ORIGINAL ARTICLE

A C G publications

J. Chem. Metrol. 12:2 (2018) 121-127

journal of chemical metrology

Quantitative determination of some phenolics in *Origanum laevigatum* Boiss. extracts via validated LC-MS/MS method and antioxidant activity

Sema Çarıkçı[*1], Turgut Kılıç[2], Züleyha Özer[3], Tuncay Dirmenci[4], Turan Arabacı[5] and Ahmet C. Gören[6]

[1]*Department of Chemistry, Faculty of Sciences and Arts, Balıkesir University, 10145- Balıkesir, Türkiye*
[2] *Department of Science and Mathematics Education, Necatibey Faculty of Education, Balıkesir University, 10100- Balıkesir, Türkiye*
[3]*Medicinal and Aromatical Plants Programme, Altınoluk Vocational School, Balıkesir University, Altınoluk, Edremit-Balıkesir, Türkiye*
[4]*Department of Biology Education, Necatibey Faculty of Education, Balıkesir University, 10100- Balıkesir, Türkiye*
[5] *Department of Pharmaceutical Botany, Faculty of Pharmacy, , Inonu University Malatya, Türkiye*
[6]*Department of Pharmaceutical Sciences, Pharmacy Faculty, Bezmialem Vakif University, 34093, Istanbul, Türkiye*

(Received November 10, 2018; Revised December 21, 2018; Accepted December 21, 2018)

Abstract: In this study, quantities of some phenolics; caffeic acid, (*E*)-ferulic acid, rosmarinic acid, chlorogenic acid, fumaric acid, gallic acid, pyrogallol and vanillin in the directly methanol (M1), chloroform (C), acetone (Ac) and methanol (M2) extratcs obtained from *Origanum laevigatum* Boiss. from Turkey were investigated via liquid chromatography and tandem mass spectrometry (LC-MS/MS). Curcumin was used as an internal standard. Caffeic acid, chlorogenic acid, rosmarinic acid and gallic acid were determined as the most abundant phenolic acids in the studied extracts using LC-MS/MS. In LC-MS/MS measurements relative standard deviations (RSD %) for caffeic acid, (*E*)-ferulic acid, rosmarinic acid, chlorogenic acid, fumaric acid, gallic acid, pyrogallol and vanillin were found to be 8.04, 5.21, 5.45, 3.73, 5.44, 4.85, 5.47 and 6.57 % respectively. For the investigated analytes, the correlation coefficient was found in the range of 0.9803 to 0.9981. Furthermore, antioxidant properties of the extracts were determined based on 2,2-diphenyl-1- picrylhydrazyl (DPPH), *β*-carotene linoleic acid and cupric ($Cu2^{+}$) ion reducing power assay (CUPRAC). Acetone (Ac) and methanol (M2) extracts showed high activity in all test assays due to their high concentration of rosmarinic acid, caffeic acid and gallic acid in the extracts.

Keywords:*Origanum*; phenolics; LC-MS/MS; validation; antioxidant activity, rosmarinic acid. © 2018 ACG Publications. All rights reserved.

1. Introduction

The genus *Origanum* L. (Lamiaceae) comprises 43 species (50 taxa) and 19 hybrids worldwide, while 21 species (24 taxa) and 12 hybrids are presented in Turkey [1-4]. Among them, 23 taxa (including hybrids) are endemic. In traditional Turkish medicine, as well as in the world, they have been

* Corresponding author E-Mail: carikci@balikesir.edu.tr

The article was published by ACG Publications
http://www.acgpubs.org/journal/journal-of-chemical-metrology July-December 2018 EISSN:1307-6183
DOI:http://doi.org/10.25135/jcm.21.18.11.1115

used for different properties such as; sedative, diuretic, digestive, carminative and showed some activities e.g.; antispasmodic, antimicrobial, expectorant, antioxidant, anticholinesterase [4,5].Also, most of the members of *Origanum* are widely used as spice namely "kekik". *Origanum laevigatum* Boiss. belongs to Sect. *Prolaticorolla* Ietsw., and is distributed from south Turkey to north Syria and Cyprus. The essential oil composition of *O. laevigatum* was investigated in two studies and bicyclogermacrene, germacrene and β-caryophyllene were identified as major constituents [6,7]. The antimicrobial activity of ethanol extract [8], and the amoebicidal activity [9] of *O. laevigatum* were determined. *O. laevigatum* showed strong inhibition against *S. aureus* in all the tested microorganism, but the amoebicidal effect of the species was found to be weak. As far as our knowledge, no report is available for the phenolics from *O. laevigatum*.

