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EVALUATION OF THE ANTIOXIDANT EFFECTS AND CYTOTOXIC POTENTIAL OF SELECTED HERBS USED IN TRADITIONAL MEDICINE

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ABSTRACT

The present study aims to investigate the bioactive properties of five aromatic herbs: *Anethum graveolens* L., *Artemisia dracunculus* L., *Artemisia vulgaris* L., *Satureja hortensis* L. and *Vanilla planifolia* Jacks. ex Andrews. Polyphenolic and flavonoid content were determined by Folin-Ciocalteu method and $AlCl_3$ method, respectively. The antioxidant activity of the extracts was evaluated by DPPH radical scavenging assay, FRAP assay, NSSOH and SSOH assay. Additionally, their cytotoxic potential was investigated by performing the Brine shrimp lethality assay. Obtained results revealed that all samples contain significant amount of phenolic compounds, with *Artemisia dracunculus* and *Satureja hortensis* showing highest concentration of polyphenols and flavonoids. Moreover, these extracts also demonstrated strong radical scavenging ability and reducing capacity. Differences between IC_{50} values in the NSSOH and SSOH assays revealed that *Artemisia dracunculus* also demonstrated prominent chelating properties. According the Brine shrimp lethality assay, *Artemisia vulgaris* showed strong cytotoxicity against the Artemia larvae, while *Artemisia dracunculus* and *Satureja hortensis* showed moderate cytotoxic potential.
Overall, *Artemisia vulgaris*, *Artemisia dracunculus* and *Satureja hortensis* could be considered as potential sources of biologically active phytochemicals for their further utilization in prevention from many chronic conditions based on oxidative stress.

Keywords: commercial herbs, antioxidant activity, cytotoxic potential, brine shrimp lethality assay.

INTRODUCTION

Reactive oxygen species (ROS) are generated as by-products under physiological conditions, but they might be harmful if they are not eliminated. The efficiency of the antioxidant defense systems in the body is altered under pathological conditions and the ineffective scavenging or overproduction of ROS may result in disruption of the normal cellular homeostasis. Extensive research during last two decades revealed the association of oxidative stress and inflammation, which in turn is the basis of many chronic diseases including cardiovascular, neurological, autoimmune diseases, cancer, etc (Rakoff-Nahoum *et al.*, 2006; Reuter *et al.*, 2010). Herbs that were selected for testing of their antioxidant activity and cytotoxic potential have been used as natural sources of flavoring and for their healing properties as part of traditional medicine for the treatment of numerous pathological conditions (Murcia *et al.*, 2004).

As reported in ethnopharmacological studies, *Artemisia dracunculus* was traditionally used as stomachic, emmenagogue, to treat infections, cancer and circulatory disorders. On the other hand, *Artemisia vulgaris* has been highly valued as diuretic agent and a febrifuge. The medicinal importance of *Artemisia* species is mostly valued for the use of artemisinin in the treatment of malaria (Mannan *et al.*, 2010). Literature review on the traditional use of *Satureja hortensis* demonstrate that it is generally used in the form of tea for the treatment of diarrhea, stomach upsets and sore throats. Seeds, leaves, and oils from *Anethum graveolens* have a wide use for the treatment of sleep disorders, kidney and urinary tract infections. Aerial parts are valued for their antibacterial properties because of the bioactive coumarins. *Vanilla planifolia* was traditionally recognized as aphrodisiac and febrifuge. Several studies examined the bioactive profile of the main component vanillin and reported its cytolytic and cytostatic effects (Ho *et al.*, 2009).

Previous research on the phytochemical composition of medicinal plants showed that they contain a wide range of secondary metabolites that provide their therapeutic effects. A great number of plant species contain pharmacologically and biologically active ingredients, of which many were identified as phenolic compounds (Sytar *et al.*, 2016). Therefore, determination of the total phenolic and flavonoid contents were also included in the current research. Since the selected plant species are widely utilized for the treatment of numerous pathological conditions, the aim of this study was to examine their antioxidant capacity and compare their activity with standards. Moreover, insufficient data on their cytotoxic activity prompted the need to determine

their cytotoxic potential *in vivo* and compare it to well known cytotoxic agents.

MATERIALS AND METHODS

Plant material: Five commercialized plant species were tested for their bioactivity: aerial parts from *Anethum graveolens* L. (dill), *Artemisia dracunculus* L. (tarragon), *Artemisia vulgaris* L. (common wormwood), *Satureja hortensis* L. (summer savory) and fruit from *Vanilla planifolia* Jacks. ex Andrews (vanilla). Dry plant material was milled in electric grinder and stored in airtight containers at ambient temperature until use.

Chemicals: Folin-Ciocalteu reagent, 2-thiobarbituric acid, aluminum chloride, ascorbic acid, sodium carbonate, ferrous sulfate heptahydrate, EDTA, ferric chloride were purchased from Merck, Germany, while gallic acid, quercetin, 2-deoxy-D-ribose, DPPH (1,1-diphenyl-2-picrylhydrazyl) reagent, $K_2Cr_2O_7$ (potassium dichromate), thymol, Trolox, BHA (Butylated hydroxyanisole) from Sigma-Aldrich (Germany). Ethanol 96% (Ph.Eur.), hydrogen peroxide 30% (v/v), sodium nitrite, sodium hydroxide and sodium chloride were acquired from Alkaloid, Skopje, while DMSO (dimethyl sulfoxide), CCl_3COOH (trichloroacetic acid) and $K_3Fe(CN)_6$ (potassium ferricyanide) were supplied by Carlo Erba, Italy.

Preparation of extracts: Samples were prepared by extraction of 250 mg dry material with 25 mL of 96% (v/v) ethanol in ultrasonic bath (50/60 Hz, 720W) for 30 minutes at room temperature. After filtration, the volume of the extracts was adjusted to 25 mL with ethanol and stored in dark at 4 °C. Preparation of the samples for the cytotoxicity assay was performed by ultrasonification of 15 g dry material in 150 mL of water: ethanol mixture (1:1, v/v) during 1 hour at 40 °C, followed by filtration. Filtrates were evaporated until dry on vacuum rotary evaporator and later freeze dried. Obtained lyophilizates were preserved at -18 °C in a dark place and later reconstituted with DMSO (0.05%, v/v) to final concentration of 10 mg/mL.

