

RESEARCH ON ENZYME INHIBITION POTENTIAL AND PHENOLIC COMPOUNDS FROM *ORIGANUM VULGARE* SSP. *VULGARE*

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Abstract

This study assessed for the first time, the enzyme inhibitory properties of the *Origanum vulgare* ssp. *vulgare* extract. The main compounds were analysed chromatographically and spectrophotometrically. The enzymatic inhibitory potential was evaluated *in vitro*, against enzymes that play key roles in different diseases: acetylcholinesterase, urease, tyrosinase, trypsin and xanthine oxidase. A specific phenolic profile was evidenced, with large amounts of rosmarinic acid and luteolin 7-*O*-glucuronide; two phenolic diterpenes (rosmanol and rosmadial) were identified for the first time in this species. The oregano extract exhibited significant urease, tyrosinase and xanthine oxidase inhibitory effects, in line with the phenolic content. As a result of this study, new potential biological activities have been described for *Origanum vulgare* extract, which can be further studied and used in the pharmaceutical field.

Rezumat

În acest studiu se evaluează pentru prima dată potențialul inhibitor enzimatic al extractului de *Origanum vulgare* ssp. *vulgare* (șovârf). Principalii compuși chimici au fost analizați prin metode cromatografice și spectrofotometrice. Capacitatea inhibitorie a fost evaluată *in vitro*, asupra unor enzime implicate în anumite afecțiuni: acetilcolinesteraza, ureaza, tirozinaza, tripsina și xantin-oxidaza. În ceea ce privește profilul fenolic al extractului, s-au pus în evidență cantități mari de acid rozmarinic și luteolin-7-*O*-glucuronidă; două diterpene fenolice (rosmanol și rosmadial) au fost identificate pentru prima dată în această specie. Extractul de șovârf a inhibat semnificativ ureaza, tirozinaza și xantin-oxidaza, în concordanță cu conținutul mare de compuși polifenolici. Prin acest studiu s-au semnalat noi activități biologice potențiale ale extractului de *Origanum vulgare*, care deschid noi perspective de interes științific în domeniul farmaceutic.

Keywords: *Origanum vulgare* ssp. *vulgare*, polyphenols, enzyme inhibition

Introduction

Origanum vulgare L. (*Lamiaceae* family) is used worldwide for its flavour as a spice, having a long history of use in gastronomy and traditional medicine [10, 28]. At the same time, oregano is considered a medicinal plant due to its complex chemical composition which includes: essential oil, polyphenols, diterpenoids, triperpenoids. It is known as antimicrobial, antiviral, hepatoprotective, antidiabetic, antiinflammatory, anti-oxidant, antispasmodic, antiurolithic, antiproliferative, neuroprotective agent, to mention some of the scientifically proven therapeutic actions [6-8, 11, 23, 25, 26]. Natural herbal products are increasingly used in many fields: pharmaceutical, food and cosmetics. In recent years, interest in this plant, as well as the enzymatic activities of plant extracts involved in the protection of human health, have increased. The purpose of this study was to analyse the chemical composition and to evaluate the enzymatic inhibition

of *O. vulgare* ssp. *vulgaris* ethanolic extract, in order to be used in the design of new formulations with therapeutic applications in Alzheimer's disease, ulcer, gout, or hyperpigmentation.

Materials and Methods

The aerial parts of *O. vulgare* ssp. *vulgare* were harvested from Beliș (Cluj, Romania), during the blooming period (July 2018) and a voucher specimen (no. 96) was deposited in the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Cluj-Napoca, Romania. The extract was prepared in 70% vol. ethanol and the ratio between the vegetal material and the solvent was 1:10 (m/v) [2, 5, 32].

Chemicals, HPLC chromatographic conditions and quantitative analysis

Chemicals were obtained from Merck, Alfa-Aesar and Roth (Germany).

