacute oral administration in mice and rats. More than fifty secondary metabolites, including flavonoids, caffeic acid oligomers and saponins were tentatively identified in the studied extract by UHPLC-HRMS. These results could be very useful for the future in vivo and clinical study of this plant species. Chronic toxicity, mutagenicity and carcinogenicity studies are necessary to further support the safe use of this herb.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2019.110841.

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