Methods: Determination of the total phenolic content (TPC) and total flavonoid content (TFC) was carried out by Folin-Ciocalteu method and Aluminum chloride method, respectively. Antioxidant activity of the extracts was evaluated by DPPH (1,1-diphenyl-2-picrylhydrazyl) assay, FRAP (Ferric reducing antioxidant power) assay, NSSOH (Nonsite-Specific Hydroxyl Radical-mediated 2-deoxyribose degradation) assay and SSOH (Site-Specific Hydroxyl Radical-mediated 2-deoxyribose degradation) assay. Additionally, brine shrimp lethality assay was conducted for the cytotoxicity testing of the extracts.

Determination of Total Phenolic Content: Total phenolic content was determined with the Folin-Ciocalteu assay according to a procedure described by Singleton *et al.* (1999) with minor modifications. An aliquot of the extract (0.1 mL) was mixed with 2.5 mL of diluted Folin-Ciocalteu reagent (1:10) and the mixture was kept at ambient temperature, with periodical mixing. After 5 minutes incubation, 3 mL of Na_2CO_3 (7%, w/v) were added and contents were mixed. The volume of the mixture was adjusted to 10 mL with deionized water and allowed to stand for 1 h in a dark place. Absorbance of the samples was measured at 765 nm. Results were expressed as gallic acid equivalents/g crude extract, based on the equation from the callibration curve for gallic acid standard (20 – 200 μg/mL).

Determination of Total Flavonoid Content: Total flavonoid content of the crude extracts was determined with the Aluminum chloride method as described by Lallianrawna *et al*. (2013). The reaction solution was prepared by mixing 0.1 mL of extract with 0.1 mL of $NaNO_2$ (5%) and incubated for 5 minutes, followed by the addition of 0.15 mL $AlCl_3$ (10%) and kept for 6 minutes. After incubation with 0.5 mL of NaOH (1M) and vigorously shaking, the volume of the mixture was diluted with deionized water to the final volume of 2.5 mL. Absorbance of the resulting solution was measured at 510 nm. Results were expressed as quercetin equivalents/g crude extract using the equation from the calibration curve for quercetin (10 – 120 μg/mL).

DPPH assay: The radical scavenging ability of the extracts was evaluated by DPPH assay according the optimized method of Brand-Williams *et al.* (1995). Three concentrations of extract were tested: 2, 5 and 10 mg/mL. Aliquots of each concentration (0.2 mL) were mixed with 4 mL of DPPH ethanol solution (100 μM) and shaken vigorously. After 10 minutes of incubation in the dark, absorption of the mixtures was measured at 517 nm. Quercetin, Trolox, BHA and ascorbic acid were used as standards. Percentage of inhibition ($I_{\%}$) for each concentration was calculated according the equation:

$$I_{\%} = [(A_{blank} - A_{sample})/A_{blank}] \times 100\%$$

The antioxidant capacity of the extracts was expressed as IC_{50} values (inhibitory concentration of extract that reduces the absorbance of the DPPH solution by 50%):

$$IC_{50}\ (mg/mL) = (50 - b)/a$$

FRAP assay: Ferric reducing antioxidant power assay was conducted by the method of Oyaizu *et al.* (1986). The reaction mixture was prepared by adding 2.5 mL phosphate buffer (0.2 M, pH 6.6) and 2.5 mL $K_3[Fe(CN)_6]$ (1%) to 0.25 mL of different concentrations of the extract. After incubation at 50 °C for 20 minutes, the reaction was stopped by adding 2.5 mL CCl_3COOH

(10%). An aliquot of the mixture (2.5 mL) was transferred into an empty tube and mixed with 2.5 mL distilled water and 0.5 mL $FeCl_3$ (1%). The absorbance of the mixture was measured after 30 minutes at 700 nm. A calibration curve was prepared for $FeSO_4$ x $7H_2O$ (0.5 – 15 mmol/mL). Quercetin, Trolox, BHA and ascorbic acid were used as standards. The reducing power of the extracts was expressed as FRAP capacity (%) based on the obtained regression equation.

***NSSOH and SSOH assays*:** The efficiency of the extracts to inhibit the formation of thiobarbituric acid reactive substances (TBARS) as a result of the hydroxyl-induced degradation of 2-deoxy-D-ribose was quantified according the SSOH assay without chelator EDTA and according the NSSOH assay in the presence of a chelator EDTA, as explained by Halliwell *et al.* (1987). 100 µL of different concentrations of extract were mixed with 500 µL 2-deoxy-D-ribose (5.6 mM, in KH_2PO_4-NaOH buffer, pH 7.4), 200 µL premixed (1:1 v/v) $FeCl_3$ (100 µM) and phosphate buffer/EDTA (104 µM), 100 µL H_2O_2 (1 mM) and 100 µL ascorbic acid (1 mM). The tubes were vortexed and incubated at 50 °C for 30 minutes, followed by the addition of 1 mL of CCl_3COOH (2.8%) and 1 mL of thiobarbituric acid (1%). Obtained mixture was vortexed and heated at 50 °C for 30 minutes and the extent of 2-deoxyribose degradation was measured at 532 nm. Quercetin, Trolox and BHA were used as standards. The hydroxyl scavenging activity of the extracts is expressed as IC_{50} values (inhibitory concentration of extract that reduces the absorbance of TBARS complexes by 50%) and calculated using regression analysis.

Hatching of Artemia salina larvae: Brine shrimp larvae were hatched in artificial sea water prepared as NaCl solution with concentration of 38 mg/mL and pH 9.00. The medium was constantly aerated and temperature regulated in the range from 25 to 28 °C. After 48 hours of continuous light exposure, *Artemia* larvae hatched in adult shrimps and entered instar stage III, the most optimal stage for the Brine Shrimp Lethality Assay (Sorgeloos *et al.*, 1978; Vanhaecke *et al.*, 1981).

