

PRELIMINARY RESEARCH CONCERNING THE OBTAINING OF HERBAL EXTRACTS WITH POTENTIAL NEUROPROTECTIVE ACTIVITY

NOTE I. OBTAINING AND CHARACTERIZATION OF A SELECTIVE *ORIGANUM VULGARE* L. DRY EXTRACT

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Manuscript received: February 2016

Abstract

The aim of our research was obtaining, characterization and evaluation of the antioxidant activity, cytotoxic properties of a selective dry extract from indigenous *Origanum vulgare* L. (oregano), which might be used for the formulation of a phytomedicine with neuroprotective activity. Oregano phytochemical analysis was based on the HPLC determination of rosmarinic acid (identification, quantification) and spectrophotometric assays of flavonoids, phenolcarboxylic acids and total phenols. The antioxidant capacity was determined using DPPH and ferric reducing power activity methods. The dry selective extract (obtained with 50% ethanol) contains: 4.21 g flavones (expressed as rutin)/100 g dry extract, 52.67 g phenolcarboxylic acids (expressed as chlorogenic acid)/100 g dry extract, 27.30 g polyphenols (expressed as tannic acid)/100 g dry extract and 5.76 g rosmarinic acid/100 g dry extract. It has a good antioxidant activity ($EC_{50} = 2.62 \mu\text{g/mL}$ - DPPH method and $EC_{50} = 104.5 \mu\text{g/mL}$ - reducing power). Cytotoxic properties against *Daphnia magna* invertebrate were observed only at high concentrations, above 500 $\mu\text{g/mL}$. Our results offer encouraging premises for the formulation of a phytomedicine with potential neuroprotective activity.

Rezumat

Lucrarea și-a propus obținerea, caracterizarea, determinarea potențialului antioxidant și a citotoxicității unui extract selectiv din părțile aeriene ale speciei *Origanum vulgare* L., de proveniență indigenă, în perspectiva formulării unui fitopreparat cu acțiune neuroprotectoare. Materia primă vegetală a fost caracterizată fitochimic (identificarea și dozarea acidului rosmarinic printr-o metodă HPLC; dozare spectrofotometrică de flavone, acizi fenolcarboxilici și polifenoli totali) și din punct de vedere al activității antioxidante (metodele DPPH și reducerea ferului). Folosind ca solvent etanolul 50% s-a obținut un extract uscat. Acesta are un conținut de 4,21 g flavone (exprimate în rutozidă)/100 g extract uscat, 52,67 g acizi fenolcarboxilici (exprimați în acid clorogenic)/100 g extract uscat, 27,30 g polifenoli totali (exprimați în acid tanic)/100 g extract uscat și 5,76 g acid rosmarinic/100 g extract uscat. Extractul are o bună capacitate antioxidantă ($IC_{50} = 2,62 \mu\text{g/mL}$ - metoda DPPH; $IC_{50} = 104,5 \mu\text{g/mL}$ - metoda reducerii ferului) și o toxicitate marcantă asupra nevertebratului *Daphnia magna*, doar la doze mari (peste 500 $\mu\text{g/mL}$). Rezultatele obținute oferă premise încurajatoare pentru dezvoltarea unui fitopreparat cu acțiune neuroprotectoare.

Keywords: polyphenols, rosmarinic acid, antioxidant activity, *Daphnia magna*

Introduction

Origanum vulgare L. (oregano) is a perennial herb, with a wide distribution throughout Europe, Asia and Nord Africa [46]. *Origanum vulgare* L. ssp. *vulgare* is most studied regarding its phytochemical and biological activity. However related species such as *Origanum vulgare* L. ssp. *viride*, *Origanum vulgare* L. ssp. *hirtum*, *Origanum vulgare* L. ssp. *heracleoticum*, *Origanum vulgare* L. ssp. *virens*, *Origanum vulgare* L. ssp. *glandulosum*. *Origanum*

vulgare L. ssp. *gracile* have also raised scientific interest [6, 24, 46].

Aerial parts of oregano (*Origanum herba*) are used in therapeutics due to their high content of essential oil and polyphenols. According to scientific literature the herbal product is also a source of sterols, terpenes, vitamin C, carotenoids and minerals [2, 5, 21, 26, 27, 46, 48, 51, 53]. *Origanum* species show high variability in their essential oils content (in terms of quality and

quantity), due to geographical areas differences and vegetative stage. Oregano essential oil comprises a series of compounds, among which tymol, carvacrol, γ -terpineol, β -caryophyllene and p-cymene prevail [3, 5, 6, 25, 30, 31, 41, 46, 48, 49].