The compounds that are formed in the presence of at least one hydroxyl group (-OH) bound in the aromatic ring are called phenols and, the number of hydroxyl groups and their position in the ring are the most important factors that provide the antioxidant activities of phenolic compounds [10]. The phenolic compounds, due to their redox properties, act as greatly effective free radical scavengers, which mainly adsorb and neutralize free radicals [11]. Due to these important feature, the number of studies on the determination of phenolic compounds in plant extracts is increasing day by day [12-14]. These studies showed that there has been a high correlation between antioxidant activity and phenolic content [4,10-12, 15,18]. Furthermore, determination of the content of plant extracts with LC-MS/MS has recently become a popular method [4,10,16-18]. In the previous studies it has been reported that the phenolics constituents of the extracts prepared from *Origanum* species were mainly composed of phenolic acids (coumaric, rosmarinic, gallic acid etc.) and flavonoids (salvigenin, kaempferol etc.) [4,10,19,20].

The present study is aimed to identify some phenolic acids (caffeic acid, (*E*)-ferulic acid, rosmarinic acid, chlorogenic acid, fumaric acid, gallic acid) and two simple phenolics (pyrogallol and vanillin) and quantities via LC-MS/MS, and to determine antioxidant activities of the extracts of *O. laevigatum*. For this purpose, a developed method was used for the chromatographic identification and LC-MS/MS quantification of phenolic acids and simple phenolics in chloroform, acetone and methanol extracts. The method developed was validated and the uncertainties were calculated via the bottom-up method. Antioxidant activities of the extracts were determined based on DPPH, *β*-carotene linoleic acid and CUPRAC assays.

2. Experimental

2.1. Plant material

Aerial part of the *O. laevigatum* was collected from Aladağ-Adana, altitude of 1015 ft, (37º 41' 502" N, 35º 43' 247" E) in August 2014. The collected specimens were identified by Prof. Dr. Tuncay Dirmenci. The voucher specimens were deposited at the Herbarium of Balıkesir University (ISTE 115945).

2.2. Extraction

From the air-dried grinded plant samples were weighed approximately 100 grams, then directly extracted with methanol for 15 days. After filtration and evaporation, they were named **M1.** Also, another 100 gram plant sample was extracted according to increasing polarity with chloroform (**C**), acetone (**Ac**) and methanol (**M2**) for 15 days. After the extraction procedure, the extracts were filtrated and evaporated.

2.3. Antioxidant Activities

Antioxidant activities of the extracts were investigated mainly using three methods:2,2-diphenyl-1- picrylhydrazyl (DPPH) [21] , *β*-carotene linoleic acid [22] and cupric ($Cu2^{+}$) ion reducing power assay (CUPRAC)[23] . In the DPPH free radical-scavenging activity and *β*-carotene linoleic acid, BHT (butylated hydroxytoluene), and BHA (butylated hydroxyanisole) and α-toc (α-tocopherol) were used as standard, the activity tests were carried on at four different concentrations: at 10 µg/mL, 25 µg/mL, 50 µg/mL, 100 µg/mL. TEAC $_{CUPRAC}$ values of compounds were calculated by using curcumin as references. TEAC $_{CUPRAC}$ value of curcumin was calculated as 0.9 mmol TR g^{-1}, by using the formula that is given in the literature [24].

2.4. LC-MS Conditions

LC-MS experiments were performed by a Zivak® HPLC and Zivak® Tandem Gold Triple quadrupole (Istanbul, Turkey) mass spectrometry equipped with a Synergy Max C18 column (250 x 2 mm i.d., particle size: 5µm). Methanol-water mixture was found to be the best separation solvent and applied as gradient elution programs. For fragmented ion stability tests, the three-partquad-pole mass spectrometry system was chosen. The optimum ESI parameters were determined according to the previous studies in the literature [17,25,26]. Also, detailed procedure was given in the supplementary material.