Brine shrimp lethality assay: Cytotoxic potential of the extracts was determined by the method of Meyer *et al.* (1982) with slight modifications. Six concentrations were prepared from the stock solution (10 mg/mL): 5, 3, 1, 0.5, 0.1 and 0.01 mg/mL. Ten shrimps were pipetted and applied in each flask containing different concentrations of extract. Number of dead nauplii was counted after 6, 10, 24, 30, 36, 48, 54 and 60 hours of exposure and the mortality was established after 10 seconds if no movement was observed. $K_2Cr_2O_7$, thymol and BHA were used as positive controls in a concentration range from 0.01 to 5 mg/mL. Final results were expressed as LC_{50} values (lethal concentration that kills 50% of the exposed population of shrimps) calculated with probit regression analysis based on the percentage of mortality for each concentration after 24-hour exposure. The cytotoxic potential of the extracts was evaluated according their LC_{50} values and classified by two scales: Meyer's scale (Meyer *et al.*, 1982) and Clarkson's scale of toxicity (Clarkson *et al*., 2004). Meyer's scale classifies the extracts into two categories: toxic (LC_{50} < 1000 µg/mL) and non-toxic (LC_{50} > 1000 µg/mL). Additionally, extracts were classified into several subcategories by the Clarkson's scale of toxicity: high (0 – 100 µg/mL), moderate (100 – 500 µg/mL), low (500 – 1000 µg/mL) and no toxicity (> 1000 µg/mL).

Statistical analysis: All data are expressed as mean ± standard deviation of three replicate measurements. Statistical significance for the cytotoxicity studies was assigned at $p \leq 0.05$ and the probit analysis was performed using IBM SPSS 20.0 statistical software.

RESULTS AND DISCUSSION

Total phenolic and total flavonoid contents: Polyphenols comprise a great diversity of compounds such as phenolic acids, flavonoids, flavanols, flavones, proanthocyanidins, tannins. Total phenolic content was determined by the Folin-Ciocalteu method based on the reduction of phosphotungstic and phosphomolybdic acids to blue oxides of tungstene and molybdene during phenol oxidation in alkaline conditions. The amount of soluble phenolics (Table 1) varied widely in the tested samples, from highest level of 363.51 mg GAE/g for *A. dracunculus* to lowest level of 18.97 mg GAE/g for *A. vulgaris*. Significant quantities of polyphenols were also obtained for *S. hortensis* (193.01 mg GAE/g), whereas *V. planifolia* and *A. graveolens* manifested values of 58.50 and 41.68 mg GAE/g, respectively. Phenolic acids are repeatedly implicated as natural antioxidants distributed broadly in the plant kingdom. Still, the most widespread and diverse phenolics are the flavonoids such as the flavonol aglycones quercetin, myricetin, kaemferol and their glycosides. Flavonoids possess multiple hydroxyl groups which make them effective radical scavengers and reducing agents, thus providing strong antioxidant activity towards easily oxidizable compounds. The $AlCl_3$ method detects the presence of flavonoids based on the reaction between their carbonyl and hydroxyl groups and the Al(III) ions resulting in acid stable yellow complex. Determination of TFC showed notable differences among the selected samples (Table 1). Highest quantity of flavonoids was reported for *A. dracunculus* (154.08 mg QE/g), which is in accordance with the high content of polyphenols in this herb. Moreover, *S. hortensis* showed similar value (89.22 mg QE/g), while *A. graveolens* (8.53 mg QE/g) and *A. vulgaris* (1.17 mg QE/g) demonstrated lowest quantities of flavonoids.

Table 1. Total phenolics (TPC) and total flavonoids (TFC) for selected commercial herbs

Plant species	TPC (mg GAE/g)	TFC (mg QE/g)
Anethum graveolens L.	41.68±2.04	8.53±4.64
Artemisia dracunculus L.	363.51±1.02	154.08±4.17
Artemisia vulgaris L.	18.97±2.83	1.17±0.83
Satureja hortensis L.	193.01±1.13	89.22±9.17
Vanilla planifolia Jacks. ex Andrews	58.50±0.21	31.31±9.22

The considerable difference in TPC and TFC between *A. dracunculus* and *A. vulgaris* is due to variations in their chemical composition. Previously reported data on the distribution of artemisinin demonstrate a significant variability, with *A. dracunculus* leaves containing notably higher amount (0.27%) compared to *A. vulgaris* leaves (0.05%) (Mannan *et al.*, 2010). Aerial parts of *A. vulgaris* also contain coumarins, sterols and caffeoylquinic acids, but a lower content of polyphenols compared to other *Artemisia* species, which is associated with the lower antioxidant potential of *A. vulgaris* herba (Bora *et al.*, 2011). Similarly to our results, low content of total polyphenols was also observed in hydroalcoholic extracts from *A. vulgaris* in a previous study (28.04 mg GAE/g) (Oyedemi *et al.*, 2015). *A. dracunculus* leaves contain large contents of phenolic and flavonoid compounds (especially flavones and flavonols) in the extract, as well as volatile oils rich in *p*-allylanisole, (E)-β-ocimene, (Z)-β-ocimene and limonene (Eidi *et al.*, 2015; Behbahani *et al.*, 2017).

Ćavar *et al.* (2013) revealed high concentration of volatile oils, phenolic acids, flavonoids, tannins and pyrocatechol in *Satureja* species. Oke *et al.* (2009) demonstrated that *S. cuneifolia* essential oil is rich in carvacrol, γ-terpinene and *p*-cimene and a high TPC value for the methanolic extract (222.5 mg/g). Previous data showed similar results for the ethanolic extracts (128 mg GAE/g) (Bahramikia *et al.*, 2008) which is comparable to the obtained TPC value (89.22 mg QE/g).

DPPH assay: One of the mechanisms for antioxidant activity of phenolic compounds is the scavenging ability for ROS. The mechanism of action of DPPH assay is based on the transformation of the reagent 1,1-diphenyl-2-pycrylhydrazyl radical to a stable molecule hydrazine. The decrease in the absorbance of the final solutions indicates greater radical scavenging ability of the extract as a result of the present antioxidants. Based on the obtained IC_{50} values, *A. dracunculus* extract manifested a significant radical scavenging activity (IC_{50} 74.62 μg/mL), followed by *S. hortensis* (439.37 μg/mL) as the most bioactive samples (Table 2). Furthermore, *S. hortensis* demonstrated higher radical scavenging ability (57.646%) than the synthetic antioxidant BHA and similar values to quercetin, while *A. dracunculus* was identified as a better radical scavenger (94.724%) than all tested standards (Table 2).

