SUPPLEMENTARY MATERIAL

Optimization of carvacrol, rosmarinic, oleanolic and ursolic acid extraction from oregano herbs (*O. onites* L., *O. vulgare* spp. *hirtum* and *O. vulgare* L.

Justė Baranauskaitė^a, Valdas Jakštas^b, Liudas Ivanauskas^c, Dalia M. Kopustinskiienė^a, Gailutė Drakšienė^a, Ruta Masteikova^d and Jurga Bernatoniienė^{a*}

^aDepartment of Drug Technology and Social Pharmacy, Lithuanian University of Health Sciences, Eivenių str. 4, LT-50161 Kaunas, Lithuania; ^bDepartment of Pharmacognosy, Lithuanian University of Health Sciences, Kaunas, Lithuania;

^cDepartment of Drug Chemistry, Lithuanian University of Health Sciences, Kaunas, Lithuania; ^dDepartment of Pharmaceutics, Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences, Brno, Czech Republic

*Corresponding author. Email: jurgabernatoniene@yahoo.com

The aim of our study was to increase the extraction efficiency of carvacrol, rosmarinic, oleanolic and ursolic acid from the different species of oregano herbs (*O. onites* L., *O. vulgare* spp. *hirtum* and *O. vulgare* L.). Various extraction methods (ultrasound-assisted, heat-reflux, continuous stirring, maceration, percolation) and extraction conditions (different solvent, material:solvent ratio, extraction temperature, extraction time) were used, and the active substances were determined by HPLC. The lowest content of carvacrol, rosmarinic, oleanolic and ursolic acid was obtained by percolation. During heat-reflux extraction, the content of active substances depended on the solvent used: ethanol/non-aqueous solvent (glycerol or propylene glycol) mixture was more effective compared to ethanol alone. The results showed that for each species of oregano the most optimal extraction method should be selected to maximize the content of biologically active substances in the extracts.

Keywords: *Origanum*; carvacrol; ursolic acid; oleanolic acid; rosmarinic acid; HPLC; extraction

Experimental section

Plant material

Dried *Origanum vulgare* L. herb was purchased from “Svencioniu zoles”, Lithuania; dried *Origanum vulgare* subsp. *hirtum* herb – from “Çengelköy spice and herbs center”, Turkey; dried *Origanum onites* L. herb – from “İnanTarım ECO DAB”, Turkey. Voucher specimens (No. L170709, L170710 and L170711) have been deposited at the Herbarium of the Department of drug technology and social pharmacy, Lithuanian university of health sciences, Lithuania. Dried herb particle size of *O.vulgare* L. and *O.vulgare* subsp. *hirtum* and *O. onites* was 125 µm.

Instruments and chemicals

An ultrasonic bath (Bandelin electronic GmbH & Co.KG, Berlin, Germany), vacuum rotatory evaporator Heidolph Laborata 4000 (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany), Memmert WNB7 waterbath (Memmert GmbH & Co. KG, Schwabach, Germany), Waters 2695 chromatography system (Waters, Milford, USA) and magnetic stirring hotplates (Heidolph MR, Germany) were used for the study.

Ursolic acid (>98.5%), carvacrol (>98%), HPLC eluent acetic acid (99.8%), extraction solvents: ethanol (96%), glycerol (99.5%) and propylene glycol (99.5%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC eluent methanol was from Carl Roth GmbH (Karlsruhe, Germany), oleanolic acid (>99%) – from Extrasynthese (Lyon, France), and rosmarinic acid (>98%) – from ChromaDex (Santa Ana, TX, USA). The water used in HPLC and for sample preparation was produced with a Super Purity Water System (Millipore, USA).

Ultrasound assisted extraction (UAE)

Dried powdered plant material (1 g) has been extracted twice with 10 mL solvent. Next step was followed by centrifugation at $4200 \times g$ for 10 min. The obtained extracts were combined into 20 mL flask. The optimization of the extraction parameters was conducted at different experimental conditions. The extract has been passed through a $0.22 \mu\text{m}$ membrane filter for HPLC analysis (Xia EQ et al. 2012). This method was used to produce extracts No. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 56 and 57. See Supplementary Table S2 for additional information.

Heat-reflux extraction (HRE)

Dried powdered plant material (1 g) has been extracted with 100 mL of 90% (v/v) ethanol in a round bottom flask by heat-reflux extraction performed in a water bath for 6 h at 95°C . The extract has been passed through a $0.22 \mu\text{m}$ membrane filter for HPLC analysis (Fu Q et al. 2014). This method was used to produce extracts No. 20 - 42). See Supplementary Table S1 for additional information.