Among phenolcarboxylic acids, rosmarinic acid predominates. It has a wide spectrum of biological activities such as antioxidant, antiviral, anti-bacterial, anti-tumoural, anti-allergenic, anti-inflammatory. It also improves the cerebral blood flow [1, 17, 33, 40]. Moreover, according to scientific literature, chlorogenic acid, caffeic acid, ferulic acid, p-hydroxybenzoic acid, syringic acid, gallic acid, lithospermic acid, protocatechuic acid and origanoside have also been found in oregano aerial parts [22, 27, 46, 48, 51]. Aerial parts of *Origanum vulgare* L. are a source of flavones, such as glucosides of kempferol, quercetin, myricetin, apigenin, naringenin, luteolin, acacetin and taxifolin [14, 21, 27, 46, 51].

Regarding the herbal product therapeutic effects, oregano aerial parts are well known for their antioxidant [8, 9, 10, 15, 41], antibacterial, [10, 23], antifungal [12, 20], cytotoxic (upon melanoma B16, hepatocarcinoma HepG2, colorectal adenocarcinoma LoVo, breast cancer MCF-7 cell lines) [22, 26, 51], antiviral (HSV-1, RSV) [53], immunomodulatory [32], antimutagenic [14, 15] and estrogen-like [54] activities. Numerous research studies have been conducted regarding the essential oil pharmacological properties: antioxidant [6, 9, 15, 41, 45, 49, 50], antibacterial [7, 9, 18, 41, 43, 44, 45, 46], antifungal [12, 18], antiparasitic [47], inhibitory activity upon several enzymes (butyrylcholinesterase, acetylcholinesterase, tyrosinase, alpha-glucosidase, alpha-amylase) [6, 54]. Oregano essential oil also acts as a preserving agent useful for organic food products [3, 6].

Taking into consideration the scientific data, the aim of our study was obtaining, characterization and evaluation of antioxidant activity, cytotoxic properties of a selective dry extract from indigenous *Origanum vulgare* L. (oregano), which might be used for the formulation of a phytomedicine with neuroprotective activity.

Materials and Methods

Materials. Aerial parts of *Origanum vulgare* L. were harvested in August 2015 from Morărești, Argeș District (44°58' North, 24°34' East), Romania. The raw material was air-dried, packed in paper bags and stored in laboratory conditions.

Reagents and solvents. All reagents and solvents were purchased from Roth (Germany), unless otherwise stated.

The research was carried out in several stages: 1) quality assessment of the raw material; 2) evaluation of the herbal product antioxidant activity; 3) obtaining and characterization of a selective dry extract;

4) determination of extracts antioxidant capacity; 5) assessment of oregano dry extract cytotoxic properties using *Daphnia magna* bioassay.

The quality assessment of the raw material was determined through HPLC identification and quantification of rosmarinic acid and spectrophotometric assays of flavones, phenolcarboxylic acids and total phenolic content.

Preparation of stock solutions for HPLC, spectrophotometric and antioxidant activity assays. 2.00 g of herbal product were heated twice with a 50% solution of ethanol on a reflux condenser, for 30 minutes. After cooling, the solutions were filtered into a 100 mL volumetric flask and filled to mark with the same solvent.

The HPLC analysis was carried out using a Jasco HPLC MD-2015 equipped with degasser, binary gradient pump, column thermostat and UV detector, according to a previous described method [13]. The analytical data was evaluated using a Jasco data processing system (Chrompass). Rosmarinic acid was identified based on its retention time and UV spectra. The calibration curve for rosmarinic acid (2 - 20 $\mu\text{g/mL}$, $R^2 = 0.989$, $n = 5$) showed good linearity.

Spectrophotometric assays. The flavones content was determined based on the chelating reaction with aluminium chloride [52]. Phenolcarboxylic acids (PCA) were assessed based on formation of oxymes in the presence of sodium nitrite/ hydrochloric acid and sodium hydroxide [52]. Total polyphenols were evaluated based on their capacity to reduce molybdenic compounds (VI) [39]. For all spectrophotometric determinations a Jasco V-530 spectrophotometer (Jasco, Japan) was used. The following calibration curves were used to calculate the content of active compounds: rutin (linearity range: 5.0 - 35.0 $\mu\text{g/mL}$, $R^2 = 0.9998$, $n = 11$), chlorogenic acid (linearity range: 0.0113 - 0.0527 mg/mL, $R^2 = 0.9998$, $n = 6$) and tannic acid (linearity range: 2.0 - 12.0 $\mu\text{g/mL}$, $R^2 = 0.9990$, $n = 10$).