According the research of Behbahani *et al.* (2017), *A. dracunculus* ethanol extract expressed strong antioxidant activity with IC_{50} value of 65.4 μg/mL. Additionally, Khezrilu and Heidari (2014) showed that sinapic, syringic, *p*-coumaric and gallic acid identified by HPLC analysis are the dominant phenolic acids in *A. dracunculus* methanolic extracts. Several studies demonstrated high radical scavenging activity for sinapic acid and its derivatives against DPPH radical (Kikuzaki *et al.*, 2002; Chen, 2016). Moreover, positive radical scavenging effect was also determined for syringic (Cikman *et al.*, 2015), ferulic, *p*-coumaric (Kikuzaki *et al.*, 2002; Kiliç and Yeşiloğlu, 2013) and gallic acid (Abourashed and Fu, 2017). Phenolics and monoterpenes present in *A. dracunculus* exert strong antioxidant activity, which may act by synergistic and/or additive mechanisms and contribute to its overall bioactivity.

Rosmarinic acid was identified by HPLC method as the major compound present in aerial parts of *S. hortensis*. Moreover, a good correlation was found between the antioxidant activity of *S. hortensis* and amount of rosmarinic acid (Zgórka and Głowniak, 2001; Plánder *et al.*, 2012). Considerable radical scavenging activity was identified for *S. hortensis* in the current study (439.37 μg/mL) and a similar bioactivity for the essential oil was declared in literature (350 μg/mL) (Güllüce *et al.*, 2003). Additionally, the presence of monoterpenes, such as carvacrol, thymol, γ-terpinene and *p*-cymene found in *S. hortensis* oil (Mahboubi and Kazempour, 2011), *S. cuneifolia* and *S. montana* also affects the overall bioactivity of the species. Oke *et al.* (2009) demonstrated high antioxidant activity for *S. cuneifolia* (26.0 μg/mL), while Miladi *et al.* (2013) demonstrated lower activity for *S. montana* (410.5 μg/mL).

Individual antioxidant potencies of carvacrol and thymol were also presented in the study of Lin *et al.* (2009) with IC_{50} values of 52 μg/mL and 51 μg/mL, respectively. Therefore, the antioxidant properties of *S. hortensis* are considered a result of the synergistic action of highly active oxygenated monoterpenes (thymol, carvacrol) and monoterpene hydrocarbons (*p*-cimene, γ-terpinene) in the oil and the presence of rosmarinic acid.

FRAP assay: The reductive capacity of the extracts was evaluated by the transformation of Fe^{3+}/ferricyanide complex to the ferrous form detected as Perl's Prussian blue complexes that serve as indicator of the antioxidant power of extracts. The reducing properties of the extracts are generally associated with the presence of reductants, which exert antioxidant action by a hydrogen atom donation and breakage of the free radical chain. In terms of their reductive ability, the FRAP capacity of the extracts is decreasing in the following order: *Artemisia*

dracunculus > *Satureja hortensis* > *Vanilla planifolia* > *Anethum graveolens* > *Artemisia vulgaris*.

Table 2. Antioxidant activity of selected spices and standards according to DPPH and FRAP assays

Plant species	DPPH		FRAP
	$I_{\%}$	IC_{50} (μg/mL)	(%)
Anethum graveolens L.	16.406	1508.26	9.8
Artemisia dracunculus L.	94.724	74.62	25.7
Artemisia vulgaris L.	9.540	2250.45	5.7
Satureja hortensis L.	57.646	439.37	24.3
Vanilla planifolia Jacks. ex Andrews	13.515	1760.24	14.6
Quercetin[1]	45.65	2.69	8.43
Trolox[1]	20.62	6.03	3.72
BHA[1]	13.25	9.71	6.47
Ascorbic acid[1]	24.06	5.08	3.87

A. dracunculus and *S. hortensis* manifested very similar reducing capacities and are better reducants than the standards, while *A. graveolens*, BHA and quercetin had closely related values for their reducing power (Table 2).

A positive correlation between the total phenolics and the radical scavenging activity of extracts tested by DPPH assay was confirmed in literature (Piluzza and Bullitta, 2011; Boeing *et al.*, 2014). In the current work, strong positive correlation is present between the obtained TPC values and the radical scavenging activity ($I_{\%}$) (R^2 = 0.9897), as well as between TPC values and FRAP capacity (%) of the extracts (R^2 = 0.8107). Moreover, a high correlation was also obtained for TFC and the aforementioned antioxidant assays with R^2 = 0.9732 and R^2 = 0.8760, correspondingly. Furthermore, the correlation analysis revealed a positive relationship between radical scavenging activity ($I_{\%}$) and the FRAP capacity of the extracts (%) (R^2 = 0.8144) as illustrated in Figure 1. This correlation is related to the radical scavenging activity of *A. dracunculus* and *S. hortensis*, as their FRAP capacity values decline in the same order as $I_{\%}$. This is supported by literature findings for the metal chelating abilities of flavonols at the 3-hydroxy-4-keto group and/or the 5-hydroxy-4-keto group as in the case of kaempferol and myricetin (Shahidi and Wanasundara, 1992).