Continuous stirring extraction (CSE)

Dried powdered plant material (1 g) has been extracted with 100 mL of 90% (v/v) ethanol. The extraction was carried out by continuous stirring for 24 h with the magnetic stirrer in a closed conical flask. The extract has been passed through a $0.22 \mu\text{m}$ membrane filter for HPLC analysis. This method was used to produce extracts No. 43 - 46. See Supplementary Table S2 for additional information.

Maceration (ME)

Dried powdered plant material (5 g) was macerated using 50 mL of 90 % ethanol (v/v) at room temperature for 48 h. The obtained extract has been passed through a $0.22 \mu\text{m}$

membrane filter for HPLC analysis. This method was used to produce extracts No. 50 - 52.

Maceration of dried powdered plant material (1 g) has been carried out with 100 mL of 90 % (v/v) ethanol in a closed conical flask at room temperature for 24 h. The extract has been passed through a 0.22 µm membrane filter for HPLC analysis. This method was used to produce extracts No. 47 - 49. See Supplementary Table S2 for additional information.

Percolation (PE)

90 % ethanol (v/v) was used as a solvent for percolation carried out at room temperature for 48 h. Powdered plant material (5 g) was moistened with 50 mL of solvent (ratio 1:1) and allowed to swell for 3 h. The swollen material was transferred to the percolator, appropriate amount of solvent was added and macerated for 48 h. Then the first concentrated extract was collected (85% of the finished product). Extraction of plant material was continued until complete exhaustion of the constituents. After the percolation, the second volume of extract was vaporized using vacuum rotatory evaporator efficient to remove the solvent until the required volume. Both extracts were combined thoroughly and the obtained extract has been passed through a 0.22 µm membrane filter for HPLC analysis. This method was used to produce extracts No. 53 – 55. See Supplementary Table S2 for additional information.

HPLC conditions for determination of RA

HPLC analysis has been carried out using Waters 2695 chromatography system (Waters, Milford, USA) equipped with Waters 996 PDA detector. Data were collected and analysed using a PC and the Empower2 chromatographic manager system (Waters Corporation, Milford, USA).

For determination of RA the ACE 5 C18 250 × 4.6 mm column (Advanced Chromatography Technologies, Aberdeen, Scotland) was used. The mobile phase was composed of the solvent A (methanol) and the solvent B (0.5 % (v/v) acetic acid in water). The following linear gradient elution profile was used: 95 % A/5 % B – 0 min, 40 % A/60 % B – 40 min, 10 % A/90 % B – 41 - 55 min, 95 % A/5 % B – 56 min. The flow rate was 1 mL/min and injection volume was 10 µL. The effluent was determined at a wavelength of 329 nm. The quantification has been carried out by the external standard method. The linear calibration curve was made ($R^2=0.999918$), and the peak areas were used for quantification. The typical chromatogram is shown in Supplementary Figure S2.

HPLC conditions for determination of UA and OA

Determination of UA and OA has been carried out using ACE 5 C18 250×4.6 mm column (Advanced Chromatography Technologies, Aberdeen, Scotland). The mobile phase was composed of methanol and water (90/10, v/v). The flow rate was 0.6 mL/min and the injection volume was 10 µL. The absorption was measured at 203 nm. The quantification has been carried out by the external standard method. The calibration curves were made (OA $R^2=0.999193$, UA $R^2=0.999546$). The typical chromatogram is shown in Supplementary Figure S2.

HPLC conditions for determination of CA

For determination of CA ACE 5 C18 250×4.6 mm column (Advanced Chromatography Technologies, Aberdeen, Scotland) was used. The mobile phase was composed of methanol and water (60/40, v/v). The flow rate was 0.6 mL/min and injection volume was 10 µL. The absorption was measured at 275 nm. The quantification has been carried out by the external standard method. The calibration curve was made (CA

$R^2=0.999751$). The typical chromatogram is shown in Supplementary Figure S2.

Statistical analysis

Data are presented as mean $\pm$ SEM. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Dunnett's post test with the software package Prism v. 5.04 (GraphPad Software Inc., La Jolla, CA, USA). A value of $p < 0.05$ was taken as the level of significance.

References:

- Fu Q, Zhang L, Cheng N, Jia M, Zhang Y. 2014. Extraction optimization of oleanolic and ursolic acids from pomegranate (*Punica granatum* L.) flowers. Food Bioprod Process 92:321–332.
- Xia EQ, Yu YY, Xu XR, Deng GF, Guo YJ, Li HB. 2012. Ultrasound-assisted extraction of oleanolic acid and ursolic acid from Ligustrum lucidum Ait. Ultrason Sonochem. 19:772–776.