The antioxidant activity was evaluated by means of two well-known methods, DPPH radical scavenging capacity and ferric reducing power, respectively [36].

DPPH radical scavenging capacity

Briefly, the stock solution was diluted with 50% ethanol in order to obtain solutions with equivalent concentrations of 8.0 - 80.0 mg herbal product/mL (test solutions). 2.0 mL of the test solutions were treated with 2 mL of a 120.0 μM ethanolic solution of DPPH. The mixture was kept in the dark, at room temperature and the absorbance of DPPH solution was measured at $\lambda = 516$ nm, before (A_{start}) and 30 min. after adding the test solutions (A_{end}). Ethanol was used as a blank. The ability to scavenge the DPPH free radical was calculated using the following formula: DPPH radical scavenging activity (%) = $(A_{\text{start}} - A_{\text{end}})/A_{\text{start}} \times 100$.

The concentration of the test solutions that inhibited 50% of the DPPH free radical (EC_{50} , $\mu\text{g/mL}$) activity was determined graphically from the linear regression curve plotted between percent (%) of inhibition and test solutions concentration ($\mu\text{g/mL}$) [28].

Ferric reducing power

The assay was performed according to Oyaizu M. method [34]. Briefly, the stock solution was diluted with 50% ethanol in order to obtain solutions with equivalent concentrations of 65.0 - 3239.0 μg herbal drug/mL (test solutions). 2.5 mL of these test solutions were mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 10 g/L potassium ferricyanide. Samples were kept at 50°C in a water bath (Raypa, Spain) for 20 min. After that, 2.5 mL of 100g/L trichloroacetic acid was added and the mixture was centrifuged at 2500 rpm for 5 min. (Universal 16 centrifuge). The upper layer (2.5 mL) was mixed with 2.5 mL of water and 0.5 mL of a 1.0 g/L ferric chloride solution. The absorbance was measured at $\lambda = 700$ nm, after 20 min., against a blank that contained all reagents except for the test solutions. A higher absorbance indicates a stronger reducing power. The test solution concentration providing 0.5 of absorbance (EC_{50} $\mu\text{g/mL}$) was determined graphically from the linear regression curve plotted between absorbance and test solutions concentration ($\mu\text{g/mL}$).

Obtaining and characterization of a selective dry extract (Origanum extractum = OE)

Preparation of oregano dry extract (OE): 800 g of powdered oregano aerial parts were heated twice with ethanol 50% (v/v) under a reflux condenser for 60 min., using 1:10 herbal product/ solvent ratio for the first extraction and 1:5 for the second one. After cooling, the combined filtrates were concentrated using a rotary evaporator (Buchi R 210 - 215) to remove the solvent, and then freeze-dried (using a Christ Alpha 1-2/B Braun, BiotechInternational lyophilizer) to yield 162 g extract.

The same qualitative and quantitative analysis used in stage 1 of our research has been employed for the dry extract characterization. All assays were performed using a 0.1% stock solution, prepared by dissolving 0.1 g extract in 50% ethanol.

The antioxidant effect of the extract was evaluated by the same methods presented in stage 2 of our analysis. Only the concentration ranges of the samples were different: 2.0 - 250.0 μg extract/mL for DPPH method; 4.0 - 250.0 μg extract/mL for ferric reducing power method, respectively.

The cytotoxicity of the extract was assessed using *Daphnia magna* bioassay. The method consists in exposure of the invertebrates at serial dilutions of

the test substance/compound and counting the survivors after 24 and 48 h of incubation. The bioassay was performed according to the methods described by Fan *et al.* [11] and Nitulescu *et al.* [29] with some modifications [42].

Preparation of samples (ED). 50.0 mg extract was dissolved in 10 mL of 5% DMSO solution. Six different volumes of this solution were diluted with artificial water, in order to obtain six serial dilutions, having concentrations equivalent to 20 μg extract/mL (noted ED1), 50 μg extract/mL (ED2), 250 μg extract/mL (ED3), 500 μg extract/mL (ED4), 1000 μg extract/mL (ED5) and 1500 μg extract/mL (ED6).