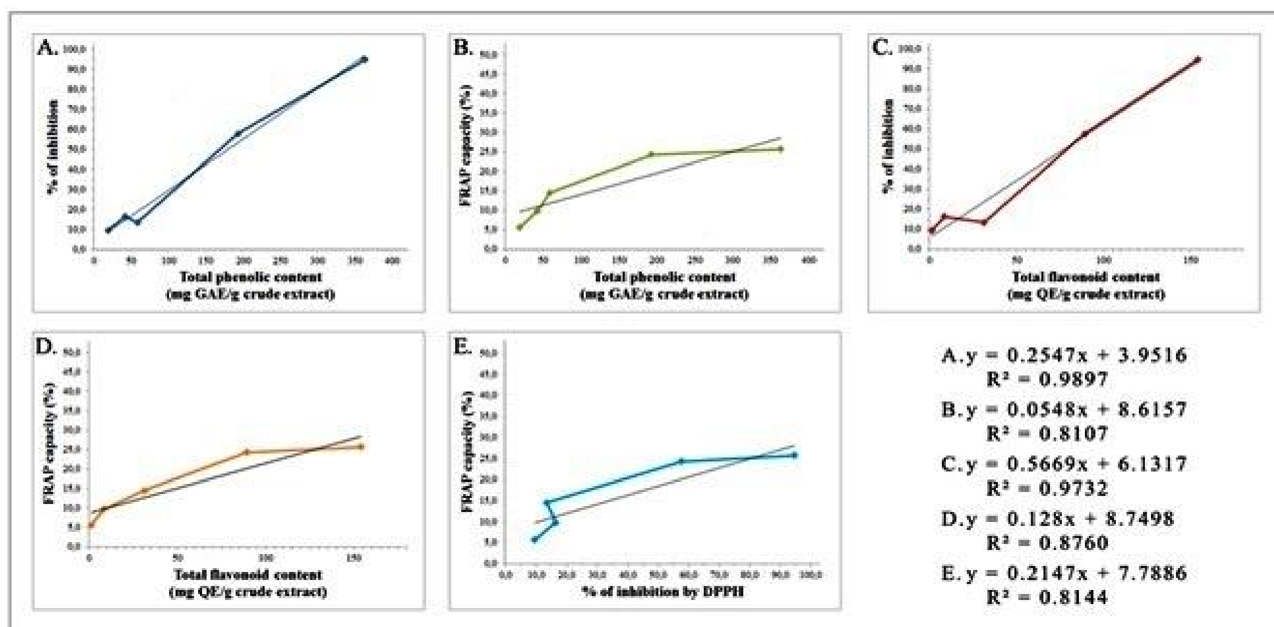

Figure 1. Correlation between A. TPC and I% (DPPH), B. TPC and % FRAP capacity; C. TFC and I% (DPPH); D. TFC and % FRAP capacity; E. I% (DPPH) and % FRAP capacity

NSSOH and SSOH assays: In the NSSOH assay, EDTA forms complex with the ferric ions, therefore only the ferrous ions take part in generating hydroxyl radicals as a result of the Fenton reaction. The absence of EDTA chelator in SSOH procedure results in a larger amount of free ferrous ions that take part in the process of 2-deoxyribose degradation and the production of TBARS, which is a good indicator of any chelating activity of present bioactive compounds. Extracts with lower values for IC_{50} in SSOH assay are considered potent chelators due to their effective binding of ferrous ions, therefore reducing the free hydroxyl radical production. Figure 2 presents the obtained results for the antioxidant activity of extracts tested by NSSOH and SSOH assays.

Analysis of the hydroxyl scavenging properties revealed that *A. dracunculus* and *A. vulgaris* performed better activity in the absence of EDTA, suggesting of their chelating abilities for the excess ferrous ions in the reaction mixture. Iron chelating compounds can reduce the extent of deoxyribose degradation even if they are not effective hydroxyl radical-scavengers. This observation may give explanation of why some extracts are successful iron chelators, despite their low radical scavenging activity and reducing power, as it was demonstrated for *A. vulgaris*. Moreover, a single antioxidant method cannot offer accurate value for the antioxidant capacity of a sample due to the involvement of multiple mechanisms and the complex nature of phytochemicals. Therefore, broader studies with more samples are needed to examine the relation between the chelating abilities of extracts and the total content of polyphenols.

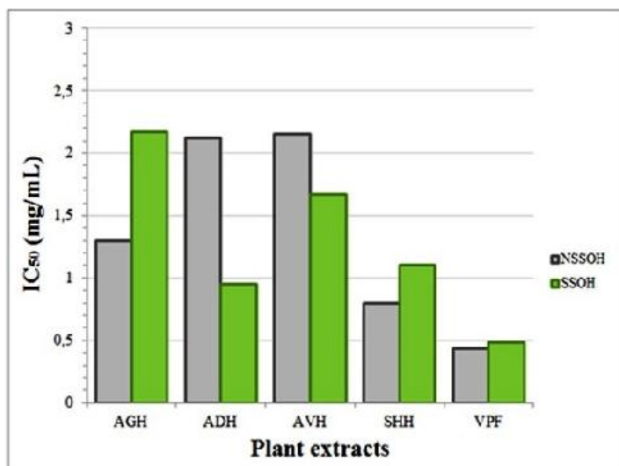

Figure 2. Comparison of IC_{50} values obtained with EDTA (NSSOH) and without EDTA (SSOH) in the reaction mixture

Brine shrimp lethality assay: The cytotoxic potential of the extracts was identified based on their toxicity against *Artemia salina* larvae in the Brine Shrimp Lethality Assay. Number of dead larvae for each concentration of extract (5, 3, 1, 0.5, 0.1 and 0.01 mg/mL) was counted after 24 hours of exposure of the brine shrimps, and the results were transformed in percentages of mortality. Figure 3 shows the trend of mortality rate for each sample and the positive controls. Strong toxic effects (50 - 60%) in the lower concentration range (0.01 – 0.5 mg/mL) were observed for *A. vulgaris*, while *A. graveolens* and *V. planifolia* demonstrated weak toxicity (0 - 20%) in the corresponding concentration range.

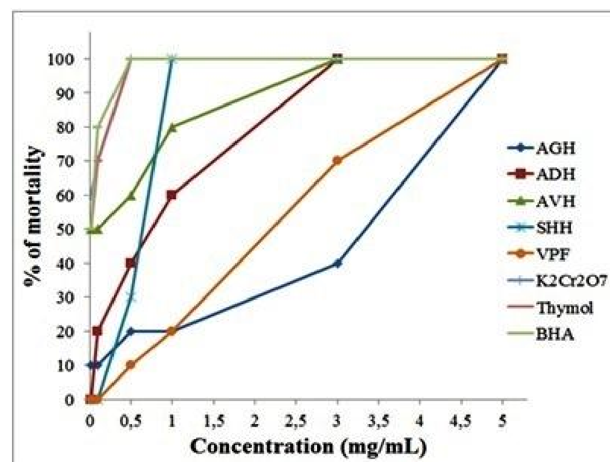

Figure 3. Trend of mortality for *A. salina* against concentration for the extracts and positive controls