The HPLC analysis was performed using an Agilent HPLC system series 1200 with quaternary pump, solvent degassing system, auto-sampler, DAD spectrophotometric detector and single quadruple MS detector type Agilent 6110 (Agilent Technologies, CA, USA). The separation was performed on an Eclipse XDB C18 column, 4.6 x 150 mm with particles of 5 μ m (Agilent Technologies, USA), at 25°C, in gradient elution. The mobile phases were water: 0.1% acetic acid in acetonitrile (99:1) (solvent A) and 0.1% acetic acid in acetonitrile (solvent B) at a flow of 0.5 mL/min. The gradient applied was as follows: % B = 5% (0 - 2 min), from 5% to 40% (2 - 18 min), from 40% to 90% (18 - 20 min), then isocratic 4 min and decrease from 90% to 5% (24 - 25 min). The MS was used in ESI positive mode, in the following scanning conditions: capillary voltage at 3.000 V, temperature at 300°C, nitrogen flow at 8 L/min, m/z ranges from 100 to 1000, full-scan mode. The DAD spectrophotometric detection was performed at 340 nm. The data acquisition and the results processing were performed using ChemStation software from Agilent Technologies, USA. The identification was performed by comparison of obtained MS spectra with the ones from the library, meanwhile for the quantitative determination was used the DAD detection [3].

Determination of total polyphenols content

The total polyphenols content (TPC) was determined using the Folin-Ciocalteu method, with a calibration curve of gallic acid ($R^2 = 0.999$), and the results were expressed as mg of gallic acid equivalents (GAE)/g dry plant material [2, 4, 14, 32].

Enzyme inhibitory activity

Urease inhibition assay. This assay is based on ammonia quantification after urea hydrolysis in the presence of urease. Briefly, 0.2 mL urease (0.1 mg/mL) were treated with 4 mL Tris-HCl acid buffer (pH 8), 0.2 mL urea (60 mM) and 0.02 mL extract. The mixture was incubated at 30°C, for 20 min and then the reaction was stopped by addition of 1 mL 10% trichloroacetic acid. The ammonia content was evaluated by using 0.5 mL Nessler reagent. Absorbance was determined at 436 nm. Thiourea (200 μ g/mL) was used as a standard inhibitor [24].

Tyrosinase inhibition assay. The activity of this enzyme was spectrometrically evaluated using L-DOPA as a substrate. To 0.4 mL tyrosinase (250 UI/mL), 7.4 mL phosphate buffer (pH 7) and 0.04 mL extract were added; then the mixture was incubated 15 minutes at 30°C. At the end 0.2 mL 10 mM L-DOPA was added. Absorbance was determined at 475 nm and the ascorbic acid (340 μ g/mL) was used as standard [13, 20].

Acetylcholinesterase inhibition assay. The extract (1 μ L) was treated with 6 mL Tris-HCl buffer (pH 8) and with 0.05 mL acetylcholinesterase (6 UI/mL), then the mixture was incubated 15 minutes at 30°C. Subsequently, 0.1 mL 5,5-dithiobis-2-nitrobenzoic acid (3 mM) and 0.1 mL acetylthiocholine iodide

(15 mM) were added. The hydrolysis of the substrate was monitored by the formation of 5-thio-2-nitrobenzoate anion as the result of the reaction of 5,5-dithiobis-2-nitrobenzoic acid with thiocholine, released by enzymatic hydrolysis of acetyl thiocholine iodide. Absorbance was measured at 405 nm and galantamine (1.5 mg/mL) was used as a standard [20, 30, 31].

Trypsin inhibition assay. The assay was based on the spectrophotometric evaluation of trypsin inhibition using bovine serum albumin as a sub-substrate. To 0.1 mL extract, 0.2 mL trypsin (0.05%) were added and the mixture was incubated at 37°C, for 20 min. Then, 2 mL bovine serum albumin (10 mg/mL) and 4 mL phosphate buffer (pH 7.6) were added. The reaction was stopped by adding 2 mL trichloroacetic acid (10%). The mixture was treated with 1 mL alkaline cupric reagent and 0.1 mL phosphomolybdenic reagent. The absorbance was measured at 750 nm. Salicylic acid (5 mg/mL) was used as standard [12, 16, 21, 29].

Xanthine oxidase inhibition assay. To 0.15 mL extract, 3.9 mL phosphate buffer (pH 7.4) and 0.6 mL xanthine oxidase (0.2 UI/mL) were added and then the mixture was incubated 10 minutes at 25°C. Finally, 4.5 mL 0.15 mM xanthine was added and this was followed by 30 min incubation at 25°C. Allopurinol (0.3 mg/mL) was used as standard [1, 18, 22].