The invertebrates were selected from a parthenogenetically culture from 2012 (maintained at "Carol Davila" University, Bucharest, Romania, Department of Pharmaceutical Botany and Cell Biology). 10 daphnids were inserted in graduated test tubes and the ED dilutions were added. Synthetic water with and without 1% DMSO were used as controls. The daphnids were maintained at $25 \pm 1^\circ\text{C}$ in a plant growth chamber (Sanyo MLR-351 H, USA) [29, 42]. The tests were performed in duplicate. The number of survivors was counted at 24 h and 48 h, and lethality percentages were calculated.

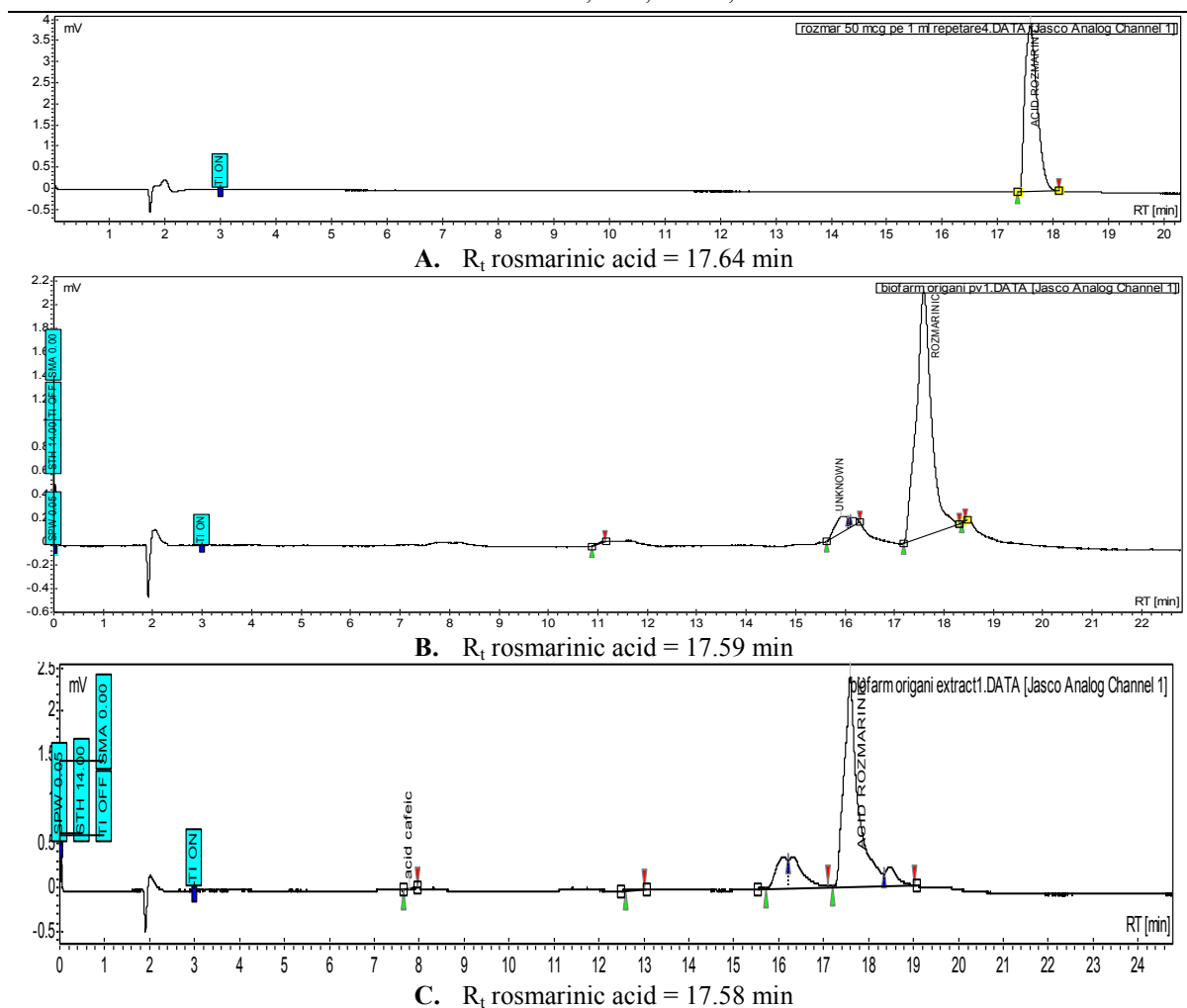
Statistical analysis. The data analysis was performed using Microsoft Excel 2010 software (Microsoft Corp., USA) and GraphPad Prism v. 5.0. (GraphPad Software, USA).