According the LC_{50} values calculated by probit regression analysis (Table 3) *A. vulgaris* is classified as highly toxic extract based on Meyer's scale and Clarkson's scale, and the results were in accordance with the *in vitro* cytotoxicity on cell lines demonstrated by several authors. The ethylacetate extract of *A. vulgaris* manifested very high cytotoxic potential (LC_{50} 57 μg/mL) against MCF-7 cells (Rabe *et al.*, 2011), and similar results were also obtained for the selective inhibition of HL-60 cells by caspase-dependent apoptosis as a result of treatment of the cancerous cells with *A. vulgaris* oil (Saleh *et al.*, 2016). Additionally, in the study of Abdelhamed *et al.* (2013), treatment with *A. vulgaris* extracts resulted in sensitization of MDA-MB-231 and MDA-MB-468 breast cancer cells to TRAIL (tumor necrosis factor-related apoptosis-inducing ligand), improving the efficacy of the treatment. The observed cytotoxicity in *A. vulgaris* might be due to the presence of numerous bioactive components, among which the monoterpenes camphor and α- and β-thujone detected in the volatile oil by GC/MS were declared as most promising (Govindaraj *et al.*, 2008). This observation is also supported by previous data on their antiproliferative and antitumor activity (Tamil Selvi *et al.*, 2015).

A moderate toxicity was observed for *S. hortensis* (LC_{50} 324 μg/mL) and *A. dracunculus* (LC_{50} 454 μg/mL), while *V. planifolia* and *A. graveolens* showed no toxic effect against the brine shrimps (1136

and 1235 μg/mL, respectively) as presented in Table 3. Comparable results to these findings for the moderate toxicity of *S. hortensis* were obtained in the study of Gohari *et al.* (2005) by testing the cytotoxic activity of diethyl ether extracts and isolated terpenoids from *S. macrantha* against *Artemia salina* larvae. The observed LC_{50} values for the extract (LC_{50} 186 μg/mL) demonstrated a moderate cytotoxic potential that could be attributed to the isolated terpenoids thymol, oleanoic acid and ursolic acid. In the current study, thymol demonstrated a very strong cytotoxic potential (LC_{50} 8 μg/mL). Similarly, previous studies demonstrated that carvacrol manifested strong antitumor and antiproliferative activity on human metastatic breast cancer cells MDA-MB231 in a dose-dependent manner (Arunasree, 2010) and a very potent cell growth inhibition of A549 cell line (Miladi *et al.*, 2013). Additionally, isolated chalcone and flavanones from *S. spicigera* aerial parts demonstrated strong cytotoxic potential against *A. salina* shrimps (LC_{50} 2 μg/mL) (Gohari *et al.*, 2012). Moreover, Berdowska *et al.* (2013) showed that rosmarinic acid exhibited the strongest cytotoxicity against MCF-7 cell line, and the overall conclusion was that the tested phenolics exert more beneficial properties when they are applied in the form of extracts comprising their mixtures.

Moderate cytotoxic potential was also obtained for *A. dracunculus*. Antitumor effects of *A. dracunculus* extracts observed in L5178Y lymphoma cells (IC_{50} 100 μg/mL) were reported as a result of the present polyphenols and possibly a flavone that also possesses antioxidant activity (Navarro-Salcedo *et al.*, 2017). Moreover, the antitumor effect of *A. dracunculus* ethanolic extract was examined against cultured EC-109 cells in a previous study and the observed toxicity (LC_{50} 80 μg/mL) showed mild to potent cell proliferation inhibitory activities against the esophageal cell line. Isolated sakuranetin from the extract was also examined and the authors reported high potency against EC-109 cancer cells, suggesting that certain flavonoids present in *A. dracunculus* may express significant cytotoxicity (Hong and Ying, 2014). The chemopreventive effects of *A. dracunculus* methanolic extracts were demonstrated against 7,12-DMBA-induced skin cancer (Ibrahem, 2017) and possibly contributed by the presence of quercetin, rutin and kaemferol that were identified in the leaves. The chemopreventive effect of rutin is also associated with the significant antioxidant activity of *A. dracunculus* (Dixit, 2014).

Table 3. Cytotoxic potential of hydroalcoholic extracts of 5 commercial spices according the Brine Shrimp Lethality Assay

Plant species	Abbr.	LC_{50} (μg/mL)	Confidence intervals (95%)	Meyer's scale	Clarkson's scale
Anethum graveolens L.	AGH	1235	888 – 2048	non-toxic	non-toxic
Artemisia dracunculus L.	ADH	454	233 – 975	toxic	moderately toxic
Artemisia vulgaris L.	AVH	80	29 – 174	toxic	highly toxic
Satureja hortensis L.	SHH	324	153 – 567	toxic	moderately toxic
Vanilla planifolia Jacks. ex Andrews	VPF	1136	819 – 1888	non-toxic	non-toxic

Obtained results were also compared with the cytotoxic potential for the positive controls $K_2Cr_2O_7$, thymol and BHA. Potassium dichromate is a commonly used oxidizing reagent and well known cytotoxic agent in laboratory applications, while BHA is a synthetic antioxidant that is commonly used in food preservation. Thymol is a monoterpene frequently present in essential oils of many plant species therefore it is a suitable marker for their bioactive properties. Based on the obtained results (Table 4), low concentration of all positive controls exerted significant cytotoxic effects, therefore they were identified as highly cytotoxic agents against the *Artemia* larvae.

Table 4. Cytotoxic potential of positive controls according the Brine Shrimp Lethality Assay

Positive controls	LC_{50} (μg/mL)	Confidence intervals (95%)	Meyer's scale	Clarkson's scale
$K_2Cr_2O_7$	10	1 - 41	Toxic	highly toxic
Thymol	8	0 - 25	Toxic	highly toxic
BHA	12	3 - 38	Toxic	highly toxic

Conclusion: All examined spices demonstrated antioxidant activity in different extent. *A. dracunculus* and *S. hortensis* exhibited strong radical scavenging activity and reducing capacity, whereas *A. dracunculus*

has also shown prominent chelating abilities. The significant amount of polyphenols and flavonoids that was obtained for all samples is possibly the major contributor to their antioxidant properties, which was confirmed by the positive correlation established between the polyphenols and the antioxidant activity.

