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# Exploring the antioxidant potential of *Teucrium polium* extracts by HPLC–SPE–NMR and on-line radical-scavenging activity detection

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ABSTRACT

Teucrium polium is a popular medicinal plant that is used in the daily diet and the in vitro and in vivo antioxidant potency of its extracts has been widely investigated. In the present work, polar extracts from *T. polium* were analyzed in terms of its composition and radical-scavenging activity with the employment of the state-of-the-art HPLC-SPE-NMR and HPLC-DPPH techniques in the search of new antioxidant agents for food industry. NMR and MS data revealed the presence of phenylpropanoid glycosides verbascoside and poliumoside, the flavones apigenin and its derivatives and two methoxyflavones. The on-line DPPH experiments showed that poliumoside is the most active component of the extracts and the antioxidant potential of *T. polium* polar extracts is mainly attributed to phenylpropanoid glycosides (66–80%). Our results suggested that T. polium extracts could be a promising source of natural antioxidants. This holistic approach also revealed that poliumoside or polar extracts of T. polium can be used in food industry as antioxidant agents with natural origin. The HPLC-SPE-NMR and HPLC-DPPH analysis of the extracts was performed for first time in the same device without any modification of the instrumentation, avoiding the need for isolation of the individual components.



## 1. Introduction

Currently, there is a growing interest in replacing synthetic with natural food additives which is mainly attributed to: (i) the plethora of epidemiological studies that show the inverse association between risk of cancer, cardiovascular diseases, diabetes, several age related chronic diseases and consumption of fruits and vegetables; (ii) the concerns regarding the safety of the chronic consumption of synthetic compounds traditionally used as preservatives in foods and beverages; and (iii) the public's conviction that natural antioxidants are safer than the synthetic analogs. Thus, the food industry is strongly activated in discovering safe and low cost antioxidants of natural origin. In this effort, herbs, spices and medicinal plants are an extraordinary reservoir of novel chemodiverse molecules (Rehecho et al., 2011; Tsakona, Galanakis, & Gekas, in press).

*Teucrium polium* is frequently used in the daily diet as a spice, appetizer, herbal tea and refreshing beverage as well as for medicinal purposes (Sharififar, Dehghn-Nudeh, & Mirtajaldini, 2009). The antioxidant activity of *T. polium* is well documented by different assays using hydrophilic (DPPH, ABTS, phosphomolybdenum assay) and lipophilic model systems (β-carotene bleaching inhibition and iron induced lipid peroxidation in rat liver microsomes) (Ardestani & Yazdanparast, 2007; Ljubuncic et al., 2006; Sharififar et al., 2009), and therefore it might be a good candidate for potential antioxidant agent in the food industry. Previous studies on the composition of *T. polium* extracts demonstrated that the major components of its polar extracts belong to the groups of phenylpropanoid glycosides, which are characteristic for *Teucrium* species, and flavonoids, mainly in the form of its methoxy-derivatives (Kawashty, El-Din, & Saleh, 1999; Serrilli et al., 2007; Sharififar et al., 2009). Recently, the use of *T. polium* extracts as food additives replacing synthetic antioxidants has been recommended due to their rich flavonoid content (Krishnaiah, Sarbatly, & Nithyanandam, 2011; Sharififar et al., 2009).The antioxidant potency of individual constituents of *T. polium* extracts has been evaluated such as apigenin, rutin, dimethoxyapigenin, verbascoside and poliumoside but their contribution to the overall antiradical-scavenging activity has not been thorough evaluated (Funes, Laporta, Cerdán-Calero, & Micol, 2010; Sharififar et al., 2009; Yamasaki, Masuoka, Nohara, & Ono, 2007).

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The main analytical methods currently used for the phytochemical study of plant extracts are based on HPLC-multi-hyphenated strategy. The coupling of HPLC with spectroscopic techniques such as UV, MS or NMR provides a useful tool for rapid data collection and structure elucidation for known and unknown constituents of complex mixtures. Recent publications also demonstrated the potential of merging the efficiency of HPLC separation with the convenience of on-line post-column detection of radical-scavenging compounds based on a model oxidation system, i.e. luminolchemiluminescence inhibition and DPPH• reduction (Goulas, Papoti, Exarchou, Tsimidou, & Gerothanassis, 2010; Niederlander, van Beek, Bartasiute, & Koleva, 2008; Van Beek et al., 2009). The hyphenated techniques and on-line DPPH radical-scavenging activity has been applied in large-scale routine analysis of complex plant extract (Exarchou, Fiamegos, van Beek, Nanos, & Vervoort, 2006).

The aim of the present study was to decode the potent antioxidant activity of polar extracts of *T. polium* in the search of new antioxidant agents of natural origin for food industry. The phytochemical content of *T. polium* extracts was investigated using the sophisticated HPLC–SPE–NMR technique, where the structure of the substances was elucidated. Using the same instrumentation and without merging an additional pump to introduce the radical solution or an auxiliary UV detector the on-line HPLC–DPPH assay was performed. This is the first time that use of HPLC–SPE–NMR and on-line DPPH assay in one configuration without any important modification is described; this provides a rapid and efficient tool for exploring antioxidants present in herbal extracts without the use of laborious and time-consuming fractionation and isolation steps.