For all assays the standards and the control samples were prepared in the same manner. The inhibition potency (I%) was calculated in all cases with the following formula:

$$I\% = [1 - A_{\text{sample}}/A_{\text{control}}] \times 100,$$

where A_{sample} is the absorbance for sample (with the extract or standard) and A_{control} is the absorbance for control (without the extract or standard).

Statistical analysis. The samples were analysed in triplicate; the average and the relative SD were calculated using the Excel software package.

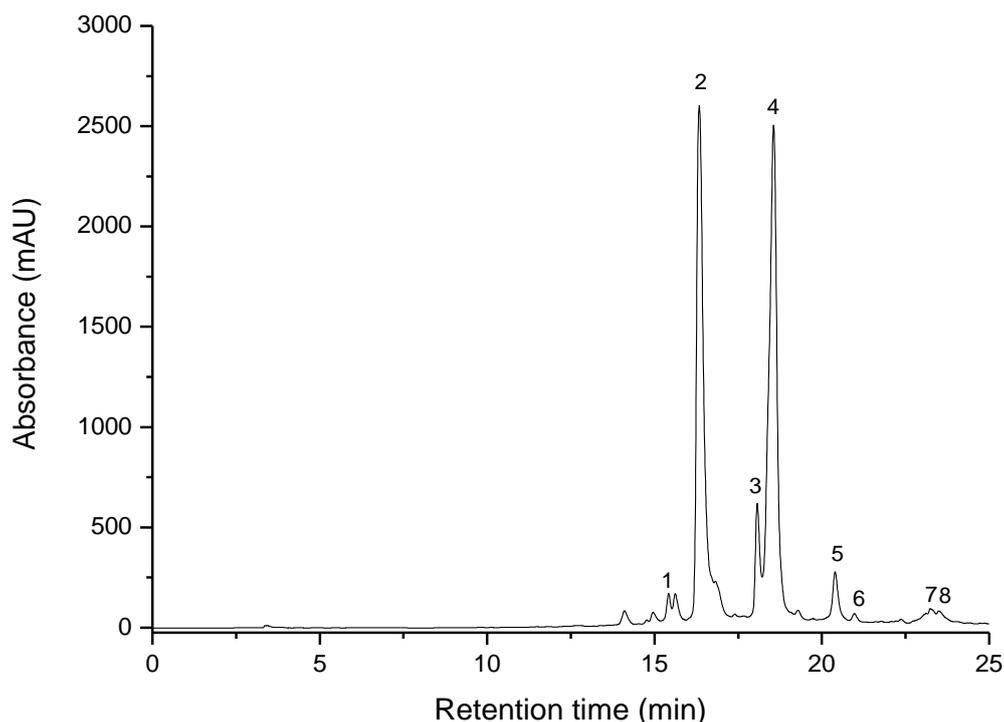
Results and Discussion

In the *Origanum* ethanolic extract a high content of total polyphenols (89.21 ± 2.79 mg GAE/g dry plant material) was determined. The results were similar to those previously obtained [6, 23].

With respect to HPLC analysis, Table I and Figure 1 show the phenolic compounds found in the ethanolic extract of *O. vulgare* ssp. *vulgare*, meaning 8 main phenolic compounds: 5 flavonoids of which 3 glycosides: of quercetin (rutin form), luteolin (glucuronide form) and kaempferol (glucoside form), and 2 free aglycons (luteolin and kaempferol). Rosmarinic acid was found in a large quantity, in accordance with similar results presented by other authors [6, 23, 28]. Two phenolic lactones diterpenes, rosmanol and rosmadial, were determined for the first time in the oregano extract. These antioxidant diterpenic compounds have been also identified in rosemary [9, 27].