Beside their prominent antioxidant potency, plant extracts also demonstrated cytotoxic effects in various levels. *A. vulgaris* exhibited high cytotoxicity against *Artemia* larvae, while *A. dracunculus* and *S. hortensis* were identified as extracts with moderate cytotoxicity.

The significant bioactive properties of the examined extracts in this research provide a new insight by their utilization as natural sources for preventive purposes against pathological conditions based on oxidative stress, as well as promising candidates for further detailed studies on the isolation and identification of their biologically active compounds.

REFERENCES

Abdelhamed, S., S. Yokoyama, L.Hafiyani, S.Kalauni, Y. Hayakawa, S. Awale, and I. Saiki (2013). Identification of plant extracts sensitizing breast cancer cells to trail. Oncol Rep. 29(5): 1991-1998.

Abourashed, E. and H. Fu (2017). Hydroxybenzoic acids are significant contributors to the antioxidant effect of borututu bark, Cochlospermum angolensis Welw. ex Oliv. Antiox. 6(1): 9.

Arunasree, K. (2010). Anti-proliferative effects of carvacrol on a human metastatic breast cancer cell line, MDA-MB 231. Phytomed. 17(8-9): 581-588.

Bahramikia, S., R. Yazdanparast and N. Nosrati (2008). A comparison of antioxidant capacities of ethanol extracts of Satureja hortensis and Artemisia dracunculus leaves. Pharmacology online 2: 694-704.

Behbahani, B., F. Shahidi, F. Yazdi, S. Mortazavi and M. Mohebbi (2017). Antioxidant activity and antimicrobial effect of tarragon (Artemisia dracunculus) extract and chemical composition of its essential oil. J Food Meas Charact. 11(2): 847-863.

Berdowska, I., B. Zieliński, I. Fecka, J. Kulbacka, J. Saczko and A. Gamian (2013). Cytotoxic impact of phenolics from Lamiaceae species on human breast cancer cells. Food Chem. 141(2): 1313-1321.

Boeing, J.S., E.O. Barizão, B.C. Silva, P.F. Montanher,V. de Cinque Almeida and J.V. Visentainer (2014). Evaluation of solvent effect on the extraction of phenolic compounds and antioxidant capacities from the berries: application of principal component analysis. Chem Cent J. 8(1): 48.

Bora, K.S. and A. Sharma (2011). The genus Artemisia: A comprehensive review. Pharm Biol. 49(1): 101-109.

Brand-Williams, W., M. Cuvelier and C. Berset (1995). Use of a free radical method to evaluate antioxidant activity. LWT - Food Sci Technol. 28(1): 25-30.

Ćavar, S., M.E. Šolić and M. Maksimović (2013). Chemical composition and antioxidant activity of two Satureja species from Mt. Biokovo. Bot serb. 37(2): 159-165.

Chen, C. (2016). Sinapic acid and its derivatives as medicine in oxidative stress-induced diseases and aging. Oxidative Med Cell Longev. doi:10.1155/2016/3571614.

Cikman, O., O. Soylemez, O. Ozkan, H. Kiraz, I. Sayar, S. Ademoglu, S. Taysi and M. Karaayvaz (2015). Antioxidant activity of syringic acid prevents oxidative stress in l-arginine–induced acute pancreatitis: an experimental study on rats. Int Surg. 100(5): 891-896.

Clarkson, C., V.J. Maharaj, N.R. Crouch, O.M. Grace, P. Pillay, M.G. Matsabisa, N. Bhagwandin, P.J. Smith and P.I. Folb (2004). In vitro antiplasmodial activity of medicinal plants native to or naturalised in South Africa. J Ethnopharmacol. 92(2-3): 177-191.

Dixit, S. (2014). Anticancer effect of rutin isolated from the methanolic extract of Triticum aestivum straw in mice. Med Sci. 2(4): 153-160.

Eidi, A., S. Oryan, J. Zaringhalam and M. Rad (2015). Antinociceptive and anti-inflammatory effects of the aerial parts of Artemisia dracunculus in mice. Pharm Biol. 54(3): 549-554.

Gohari, A.R., A. Hadjiakhoondi, S.E. Sadat-Ebrahimi, S. Saeidnia and A. Shafiee (2005). Cytotoxic terpenoids from Satureja macrantha C.A. Mey. DARU J Pharm Sci. 13(4): 177-181.

Gohari, A.R., S.N. Ostad, F. Moradi-Afrapoli, M. Malmir, S. Tavajohi, H. Akbari and S. Saeidnia (2012). Evaluation of the cytotoxicity of Satureja spicigera and its main compounds. Sci World J. doi: 10.1100/2012/203861.

Govindaraj, S., B. Kumari, P. Cioni and G. Flamini (2008). Mass propagation and essential oil analysis of Artemisia vulgaris. J Biosci Bioeng. 105(3): 176-183.

Güllüce, M., M. Sökmen, D. Daferera, G. Ağar, H. Özkan, N. Kartal, M. Polissiou, A. Sökmen and F. Sahin (2003). In vitro antibacterial, antifungal, and antioxidant activities of the essential oil and methanol extracts of herbal parts and callus cultures of Satureja hortensis L. J Agric Food Chem. 51(14): 3958-3965.

Halliwell, B., J. Gutteridge and O. Aruoma (1987). The deoxyribose method: A simple “test-tube” assay for determination of rate constants for reactions of hydroxyl radicals. Anal Biochem. 165(1): 215-219.

Ho, K., L. Yazan, N. Ismail and M. Ismail (2009). Apoptosis and cell cycle arrest of human colorectal cancer cell line HT-29 induced by vanillin. Cancer Epidemiol. 33(2): 155-160.

Hong, L. and S. Ying (2014). Ethanol extract and isolated constituents from Artemisia dracunculus inhibit esophageal squamous cell carcinoma and induce apoptotic cell death. Drug Res. 65(02): 101-106.

Ibrahem, N.M. (2017). Extraction and characterization of Iraqi Artemisia dracunculus dried aerial parts extract though HPLC and GC-MS analysis with evaluation of its antitumor activity against 7,12-dimethylbenz(a)anthracene induced skin cancer in mice. Int J Pharm Pharm Sci. 9(5): 34.

Khezrilu, B.J. and R. Heidari (2014). The evaluation of antioxidant activities and phenolic compounds in leaves and inflorescence of Artemisia dracunculus L. by HPLC. J Med Plants. 13(51): 41-50.