Supplementary Table S1. Influence of extraction methods on the maximal extraction yield of rosmarinic, oleanolic and ursolic acids and carvacrol.

Extraction conditions	Rosmarinic (RA), oleanolic (OA), ursolic (UA) acids and carvacrol (CA) in different species of oregano herb, µg/g						
		<i>O. vulgare</i> L.	Extract No.#	<i>O. vulgare</i> spp. <i>hirtum</i>	Extract No.#	<i>O. onites</i> L.	Extract No.#
Ultrasound-assisted	RA	6425±54*	4a ¹	29648±70*	6a ²	12021±51**	7a ³
	OA	627±10*	14a ¹	1628±24*#	9a ²	1647±41	18a ³
	UA	1001±15*	14a ¹	3748±32*#	12a ²	4450±66	18a ³
	CA	1999±20##	14a ¹	22642±65	15a ²	22210±98***	18a ³
Heat-reflux	RA	9380±46	20b ¹	36231±50	21b ²	14800±74**	22b ³
	OA	877±24	20b ¹	1109±11*#	21b ²	1510±45***	22b ³
	UA	1281±13	20b ¹	4527±41*#	21b ²	3863±58	22b ³
	CA	7552±35##	20b ¹	19891±45**	21b ²	22618±89***	22b ³
Continuous stirring	RA	7225±23*	43c ¹	30448±15*	44c ²	15648±32	46c ³
	OA	655±10*	43c ¹	1475±44*#	44c ²	1027±36***	45c ³
	UA	1049±16*	43c ¹	4109±123*#	44c ²	2822±26	45c ³
	CA	7886±41	43c ¹	22667±80	44c ²	25753±85***	46c ³
Maceration 24 h	RA	4399±27*	47d ¹	21548±13*	48d ²	11593±15**	49d ³
	OA	517±2*	47d ¹	1217±24*#	48d ²	1317±26***	49d ³
	UA	801±7*	47d ¹	3761±73*#	48d ²	3678±48	49d ³
	CA	7459±38##	47d ¹	21893±59**	48d ²	27523±74	49d ³
Maceration 48 h	RA	4408±15*	50e ¹	19685±12*	51e ²	8945±4**	52e ³
	OA	718±14*	50e ¹	2268±45	51e ²	1100±45***	52e ³
	UA	1067±21*	50e ¹	8574±171	51e ²	3200±37	52e ³
	CA	1957±24##	50e ¹	16833±69**	51e ²	20439±68***	52e ³
Percolation	RA	229±2*	53f ¹	7908±79*	54f ²	5824±34**	55f ³
	OA	202±4*	53f ¹	367±1*#	54f ²	387±1***	55f ³
	UA	445±6*	53f ¹	1522±12*#	54f ²	1100±15	55f ³
	CA	1400±18##	53f ¹	6028±30**	54f ²	9025±57***	55f ³

* $p < 0.05$ vs heat-reflux, ** $p < 0.05$ vs continuous stirring, *[#] $p < 0.05$ vs maceration 48 h,
*** $p < 0.05$ vs ultrasound, ^{##} $p < 0.05$ vs continuous stirring, [#]* $p < 0.05$ vs continuous stirring
and ultrasound, **[#]* $p < 0.05$ vs maceration 24 h. n =3-6.

#See Supplementary Table S2 for additional explanation.

Supplementary Table S2. Conditions for extract production from Oregano herb (*O. onites* L., *O. vulgare* spp.*hirtum* and *O. vulgare* L.).

Extract number	Material:solvent ratio (g/mL)	Extraction temperature, °C	Extraction time, min	Solvent
1 ^{a1}	1:20	25	10	Ethanol, 30% v/v
2 ^{a1}	1:20	25	10	Ethanol, 40% v/v
3 ^{a1}	1:20	25	10	Ethanol, 50% v/v
4 ^{a1}	1:20	25	10	Ethanol, 60% v/v
5 ^{a1}	1:20	25	10	Ethanol, 70% v/v
6 ^{a2}	1:20	25	10	Ethanol, 70% v/v
7 ^{a3}	1:20	25	10	Ethanol, 70% v/v
8 ^{a1}	1:20	25	10	Ethanol, 80% v/v
9 ^{a2}	1:20	25	10	Ethanol, 80% v/v
10 ^{a3}	1:20	25	10	Ethanol, 80% v/v
11 ^{a1}	1:20	25	10	Ethanol, 90% v/v
12 ^{a2}	1:20	25	10	Ethanol, 90% v/v
13 ^{a3}	1:20	25	10	Ethanol, 90% v/v
14 ^{a1}	1:20	25	10	Ethanol, 96% v/v
15 ^{a2}	1:20	25	10	Ethanol, 96% v/v
16 ^{a3}	1:20	25	10	Ethanol, 96% v/v
17 ^{a3}	1:20	30	10	Ethanol, 90% v/v
18 ^{a3}	1:20	45	10	Ethanol, 90% v/v
19 ^{a3}	1:20	60	10	Ethanol, 90% v/v
20 ^{b1}	1:100	95	360	Ethanol, 90% v/v