The experiment parameters of the phenolics are given in Table 1.

Table 1. LC-MS/MS parameters of phenolics

	Compounds	***Parent ion***	**Daughter ion**	**Collision energy (V)**	**ESI mode**
1	Caffeic acid	179	135	14	negative
2	(*E*)-ferulic acid	193	133	15	negative
3	Chlorogenic acid	353	191	14	negative
4	Rosmarinic acid	359.2	160.5	15	negative
5	Fumaric acid	115	71	8	negative
6	Gallic acid	168.6	124	13	negative
7	Pyrogallol	125	80	16	negative
8	Vanillin	150.7	135.4	12	negative
	*Curcumin	369.3	176.9	20	negative

*Used as internal standard

2.5. Chemicals

For LC-MS/MS analysis of phenolics, caffeic acid (98%, Sigma-Aldrich), (*E*)-ferulic acid (99%, Sigma-Aldrich), chlorogenic acid (95%, Sigma-Aldrich), rosmarinic acid (96%, Sigma-Aldrich), fumaric acid (99%, Sigma-Aldrich), gallic acid (99%, Merck), pyrogallol (98%, Sigma-Aldrich) and vanillin (99%, Sigma-Aldrich) were used as standards. HPLC grade methanol (Merck) was used for the preparation of stock solutions. The freshly prepared 100 mg/L curcumin solution was used as an Internal Standard (IS) in all experiments. The detailed procedure for the preparation of the test solutions was given in the supplementary material.

2.6. Method validation

LOD (limit of detection) and LOQ (limit of quantification) of the LC-MS/MS methods of the indicated phenolics were calculated to be 0.5-50 mg/L. The LODs and LOQs were determined to be three and ten times bigger than standard deviation respectively. In Table 2, the LOD and LOQ values of phenolics for LC-MS/MS method are summarized.

The linearity was determined by analyzing the standard solution. Linearity ranges and linear regression equations of the phenolics are given in Table 2. The correlation coefficients (r^2) were found to be ≥0.98.

Table 2. Validation and uncertainty parameters for the LC/MSMS method developed for the phenolics

	Compounds	**Linear regression equation**	R^2	**LOD (mg/L)**	**LOQ (mg/L)**	**RSD (%)**
1	Caffeic acid	y=0.3300x+0.0036	0.9924	0.028	0.093	8.04
2	(*E*)-ferulic acid	y=0.0655x+0.0266	0.9925	0.047	0.158	5.21
3	Chlorogenic acid	y=0.2620x+0.0674	0.9980	0.445	1.483	5.45
4	Rosmarinic acid	y=0.1960x+0.0043	0.9982	0.022	0.072	3.73
5	Fumaric acid	y=0.0569x+0.0177	0.9912	0.003	0.010	5.44
6	Gallic acid	y=0.0569x+0.0177	0.9912	0.002	0.008	4.85
7	Pyrogallol	y=0.0438x+0.0073	0.9803	0.001	0.002	5.47
8	Vanillin	y=0.0982x+0.0158	0.9982	0.019	0.064	6.57

The EURACHEM/CITAC guide was used for evaluation of sources and quantification of uncertainty of LC-MS/MS method [24]. Also, some details of the procedures of uncertainty evaluation are reported previously in the literature [25,26] and given in the supplementary material.

3. Results and discussion

The chloroform, acetone and methanol extracts of the aerial parts of *O. laevigatum* were analyzed using LC-MS/MS. Quantities of six phenolic acids and two simple phenolics were determine in the extracts. The acetone and methanol 2 extracts were found to be rich. The amounts of phenolic compounds in Ac was found to be 1686.95mg/kg dried herba whereas 1389.73mg/kg dried herba for M2. In the extracts, the most abundant compounds are as in the following: in the M1 extract caffeic acid (87.96±17.41mg/kg dried herba); in the C extract chlorogenic acid (21.54±2.98 mg/kg dried herba); in the Ac extract rosmarinic acid (1623.83±124.51 mg/kg dried herba); in the M2 extract gallic acid (743.95±51.59 mg/kg dried herba). The results are given in the Table 3. The LC-MS/MS chromatogram and the structure of the determined phenolics are given in Figure1 and Figure 2 in the supplementary material.