Table IHPLC analysis of the phenolic compounds from *O. vulgare* ssp. *vulgare*

Compounds	m/z value	R _t (min)	Peak No.	Concentration (mg/g plant product)
Rutin (quercetin-3- <i>O</i> -rutinoside)	611	15.42	1	0.67 ± 0.02
Luteolin 7- <i>O</i> -glucuronide	463	16.03	2	15.20 ± 0.70
Kaempferol- <i>O</i> -glucoside	448	18.07	3	2.58 ± 0.42
Rosmarinic acid	361	18.55	4	16.89 ± 0.11
Rosmanol	347	20.39	5	1.79 ± 0.02
Luteolin	287	21.57	6	0.55 ± 0.06
Rosmadiol	345	22.98	7	0.45 ± 0.04
Kaempferol	287	23.49	8	0.62 ± 0.08

**Figure 1.**HPLC chromatograms for *Origanum vulgare* extract

Chromatographic conditions were as given in the Material and Methods section. The identified compounds: 1. Rutin; 2. luteolin 7-*O*-glucuronide; 3. kaempferol-*O*-glucoside; 4. rosmarinic acid; 5. Rosmanol; 6. Luteolin; 7. Rosmadiol; 8. kaempferol

In order to determine the enzyme inhibitory activity, our extract was tested against urease, tyrosinase, acetylcholinesterase, trypsin and xanthine oxidase (Table II).

There were correlated the total polyphenols content with the individual polyphenols concentrations. The Figure 2 shows that the main identified polyphenols are the rosmarinic acid respectively the luteoline-7-

O-glucuronide, representing 18.9 respectively 17 % from the total polyphenols content, while the others have less than 3 %. These polyphenols induce to the *O. vulgare* extract more antioxidant effect, that lead us to foresee mostly an inhibition on oxidative enzymes, like xanthinoxidase or tyrosinase, and less inhibition on hydrolytic enzymes, like acetylcholinesterase or trypsin.

Table IIEnzyme inhibition activity of *O. vulgare* ssp. *vulgare* extract

Sample	URE inhibition (%)	TYRE inhibition (%)	AChE inhibition (%)	TRY inhibition (%)	XO inhibitory (%)
<i>O. vulgare</i> extract	90.47 ± 6.07	58.63 ± 8.60	40.67 ± 3.00	17.1 ± 0.09	85.19 ± 5.10
Standards	Thi	Aa	Ga	Sa	Al
	93.26 ± 5.85	96.53 ± 5.14	99.83 ± 1.52	90.2 ± 0.13	91.05 ± 2.35

Each value is the mean ± SD of three independent measurements. URE: urease; α-CHYM: α-chymotrypsin; TYRE: tyrosinase; AChE: acetylcholinesterase; TRY: trypsin; XO: xanthine oxidase. Thi: thiourea; Aa: ascorbic acid; Ga: galantamine; Sa: salicylic acid; Al: allopurinol