Results and Discussion

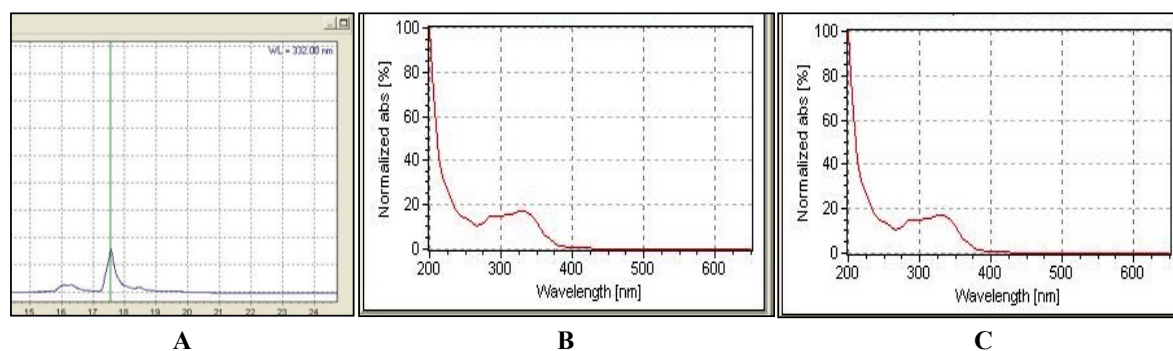
Retention times (R_t) and UV spectra confirmed the presence of rosmarinic acid in both *Origanum herba* and *Origanum extractum*. (Figure 1.A-C; Figure 2.A-C). Our spectrophotometric results (Table I) revealed a significant amount of flavones in oregano aerial parts (1.66 g rutin/100 g herbal material, equivalent to 0.82 g quercetin/100 g herbal material), higher compared to other authors results, that reported 156.93 μg flavones/g [19].

The herbal product and dry extract PCA content (Table I) was higher compared to scientific literature data, that reports a content of 7% hydroxycinnamic acid derivatives for oregano aerial parts [46].

The content of total polyphenols determined in the present study (11.63 g tannic acid/100 g herbal material) is lower than the one cited by other authors [38]. We assume that both herbal product source and experimental protocol are responsible for these differences.

**Figure 1.**

HPLC chromatograms: A – rosmarinic acid standard solution; B – *Origanum herba*; C – *Origanum extractum*

**Figure 2.**

UV spectra of rosmarinic acid: A – standard solution; B – *Origanum herba*; C – *Origanum extractum*

Our results emphasized an important content of rosmarinic acid in aerial parts of *Origanum vulgare* L. (2.45 mg/g) (Table I). Scientific data regarding the rosmarinic acid content are both controversial and varied. For instance Valero A. *et al.* found 52 µg/g, Lukas B. *et al.* – 0.6 - 37.2 mg/g, Shekarchi M. *et al.* – 25.0 mg/g, and Shan B. *et al.* – 25.63 mg/g [25, 38, 40, 47]. As far as flavones content is concerned, our results are similar to

Spiridon I. *et al.*, but higher than ones found by Marelli M. *et al.* [26, 43]. We consider that differences among our results and other authors' research are the consequence of geographic influences upon chemical composition together with different experimental conditions. Total phenolic content determined by us is lower compared to other author research, since different extraction methods have been used [10, 43].

Table I

Quantitative analysis results

Active substances	Method	<i>Origani herba</i>	<i>Origani extractum</i>
Flavones	spectrophotometry	1.6607 ± 0.217 (g rutin/100 g herbal material)	4.2132 ± 0.127 (g rutin/100 g dry extract)
PCA		28.7075 ± 1.759 (g chlorogenic acid/100 g herbal material)	52.758 ± 3.492 (g chlorogenic acid/100 g dry extract)
Total phenolic content		11.63295 ± 0.150 (g tannic acid/100 g herbal material)	27.29572 ± 2.468 (g tannic acid/100 g dry extract)
Rosmarinic acid	HPLC	0.245 g/100 g herbal material	5.762 g/100 g dry extract

Results are mean ± SD (n = 5)

As regards the antioxidant activity (Table II), oregano extract displays a better antioxidant capacity compared to the raw material (pointed out by a lower EC₅₀ value). Our EC₅₀ values (µg/mL) are lower compared to other authors' results, which suggest a better antioxidant activity [6, 23, 45]. However the comparison with scientific literature was difficult, since different extraction methods and experimental protocols have been used.