The activity results of the extracts, which are the richest in terms of the amount of phenolic compounds, were found to be quite remarkable. When the activity results of these extracts were analyzed, with DPPH method, except for 10 µg/mL, activity values were found higher than their standards in all concentrations. At a concentration of 100 µg/mL Ac was determined as 86.02% while BHA was 83.66%, BHT was 83.05% and α-Toc was 83.92%. The C extract, which had the minimum quantities of the phenolics, showed the weakest antioxidant properties.

Table 3.Amount of phenolic acids in the extracts of *Origanum laevigatum*

Phenolics	**M1**	**C**	**Ac**	**M2**
Caffeic acid	**87.96±17.41**	5.21±1.03	39.02±7.72	645.78±127.79
(*E*)-Ferulic acid	75.65±5.29	-	6.68±0.47	-
Chlorogenic acid	12.73±1.76	**21.54±2.98**	8.36±1.16	-
Rosmarinic acid	-	4.08±0.31	**1623.83±124.51**	-
Fumaric acid	74.89±5.19	-	-	-
Gallic acid	5.39±0.37	-	6.38±0.44	**743.95±51.59**
Pyrogallol	11.05±0.74	-	-	-
Vannilin	-	0.57±0.05	2.68±0.18	-
Total (mg/kg dried herba)	**267.67**	**31.4**	**1686.95**	**1389.73**

M1: Methanol extract (directly) **C:**Chloroform extract **Ac:**Acetone extract **M2:**Methanol extract (successively)

For the *β*-carotene linoleic acid and CUPRAC method, the results correlated with DPPH results. The Ac extract has the best activity values. The activity results are given in Figure 1.

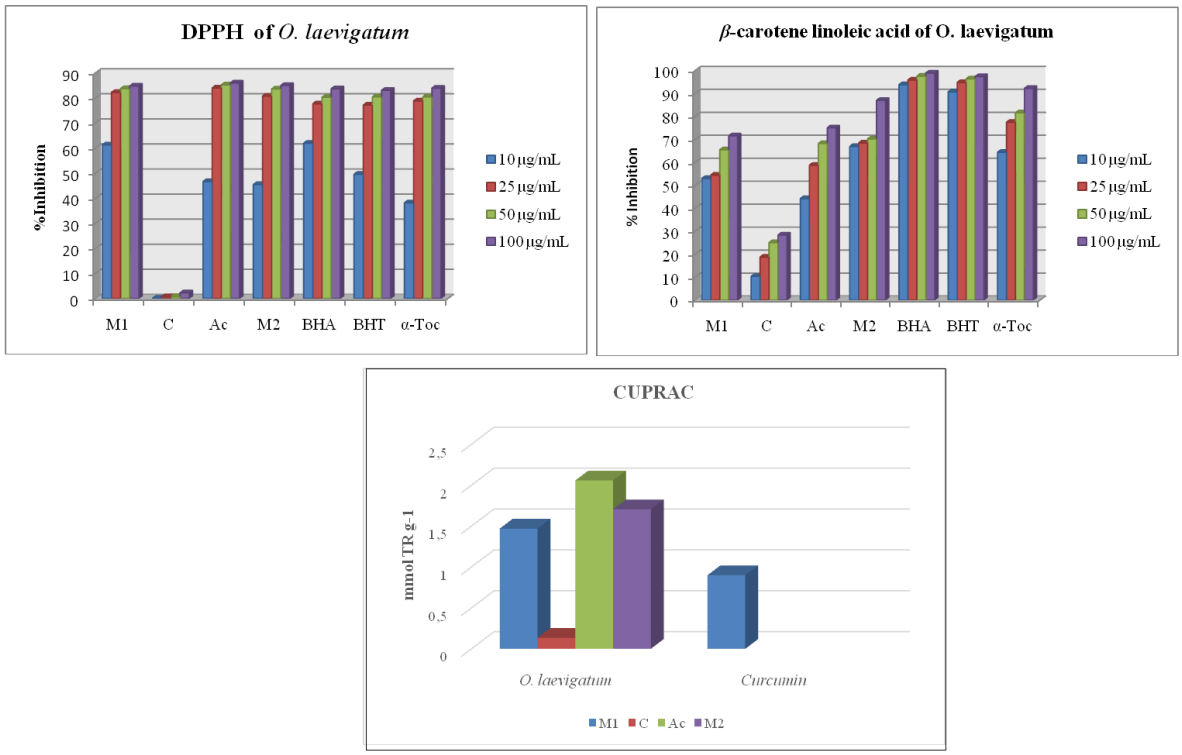

Figure 1. Antioxidant activity results of the *O. laevigatum* extracts.
M1: Methanol extract **C**: Chloform extract **Ac**: Acetone extract; **M2**: methanol 2 (successively)