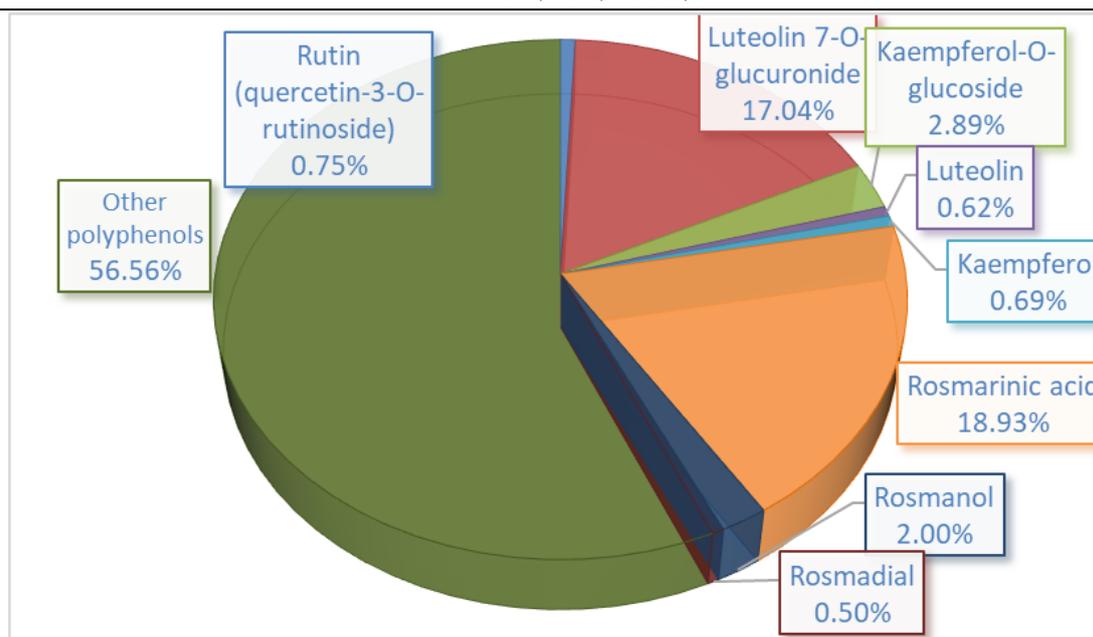


Figure 2.

Percentage distribution of individual polyphenols from total polyphenol content

Data on the enzymatic inhibitory properties of the ethanolic extract obtained from spontaneous *O. vulgare* ssp. *vulgare* are limited. Our results confirm the foresee upon the phytochemical profile, a better inhibition being obtained on oxidative enzymes, xanthinoxidase and tyrosinase, 85.19 respectively 58.63 %.

Urease is a Ni-containing enzyme related to gastro-duodenal diseases (gastric and peptic ulcers). It catalyses the hydrolysis of urea to ammonia and carbonic acid, and allows bacteria like *Helicobacter pylori* to survive at low pH values of the stomach [19, 24]. The results of this study suggest that at the tested dose, *O. vulgare* extract revealed a strong *in vitro* urease inhibitory activity (90.47%), very close to inhibition of the standard (Thi: 93.26% at 200 µg/mL). So, the oregano extract can be a potential anti-ulcer agent because of the polyphenolic compounds that are involved in the inhibition of *Helicobacter pylori* [19, 24].

Tyrosinase is an oxidase that converts L-tyrosine to L-DOPA and oxidizes DOPA to dopaquinone, which induces melanin biosynthesis. Tyrosinase inhibitors can control the melanin synthesis and could be useful in therapy (in hyperpigmentation, age spots etc.). In addition, polyphenols structurally similar to DOPA and tyrosine could block the synthesis of melanin. In the same time the lack of dopamine, obtained from L-DOPA, is involved in the pathogenesis of Parkinson's disease. Recently the tyrosinase inhibitors were tested to prevent or lower the evolution of this neuro-degenerative disease [17]. The tested extract of *O. vulgare* ssp. *vulgare* showed moderate tyrosinase inhibition activity (58.63%). Other authors have reported

similar anti-tyrosinase activity and rosmarinic acid is probably involved in this effect [13, 15].

Cholinesterase inhibition has become the most widely employed clinical approach for treating the symptoms of Alzheimer's disease [31]. In our study, the *O. vulgare* extract had a moderate inhibition activity on AChE (40.67%). Other authors have achieved comparable results [15].

Trypsin plays an important role in the virulence of many human, plant and insect pathogens. The trypsin inhibitors of plant origin have been reported widely from many plant species. *O. vulgare* extract exhibited low trypsin inhibition activity (17.1%). Naturally occurring trypsin inhibitors are proteins and this could explain the low activity of the ethanolic extract [12].

A good xanthine oxidase inhibition effect may be associated with a decrease in the production of uric acid. Our extract showed a good xanthine oxidase inhibitory activity (85.19%), so *O. vulgare* extract can provide encouraging premise for new anti-hyperuricemic natural products [18].

Conclusions

This study is the first attempt to evaluate the inhibitory action of ethanolic extracts of the indigenous species *Origanum vulgare* ssp. *vulgare* on some key enzymes involved in chronic diseases. A specific phenolic profile was highlighted by the presence of rosmarinic acid and luteolin-7-*O*-glucuronide, as well as rosmanol and rosmadiol, two phenolic diterpenes, which are reported for the first time. Our results showed that *O. vulgare* extract revealed a good urease, xanthine oxidase and tyrosinase *in vitro* inhibitory activity. These preliminary results could be useful for future

experimental studies, in order to define the efficiency of the *O. vulgare* extract in the treatment of different diseases.

Conflict of interest

The authors declare no conflict of interest.

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