Kikuzaki, H., M. Hisamoto, K. Hirose, K. Akiyama and H. Taniguchi (2002). Antioxidant properties of ferulic acid and its related compounds. J Agric Food Chem. 50(7): 2161-2168.

Kiliç, I. and Y. Yeşiloğlu (2013). Spectroscopic studies on the antioxidant activity of p-coumaric acid. Spectrochim Acta Mol Biomol Spectrosc. 115: 719-724.

Lallianrawna, S., R. Muthakumaran, V. Ralte, G. Gurusubramanian and N. Senthil Kumar (2013). Determination of total phenolic content, total flavonoid content and total antioxidant capacity of Ageratina adenophora (Spreng.) King & H. Rob. Sci Vis. 13(4): 149-156.

Lin, C., C. Yu, S. Wu and K. Yih (2009). DPPH free-radical scavenging activity, total phenolic contents and chemical composition analysis of forty-two kinds of essential oils. J Food Drug Anal. 17(5): 386-395.

Mahboubi, M. and N. Kazempour (2011). Chemical composition and antimicrobial activity of Satureja hortensis and Trachyspermum copticum essential oil. Iran J Microbiol. 3(4): 194-200.

Mannan, A., I. Ahmed, W. Arshad, M.F. Asim, R.A. Qureshi, I. Hussain and B. Mirza (2010). Survey of artemisinin production by diverse Artemisia species in northern Pakistan. Malar J. 9(1): 310.

Meyer, B., N. Ferrigni, J. Putnam, L. Jacobsen, D. Nichols and J. McLaughlin (1982). Brine shrimp: A convenient general bioassay for active plant constituents. Planta Med. 45(05): 31-34.

Miladi, H., R. Ben Slama, D. Mili, S. Zouari, A. Bakhrouf and E. Ammar (2013). Chemical composition and cytotoxic and antioxidant activities of Satureja montana L. essential oil and its antibacterial potential against Salmonella spp. strains. J Chem. 1-9.

Murcia, M., I. Egea, F. Romojaro, P. Parras, A. Jiménez and M. Martínez-Tomé (2004). Antioxidant evaluation in dessert spices compared with common food additives. Influence of irradiation procedure. J Agric Food Chem. 52(7): 1872-1881.

Navarro-Salcedo, M.H., J.I. Delgado-Saucedo, V.H. Siordia Sanchez, L.J. Gonzalez-Ortiz, G.A. Castillo-Herrera and A.M. Puebla Perez (2017). Artemisia dracunculus extracts obtained by organic solvents and supercritical CO_2 produce cytotoxic and antitumor effects in mice with L5178Y lymphoma. J Med Food. doi: 10.1089/jmf.2017.0044.

Oke, F., B. Aslim, S. Ozturk and S. Altundag (2009). Essential oil composition, antimicrobial and antioxidant activities of Satureja cuneifolia Ten. Food Chem. 112(4): 874-879.

Oyaizu, M. (1986). Studies on product of browning reaction prepared from glucose amine. Jpn J Nutr. 44: 307-315.

Oyedemi, S. and R. Coopoosamy (2015). Preliminary studies on the antibacterial and antioxidative potentials of hydroalcoholic extract from the whole parts of Artemisia vulgaris L. Int J Pharmacol. 11(6): 561-569.

Piluzza, G. and S. Bullitta (2011). Correlations between phenolic content and antioxidant properties in twenty-four plant species of traditional ethnoveterinary use in the Mediterranean area. Pharm Biol. 49(3): 240-247.

Plánder, S., L. Gontaru, B. Blazics, K. Veres, Á. Kéry, S. Kareth and B. Simándi (2012). Major antioxidant constituents from Satureja hortensis L. extracts obtained with different solvents. Eur J Lipid Sci Technol. 114(7): 772-779.

Rabe, S.Z.T., M. Mahmoudi, A. Ahi and S.A. Emami (2011). Antiproliferative effects of extracts from Iranian Artemisia species on cancer cell lines. Pharm Biol. 49(9): 962–969.

Rakoff-Nahoum, S. (2006). Why Cancer and inflammation? Yale J Biol Med. 79(3-4): 123–130.

Reuter, S., S. Gupta, M. Chaturvedi and B. Aggarwal (2010). Oxidative stress, inflammation, and cancer: How are they linked? Free Radic Biol and Med. 49(11): 1603-1616.

Saleh, A.M., A. Aljada, S.A. Rizvi, A. Nasr, A.S. Alaskar and J.D. Williams (2014). In vitro cytotoxicity of Artemisia vulgaris L. essential oil is mediated

by a mitochondria-dependent apoptosis in HL-60 leukemic cell line. BMC Complement Altern Med. 14(1): 2016.

Shahidi, F. and P.K.J. Wanasundara (1992). Phenolic antioxidants. Crit Rev Food Sci Nutr. 32(l): 67-103.

Singleton, V.L., R. Orthofer and R.M. Lamuela-Raventos (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. Methods Enzymol. 299: 152-178.

Sorgeloos, P., C. Remiche-Van Der Wielen and G. Persoone (1978). The use of Artemia nauplii for toxicity tests—A critical analysis. Ecotoxicol Environ Saf. 2(3-4): 249-255.

Sytar, O., I. Hemmerich, M. Zivcak, C. Rauh and M. Brestic (2016). Comparative analysis of bioactive phenolic compounds composition from 26 medicinal plants. Saudi J Biol Sci. doi: http://dx.doi.org/10.1016/j.sjbs.2016.01.036.

Tamil Selvi, M., R. Thirugnanasampandan and S. Sundarammal (2015). Antioxidant and cytotoxic activities of essential oil of Ocimum canum Sims. from India. J Saudi Chem Soc. 19(1): 97-100.

Vanhaecke, P., G. Persoone, C. Claus and P. Sorgeloos (1981). Proposal for a short-term toxicity test with Artemia nauplii. Ecotoxicol Environ Saf. 5(3): 382-387.

Zgórka, G. and K. Głowniak (2001). Variation of free phenolic acids in medicinal plants belonging to the Lamiaceae family. J Pharm Biomed Anal. 26(1): 79-87.