## 2. Materials and methods

### 2.1. Chemicals

Methanol, hexane and ethyl acetate for Soxhlet extraction were obtained from Riedel de Haen (Seelze, Germany). Acetonitrile-$d_3$ (NMR quality) was purchased from Deutero (Kastellaun, Germany). DPPH• and Folin–Ciocalteu phenol reagent were purchased from Sigma (St. Louis, MO) and Merck (Darmstadt, Germany) respectively. Acetonitrile and water were of HPLC grade and were obtained from Scharlau (Barcelona, Spain). Acetic acid was analytical grade provided by Merck (Darmstadt, Germany).

### 2.2. Preparation of extracts

Dried aerial parts of *T. polium* in flowering stage were collected from Prosilio of Tzoumerka (Epirus, Greece). The plant material was air-dried at room temperature in the dark and extracted with different solvents. The plant was kindly characterized in the Laboratory of Botany, Department of Biological Applications and Technologies (Dr A. Kyparissis).

Twenty g of plant material were boiled with 100 mL distilled water for 1 h, filtered, and lyophilized in order to obtain aqueous extract (ATP). Methanol extract (MTP1) was prepared by extracting 10 g of plant material with 200 mL methanol in a Soxhlet apparatus for 6 h. The extract was concentrated in a rotary evaporator and kept in sealed dark flasks after minutes of nitrogen flushing. The same procedure was repeated and subsequent extraction with solvents of increasing polarity in a Soxhlet apparatus was performed. The sequence of the solvents was hexane, ethyl acetate and methanol and, thus, the ethyl acetate (EATP) and a second methanol extract (MTP2) were obtained.

### 2.3. Determination of total phenolics

The determination of total phenolics was performed with Folin–Ciocalteu according to previous work (Goulas & Manganaris, 2011). Each measurement was repeated in triplicate and total phenolic content was expressed as gallic acid equivalent (mg/g plant extract).

### 2.4. Evaluation of radical-scavenging activity using off-line DPPH assay

Two mL of each sample (5 mg/mL) were mixed with 1 mL of 0.3 mmol/L solution of DPPH• in methanol, and the absorbance of the mixture was measured after 30 min incubation time in the dark at 517 nm. Several concentrations of each sample were tested and the % of free radical-scavenging activity was determined by the following equation:

$$\%\ \text{scavenging activity} = 100 - [(\text{Ab of sample} - \text{Ab of blank}) \times 100/\text{Ab of control}]$$

$EC_{50}$ values are referred to the lower concentration of the extracts required for the 50% of the antioxidant activity (Goulas et al., 2009).

### 2.5. Instrumentation and chemical analysis using HPLC–SPE–NMR and ESI-MS

HPLC–UV–SPE–NMR measurements were carried out on a chromatographic separation system consisting of an Agilent G13311A solvent delivery pump and a Bruker DAD UV detector (Bruker BioSpin, Rheinstetten, Germany). The samples were injected using an Agilent G1311A autosampler with a 30 μL loop. The Bruker/Spark Prospect 2 solid phase extraction unit (Bruker BioSpin and Spark, Emmen, The Netherlands) was used to trap the chromatographic peaks on Hysphere General Phase cartridges (2 mm i.d, 10–12 μm) after post-column addition of water using a Knauer K 120 HPLC pump (Berlin, Germany). The trapped peaks were dried with dry nitrogen gas and eluted with deuterated acetonitrile into a Bruker AV-500 NMR spectrometer equipped with 3 mm LC SEI $^{13}C-^{1}H$ probe head with an active volume of 60 μL from Bruker BioSpin (Fig. 1). NMR system was controlled by software TopSpin 1.3. NMR data acquisition was carried out on a Bruker AV-500 spectrometer (Bruker BioSpin, Rheinstetten, Germany). Chemical shifts are expressed in $\delta$ (parts per million) referenced to the solvent peak (1.94 ppm for $CD_3CN$). $^{1}H$ NMR spectra were acquired for all compounds trapped; 2D $^{1}H-^{1}H$ COSY, $^{1}H-^{1}H$ total correlated spectroscopy ($^{1}H-^{1}H$ TOCSY), $^{1}H-^{13}C$ heteronuclear single quantum correlation ($^{1}H-^{13}C$ HSQC) and $^{1}H-^{13}C$ heteronuclear multiple bond correlation ($^{1}H-^{13}C$ HMBC) were obtained when necessary.

The chromatographic separation was carried out on a 250 mm × 4.6 mm i.d., 5-μm, Discovery C18 column from Supelco (Pennsylvania, USA). The flow rate was 0.6 mL/min, and the

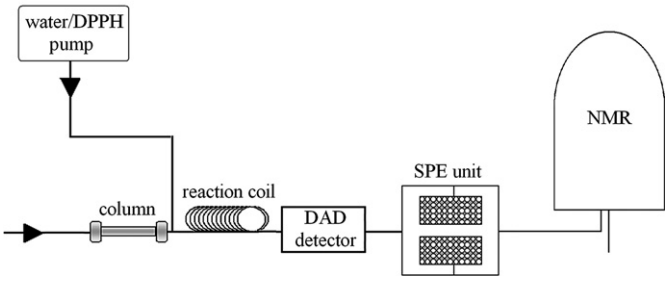


**Fig. 1.** Schematic representation of the HPLC–SPE–NMR and HPLC–DPPH set-up.