As a conclusion, quantities of relatively small phenolics were successfully characterized by using LC-MS/MS and chemical composition and antioxidant activity relationship evaluated of the various extracts of *O. laevigatum*. The highest antioxidant activity was determined in the acetone extract and this activity mainly comes from rosmarinic acid. As a new source of rosmarinic acid the species can be used as an antioxidant agent in many food supplements in the future.

Acknowledgments

This work was supported by The Scientific and Technological Research Council of Turkey (TUBITAK) [grant number 113Z710].

Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/journal/journal-of-chemical-metrology

ORCID
Sema Çarıkçı: 0000-0003-3657-9926
Turgut Kılıç: 0000-0002-6842-3160
Züleyha Özer: 0000-0003-4957-5756
Tuncay Dirmenci: 0000-0003-3038-6904
Turan Arabacı: 0000-0003-3869-4557
Ahmet C. Gören: 0000-0002-5470-130X

References

[1] T. Dirmenci, T. Yazıcı, T. Özcan, Ç. Çelenk and E. Martin (2018). A new species and a new natural hybrid of *Origanum* L. (Lamiaceae) from the west of Turkey, *Turk. J. Bot.***42**, 73–90.

[2] T. Dirmenci, T. Özcan, T. Yazıcı, T. Arabacı, and E. Martin (2018). Morphological, cytological, palynological and molecular evidence on two new hybrids: an example of homoploid hybridization in *Origanum* (Lamiaceae), *Phytotaxa*, **371(3),** 145–167.

[3] Z. Özer, T. Kiliç, S. Selvi, and C. Pasa (2018). Effect of different drying methods and development stages on the essential oil chemical composition of aerial parts of *Origanum vulgare* L. subsp. *hirtum* (link) Letsw, *J. Essent. Oil Bear. Pl.* **21(5),** 1403-1409

[4] H. Yılmaz, S. Çarıkçı, T. Kılıç, T. Dirmenci, T. Arabacı, and A.C. Gören (2017). Screening of chemical composition, antioxidant and anticholinesterase activity of section *Brevifilamentum* of *Origanum* (L.) species, *Rec. Nat. Prod.***11(5),** 439-455.

[5] H. Özbek, Z. Güvenalp, T. Özek, H.G. Sevindik, H. Yuca, K.Ö. Yerdelen, and L.Ö. Demirezer (2017). Chemical composition, antioxidant and anticholinesterase activities of the essential oil of *Origanum rotundifolium* Boiss. from Turkey, *Rec. Nat. Prod.* **11(5)**, 485-490.

[6] K.H.C. Baser, T. Özek, M. Kürkçüoglu and G. Tümen (1996). The essential oil of *Origanum laevigatum* Boiss., *J. Essent. Oil. Res.***8,** 185-186.

[7] A.O. Tucker and M.J. Maciarello (1992). The essential oil of *Origanum laevigatum* Boiss. (Labiatae), *J. Essent. Oil Res.***4,** 419-420.

[8] B. Sariboga and H. Korkmaz (2009). Antimicrobial activity of *Thymus leucotrichus* and *Origanum laevigatum*, *Asian J. Chem*. **21(4)**, 2777-2781.

[9] S. Degerli, B. Tepe, A. Celiksoz, S. Berk and E. Malatyali (2012). In vitro amoebicidal activity of *Origanum syriacum* and *Origanum laevigatum* on *Acanthamoeba castellanii* cysts and trophozoites, *Exp. Parasitol.* **131(1)**, 20-24.

