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Total phenolic content, antioxidant activity and toxicity of aqueous extracts from selected Greek medicinal and aromatic plants

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ABSTRACT

In this study, the total phenolic content, the antioxidant activity and toxicity of selected Greek medicinal aromatic namely *Melissa officinalis* L., *Origanum vulgare* L., *Origanum dictamnus* L., *Salvia officinalis* L. and *Hyssopus officinalis* L., were investigated under three different extraction processes. Among the plants studied, *Melissa officinalis* L. showed the highest values in total phenolic content (0.985 ± 0.001 mg caffeic acid/mL) and antioxidant activity (6.61 ± 0.04 μmol Trolox/mL) independently of the extraction process. Toxicity evaluation of the infusions prepared in concentrations contained in commercial portions showed that inhibition values of *Origanum vulgare* L. and *Origanum dictamnus* L. were greater than 20% thus further research is necessary. Although toxicity of plant extracts examined is not correlated to their total phenolic content and antioxidant activity, it was linked to the interaction of water soluble and volatile organic compounds. Interaction of water soluble and volatile organic compounds was evaluated through the estimation of synergism ratios (SR), where *Origanum vulgare* L., presented the maximum synergism (SR = 4.2) in toxicity.

1. Introduction

The importance of dietary antioxidant components for the prevention of some diseases and health quality improvement has attracted much research attention through the last decade (Alvarado et al., 2006; Blasa et al., 2010; Pérez-Jiménez et al., 2008; Turkmen et al., 2005; Wootton-Beard and Ryan, 2011). Vegetables and herbal infusions have been recognized as important sources of antioxidants (Alarcón et al., 2008; Almajano et al., 2008; Chan et al., 2010). Total phenolic content, antioxidant activity and antimicrobial activity of herbal extracts are of particular interest to food industry which is looking for plant extracts with significant antimicrobial activity to be used as alternatives to conventional food preservatives (Almajano et al., 2008; Madsen and Bertelsen, 1995; Thorsen and Hildebrandt, 2003).

Plant extracts obtained by different solvents and several extraction techniques are mixtures of numerous compounds. Their possible biological activity as mixtures is being investigated through various screening models such as plant (Bouchagier et al., 2008; Bouchagier and Efthimiadis, 2010), bioassays (Daferera et al., 2003), tissue or cell culture (Wang et al., 2011), receptor enzyme (Peelman et al., 2006) and biochromatography (He et al., 2008; Kotecha et al., 2007; Kvalheim et al., 2011).

Aqueous herbal extracts have attracted attention since they can be consumed in a daily basis as decoctions. Many Lamiaceae extracts are extensively used in traditional diet and popular medicine (Barros et al., 2011; Carrió and Vallès, 2012; Degner et al., 2009; Liolios et al., 2010; Nedorostova et al., 2009; Noorbala et al., 2005). Their therapeutic actions are assigned to biologically active polyphenol components, such as flavonoids and phenolic acids, which possess antioxidant activities (Pietta, 2000; Surveswaran et al., 2007; Zheng and Wang, 2001). However, attention should be given in case of high doses (Tajkarimi et al., 2010), since their toxicity has not yet been fully elucidated. Conforti et al. (2008) introduced Microtox® test as an evaluation tool for toxicity estimation of dietary plants extracts.

Microtox® analyzer uses the marine bioluminescent bacteria *Vibrio fischeri* as a reference test species and provides a sensitive, rapid, cost effective and reproducible method for the estimation of toxicity of toxicity of single compounds and mixtures of organic and inorganic compounds (Backhaus et al., 2000; Fulladosa et al., 2005; Hernando et al., 2007; Park and Que Hee, 2001), biomaterials (Bulich et al., 1990; Burton et al., 1986), without raising any ethical considerations (Conforti et al., 2008; Cotou et al., 2002; Parvez et al., 2006; Shettlemore and Bundy, 2002; Zhao et al., 1998). Conforti et al. (2008) were the first to propose its use to determine toxicity of herbal infusions and decoctions and evaluate interaction between

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water soluble and volatile compounds extracted by a non-polar organic solvent.

Recently, there is, also, an increasing interest in the evaluation of potential synergism or antagonism in antimicrobial activity (Al-Bayati, 2008; Endo et al., 2010; Hemaiswarya and Doble, 2009; Moon et al., 2011; Ncube et al., 2012) and antioxidant activity (Romano et al., 2009) of single compounds or mixtures. Bioluminescence inhibition test using Vibrio sp. has been used by Zhang et al. (2011) for the evaluation of toxicity antagonism or synergism in mixtures.

In the present work, aqueous extracts of medicinal and aromatic plants of Lamiaceae family, namely *Melissa officinalis* L., *Origanum vulgare* L., *Origanum dictamnus* L., *Salvia officinalis* L. and *Hyssopus officinalis* L., were screened in reference to their total phenolic content, antioxidant activity and toxicity, as they are representative species of the Mediterranean flora. The aim of this study is to investigate a possible correlation between total phenolic content, antioxidant activity and toxicity and the interaction of water soluble and volatile organic compounds of the plant species extracts examined.