21 ^{b2}	1:100	95	360	Ethanol, 90% v/v
22 ^{b3}	1:100	95	360	Ethanol, 90% v/v
23 ^{b3}	1:100	95	60	Ethanol, 90% v/v
24 ^{b3}	1:100	95	120	Ethanol, 90% v/v
25 ^{b3}	1:100	95	180	Ethanol, 90% v/v
26 ^{b3}	1:100	95	240	Ethanol, 90% v/v
27 ^{b3}	1:100	95	300	Ethanol, 90% v/v
28 ^{b3}	1:100	95	360	Ethanol, 90% v/v + glycerol, 98%, 10%
29 ^{b3}	1:100	95	360	Ethanol, 90% v/v + propylenglycol, 99.5%, 10%
30 ^{b3}	1:100	95	360	Ethanol, 90% v/v + propylenglycol, 99.5% 400, 10%
31 ^{b3}	1:100	95	360	Ethanol, 90% v/v + glycerol, 98%, 20%
32 ^{b3}	1:100	95	360	Ethanol, 90% v/v + propylenglycol, 99,5%, 20%
33 ^{b3}	1:100	95	360	Ethanol, 90% v/v + propylenglycol, 400, 20%
34 ^{b3}	1:100	95	360	Ethanol, 90% v/v + glycerol, 98%, 30%
35 ^{b3}	1:100	95	60	Ethanol, 90% v/v + glycerol, 98%, 10%
36 ^{b3}	1:100	95	120	Ethanol, 90% v/v + glycerol, 98%, 10%

37 ^{b3}	1:100	95	180	Ethanol, 90% v/v + glycerol, 98%, 10%
38 ^{b3}	1:100	95	240	Ethanol, 90% v/v + glycerol, 98%, 10%
39 ^{b3}	1:100	95	300	Ethanol, 90% v/v + glycerol, 98%, 10%
40 ^{b3}	10:100	95	360	Ethanol, 90% v/v
41 ^{b**3}	1:100	95	360	Ethanol, 90% v/v
42 ^{b**3}	1:100	95	360	Ethanol, 90% v/v+ glycerol, 98%, 10%
43 ^{c1}	1:100	25	1440	Ethanol, 90% v/v
44 ^{c2}	1:100	25	1440	Ethanol, 90% v/v
45 ^{c3}	1:100	25	1440	Ethanol, 90% v/v
46 ^{c3}	1:100	30	1440	Ethanol, 90% v/v
47 ^{d1}	1:100	25	1440	Ethanol, 90% v/v
48 ^{d2}	1:100	25	1440	Ethanol, 90% v/v
49 ^{d3}	1:100	25	1440	Ethanol, 90% v/v
50 ^{e1}	1:5	25	2880	Ethanol, 90% v/v
51 ^{e2}	1:5	25	2880	Ethanol, 90% v/v
52 ^{e3}	1:5	25	2880	Ethanol, 90% v/v
53 ^{f1}	1:1	25	2880	Ethanol, 90% v/v
54 ^{f2}	1:1	25	2880	Ethanol, 90% v/v
55 ^{f3}	1:1	25	2880	Ethanol, 90% v/v
56 ^{a*1}	1:20	25	10	Ethanol, 96% v/v

57 ^{a1}	1:20	25	10	Methanol, 99.9 % v/v
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^a ultrasound extraction, re-extracted twice; ^{a*} material diameter 710 µm; ^b heated-reflux extraction; ^{b**} 24h freeze-shock for extract material; ^c continuous stirring extraction; ^d 24h maceration; ^e 48h maceration; ^f percoliation; ¹ *origanum vulgare* L.; ² *origanum vulgare* spp. *hirtum*; ³ *origanum onites* L.

Supplementary Figure S1. Influence of non-aqueous solvent on extraction of rosmarinic, oleanolic, ursolic acids and carvacrol from *O. onites* L. herb. *p < 0.05 vs extract 22 (control), n = 2-5. See Supplementary Table S2 for additional explanations.

Supplementary Figure S2. HPLC chromatogram of ethanol extract from *Origanum onites* L. herb sample. 1 – carvacrol – 19.743 min; 2 – rosmarinic acid – 33.146 min ; 3 – oleanolic acid -18.649 min; 4 – ursolic acid – 19.928 min.