[10] H. Han, H. Yılmaz and İ. Gülçin (2018). Antioxidant activity of flaxseed (*Linum usitatissimum* L.) shell and analysis of its polyphenol contents by LC-MS/MS, *Rec Nat Prod*, **12(4)**, 397-402.

[11] H.O. Hamad, M. H. Alma, İ. Gulcin, M.A. Yilmaz, and E. Karaogul (2017). Evaluation of phenolic contents and bioactivity of root and nutgall extracts from Iraqian *Quercus infectoria* Olivier, *Rec. Nat. Prod.* **11(2)**, 205-210.

[12] M. Li, P.W. Pare, J. Zhang, T. Kang, Z. Zhang, D. Yang, K. Wang and H. Xing (2018). Antioxidant capacity connection with phenolic and flavonoid content in chinese medicinal herbs, *Rec. Nat. Prod.* **12(3)**, 239-250.

[13] D. Ozyurt, B. Demirata, R. Apak, J.F. Hamilton, A.C. Lewis and M.Z. Ozel (2016). GC×GC-TOF/MS chromatographic analysis, antioxidant capacity and phenolic content of *Rosa Canin*a L. at different maturities, *Rec. Nat. Prod.* **10(4)**,407-425.

[14] M. Glensk, J.A. Glinski, M. Jamróz, P. Stefanowicz and S. Kazmierski (2016). Phenolic constituents from *alchornea castaneifolia*, *Rec. Nat. Prod.* **10(1)**, 32-39.

[15] L. Monzote, W.H.P. Córdova, M. García, A. Piñón and W.N. Setzer (2016). *In-vitro* and *Iin-vivo* activities of phenolic compounds against *Cutaneous leishmaniasis*, *Rec. Nat. Prod.* **10(3)**, 269-276.

[16] S.B. Oguzkan, B. Karagul, E.S. Aksoy, A. Uzun, M. Can, H. Yilmaz, H.I. Ugras, B. Binici and A.C. Goren (2018). Determination of taxanes by validated LC-MS/MS method in hazelnut collected from different regions and altitudes in Turkey, *J. Chem. Metrol.* **12(1)**, 26-33.

[17] S.D. Hatipoglu (2016). Phenolic acid contents of *Salvia poculata* Nab by LC-MS/MS, *J. Chem. Metrol.* **10(1)**, 12-15.

[18] Z.O. Sagir, S. Carikci, T. Kilic and A.C. Goren (2017). Metabolic profile and biological activity of *Sideritis brevibracteata* PH Davis endemic to Turkey, *Int. J. Food. Prop.* **20(12)**, 2994-3005.

[19] G. Özkan and M.M Özcan (2014). Some phenolic compounds of extracts obtained from *Origanum* species growing in Turkey, *Environ. Monit. Asses.***186**, 4947-4957.

[20] B. Tepe, A. Cakir and A. Sihoglu Tepe (2016). Medicinal uses, phytochemistry, and pharmacology of *Origanum onites* (L.) : A Review, *Chem. Biodivers.* **13(5)**, 504-520.

[21] M.S. Blois (1958). Antioxidant determinations by the use of a stable free radical, *Nature***181**, 1199- 1200.

[22] G.J. Marco (1968). A rapid method for evaluation of antioxidants, *J. Am. Oil. Chem. Soc*. **45**, 594- 598.

[23] R. Apak, K. Güçlü, M. Özyürek and S. E. Karademir (2008). Mechanism of antioxidant capacity assays and the CUPRAC (cupric ion reducing antioxidant capacity) assay, *Microchim. Acta*. **160**, 413-419.

[24] S.L.R Ellison and A. Williams (2012). EURACHEM/CITAC Guide CG 4: Quantifying uncertainty in analytical measurement.

[25] A.C. Goren, G. Bilsel and M. Bilsel (2007). Rapid and simultaneous determination of 25-OH-vitamin D2 and D3 in human serum by LC/MS/MS: validation and uncertainty assessment, *J. Chem. Metrol.***1**, 1-10.

[26] A.C. Gören, S. Çıkrıkçı, M. Çergel and G. Bilsel (2009). Rapid quantitation of curcumin in turmeric via NMR and LC-tandem mass spectrometry, *Food Chem.***113**, 1239-1242.

A C G
publications

© 2018 ACG Publications