2. Materials and methods

2.1. Plant material

Air dried samples (harvest 2010) of the Lamiaceae family were offered by Aetoloakarnania's Rural Cooperative of Aromatic, Pharmaceutical and Energy Plant Cultivators (Agrotikos Syneterismos Kalliergiton Aromatikon, Farmakeftikon, Energiakon Fyton Aetoloakarnanias, ASKAFEFA), Greece. These samples were, namely, lemon balm (*Melissa officinalis* L.), sage (*Salvia officinalis* L.), oregano (*Origanum vulgare* L.; chemotype carvacrol), and hyssop (*Hyssopus officinalis* L.). Dittany (*Origanum dictamnus* L.; chemotype carvacrol) was supplied from the local market in Crete, Greece. Only the leaves were used, except oregano and hyssop, for which leaves and flowers were used together. Dry plant material was stored at −20 °C until used.

2.2. Materials

2,2-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), were purchased from Sigma–Aldrich (St. Louis, MO, USA). Folin-Ciocalteau phenol reagent, potassium persulfate ($K_2S_2O_8$), sodium carbonate (Na_2CO_3), absolute ethanol and petroleum ether 40–60° were supplied by Merck KGaA (Darmstadt, Germany), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and caffeic acid were purchased from Aldrich (Steinheim, Germany).

Toxicity tests were performed using the Microtox® Toxicity analyzer model 500, equipped with 30 temperature controlled wells regulated at 15 °C in addition with a reactivation well regulated at 5 °C used to store the bacteria suspension before dilution. Acute toxicity reagents such as Diluent (sterile 2% sodium chloride), OAS (osmotic adjusting solution 22% sodium chloride), reconstitution solution and test organisms *V. fischeri*, formerly known as *Photobacterium phosphoreum*, NRRL No B-11177 were supplied by Strategic Diagnostic INC.

2.3. Preparation of extracts

For total phenolic content and antioxidant activity assessment tests, the extracts were prepared by adding 2 g of individual freshly crushed herb ($n = 5$) in 200 mL (1 cup) deionized water and steeped under three different processes. (a) at 85 °C, (b) at room temperature and, (c) at room temperature with the assistance of ultrasound water bath (35 MHz). Each treatment lasted for 15 min. The herbal infusions were then filtered through a Whatman filter No. 1. Prior to analysis, an aliquot was further filtrated with a PTFE filter with 0.45 μm pore size.

For toxicity determination, infusions were prepared as above and were then filtrated through a nylon 0.2 μm membrane filter, in order to remove any suspended material that may interfere with luminescence readings (Parvez et al., 2006).

Total phenolic content, antioxidant activity and toxicity determination was performed for all plant species. All the infusions described above were then extracted three times with petroleum ether in order to further analyze the aqueous phase derived from this extraction. Three individual preparations for each extract were prepared.

2.4. Determination of total phenolic content

Total phenolic content was determined in herbal infusions prior and after extraction with petroleum ether using Folin-Ciocalteu assay (Singleton et al., 1999), with some modifications. Thus 100 μL of each tested infusion, 500 μL of Folin-Ciocalteu reagent and 6 mL of deionized water were transferred in a 10 mL flask and mixed thoroughly. After 3 min, 1.5 mL of 20% Na_2CO_3 was added in the flask which was filled to 10 mL with water. After two hours standing, absorbance was measured at 725 nm. Caffeic acid was used as a standard (0–500 mg/L) and the results were expressed as mg caffeic acid per mL. All measurements were performed in triplicate.

2.5. Determination of antioxidant activity

Herbal infusions were examined for antioxidant activity prior and after extraction with petroleum ether applying both the ABTS assay 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (Ree et al., 1999) and the DPPH assay 2,2-diphenyl-1-picrylhydrazyl radical (Surveswaran et al., 2007) with some modifications.

The ABTS radical cation ($ABTS^{\bullet+}$) solution was prepared by the reaction of 7 mM ABTS and 2.45 mM potassium persulphate, after incubation at room temperature in the dark for 12–16 h. The ($ABTS^{\bullet+}$) solution was then diluted with ethanol to obtain an absorbance of 0.700 ± 0.020 at 734 nm. After the addition of 3.0 mL of diluted ($ABTS^{\bullet+}$) solution ($A_{734\,nm} = 0.700 \pm 0.020$) to 30 μL of extracts, the absorbance reading was acquired at 30 °C exactly $t = 6$ min after initial mixing (A_{sample}). Absorbance of control (3.0 mL ($ABTS^{\bullet+}$) solution with 30 μL water) was recorded in advance ($A_{control}$). The percentage of absorbance inhibition at 734 nm was calculated using the formula:

$$\text{Inhibition}\,(\%) = \left[\frac{A_{control} - A_{sample}}{A_{control}}\right] \times 100$$

where $A_{control}$ and A_{sample} are the absorbance values of the control and the test sample at $t = 6$ min, respectively.

A standard curve was obtained by using Trolox standard solution at various concentrations (ranging from 0 to 15 μM final concentration) in ethanol. The inhibition percentage of the test samples was compared to that of the Trolox standard and the results were expressed in terms of Trolox Equivalent Antioxidant Capacity (TEAC), as mmol Trolox equivalents per mL. All determinations were carried out three times.

The DPPH radical ($DPPH^{\bullet}$) solution (60 μM) was prepared in ethanol. The $DPPH^{\bullet}$ solution had an absorbance of 0.680 ± 0.050 at 515 nm. A volume of 3.0 mL of the $DPPH^{\bullet}$ solution was mixed with 30 μL of the tested extracts. The resulting solution was vigorously mixed, incubated at room temperature in the dark for 30 min and the absorbance was recorded at 515 nm. The percentage of absorbance inhibition at 515 nm was calculated as above.