Table IIAntioxidant activity - EC₅₀ (µg/mL)

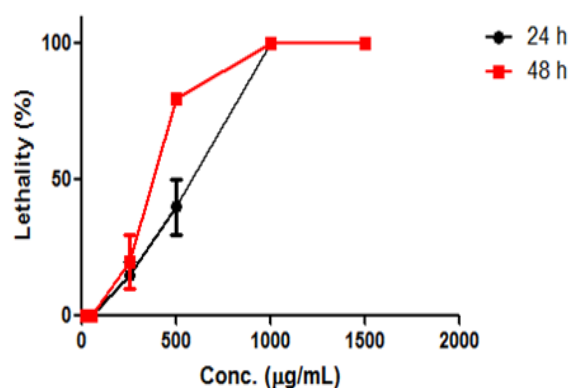
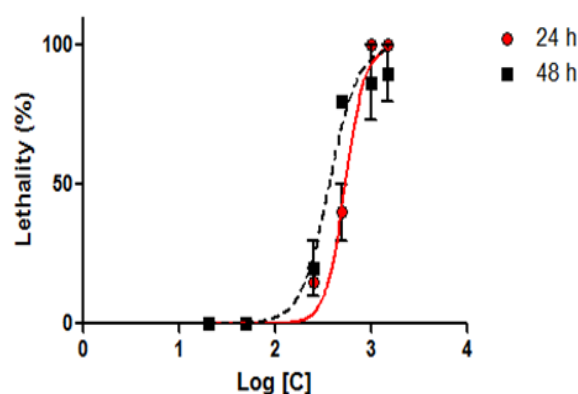
Method	<i>Origani herba</i>	<i>Origani extractum</i>
DPPH	15.83 ± 1.41	2.62 ± 0.29
Ferric reducing power	613.00 ± 0.00	104.5 ± 0.14

Results are mean ± SD (n = 3)

Considering the oxidative stress role in neurodegenerative, cardiovascular and metabolic diseases [15, 31, 34], the high content of polyphenols and rosmarinic acid correlated with a strong *in vitro* antioxidant capacity represents the basis of future research for formulation of a phytomedicine with neuroprotective activity. Our extract induced a moderate to high toxicity on *Daphnia magna*, both after 24 h and 48 h. Thus, at higher concentrations (1500 and 1000 µg/mL) the lethality is 100%, whereas at 500 µg/mL, the lethality is approximately 35% at 24 h and 80% at 48 h (Table III). At lower concentrations (20.50 µg/mL) no lethality was registered.

The LC values indicate that the toxicity of the extract is moderate at 24 h and slightly increased at 48 h. The toxicity of the extract is probably cumulative, the two lethality curves being almost parallel (Figure 3 and Figure 4). R² coefficients are satisfactory, thus showing a good correlation between concentration and biological effect (Table III). The high lethality registered at concentrations ranging from 500 to 1500 µg/mL is well correlated with a high phenolic content. These compounds behave at

higher concentrations as prooxidants and can induce apoptosis [16]. Practically, this ambivalence is a consequence of polyphenols concentration. Thus, further studies are required in order to determine the therapeutic doses.

**Figure 3.**Lethality on *Daphnia magna* versus concentration of extract**Figure 4.**Concentration-lethality curves for cytotoxic activity on *Daphnia magna***Table III**Cytotoxicity of *Origani extractum* on *Daphnia magna*

Sample	Time of determination	LC50 (µg/mL)	IC95% (µg/mL)	Goodness of fit r ²
<i>Origani extractum</i>	24 h	532.6	474.7 - 597.5	0.9727
	48 h	364.4	278.6 - 476.4	0.8977

Conclusions

Aerial parts of indigenous oregano (*Origanum herba*) were phytochemically characterised by means of qualitative and quantitative methods. The herbal product was used for obtaining a dry hydro-ethanolic extract rich in flavones, phenolcarboxylic acids, polyphenols and rosmarinic acid. The dry extract showed antioxidant activity. At higher concentrations (above 500 µg/mL) the extract showed maximum toxicity on *Daphnia magna* invertebrate.

Future pre-clinical research is needed for the determination of the pharmaco-toxicological profile, in view of obtaining a phytomedicine with potential neuroprotective activity.

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