

ANTIOXIDANT POLYPHENOLIC CONSTITUENTS OF *SATUREJA MONTANA* L. GROWING IN EGYPT

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ABSTRACT

Objective: *Satureja montana* L. is a deciduous perennial shrub known to possess a few pharmacological activities. The plant essential oil and extracts are used as folk remedies for many diseases. This study was designed to examine the antioxidant activity of the essential oil and ethanol extract of *S. montana* L. grown in Egypt, and determine their active constituents.

Method: The volatile oil content has been analyzed by GC/MS. The alcoholic extract was fractionated and the fractions were tested for their free radical scavenging activity using DPPH assay. Identification of isolated compounds was carried out by spectroscopic analysis viz., UV, NMR, TLC and PC.

Results: The GC/MS revealed the presence of carvacrol (79.75%) which represents the main compound of volatile oil. The herb has been extracted with ethanol (80%) for the study of the phenolic content. Rosmarinic acid, caffeic acid and luteolin were isolated from the ethyl acetate fraction which showed the highest antioxidant activity compared to trolox. TLC scanner system has been used for the quantitative determination of the rosmarinic and caffeic acids in the total extract. The results revealed that the concentration is 0.1 and 0.17% (w/w) for rosmarinic and caffeic acids respectively; in the dry herb of *S. montana* cultivated in Egypt.

Conclusion: Our study, allowed evidencing that *S. montana* growing in Egypt contains a high content of phenolic and flavonoid compounds which could be responsible for a remarkable radical scavenging and antioxidant properties observed.

Keywords: *Satureja montana*, Essential oil, Ethanol extract, Antioxidant activity.

INTRODUCTION

Herbal medicine has lower effects in comparison with chemical drugs. Today, attention for using herbal drugs is increasing in the concept of food that combines nutritional and medicinal benefits. Many natural compounds isolated from medicinal plants have demonstrated a wide spectrum of biological activities, among which, essential oils have proven to have various pharmacological effects, such as spasmolytic, hepatoprotective, antiviral and anticarcinogenic effects [1,2]. Also, there is a considerable interest in the polyphenolic compounds for their antioxidative efficacy which can affect the etiology of chronic diseases and the aging process [3, 4]. *Satureja montana* L. (winter savory or mountain savory), belongs to the Lamiaceae family, native to the Mediterranean regions is a perennial herb (20–30 cm tall) with white flowers and small rough leaves [5,6]. *S. montana* is a well known aromatic and medicinal plant which contains various biologically active constituents such as, essential oil, triterpenes [7], flavonoids [8], and rosmarinic acid [9]. The whole herb is mildly antiseptic, aromatic, carminative, digestive, mildly expectorant and stomachic, while its essential oil is used in the food industry, liqueurs and in perfumery. The positive effects of savory on human health are attributed to its active constituents which show high antioxidant effect [10].

The main chemical compounds of essential oil are aromatic monoterpenes which are responsible for characteristic odor and taste [11]. The typical phenolic compound of *S. montana* is carvacrol and the prevailing carvacrol chemotype occurs also in Italy and the former Yugoslavia [12,13]. In fact, the composition of the essential oil of *S. montana* depends on many factors, such as environmental conditions, harvest time, geographic origin, and storage conditions which seem to have a significant influence on the essential oil relative compounds concentration in *S. montana* [14, 15]. Various

isolates of winter savory from Croatia, Bosnia, and Herzegovina have carvacrol (up to 84.19%) as the main constituent [16]. Other important compounds were γ -terpinene, thymol, *p*-cymene, β -caryophyllene, linalool, α -terpinene, myrcene, β -bisabolene, β -Thujene, β -Myrcene and borneol were evidenced [17]. The study of the active constituents of *S. montana* growing in Egypt might be of some biological interest as well as flavor value as a food additive. The aim of this study is to determine the concentration of the main phenolic constituents of *S. montana* responsible for its antioxidant activity, as well as, investigating the composition of the essential oil.

MATERIALS AND METHODS

General

Thin layer chromatography (TLC) was carried out on pre-coated silica gel F₂₅₄ plates (Merck, Darmstadt, Germany) developed with methylene chloride: methanol (8:2, & 7:3), Whatmann 3 was used for paper chromatography (PC) developed with *n*-butanol: acetic acid: water 4:1:5 (BAW). Spots were detected using Neu's reagent (1% 2-aminoethyl diphenylborinate in methanol) Aldrich. Column chromatography (CC) was performed using silica gel (Merck) and sephadex LH-20 (Pharmacia, Merck, Darmstadt, Germany). NMR was recorded on a Delta2 spectrometer operating at 600 MHz in methanol-D₃.

Plant Material

Seeds of *S. montana* were obtained from the HEM ZADEN B.V - P.O. Box 4 - 1606 ZG Venhuizen - The Netherlands. Seeds were sown in the nursery on 25th November, 2011. On February 10, 2012, uniform seedlings were transplanted into the experimental farm of the Faculty of Pharmacy, Cairo University, Giza, Egypt, which represents clay-loamy soil. The fresh herb was collected at the end

of July. The herb was air-dried in a room (under shade) for three weeks. A voucher specimen of the plant is deposited at the National Research centre herbarium.

Gas chromatography/mass spectrometry (GC/MS)

Extraction of essential oil: Fresh herb (100 g) was minced and distilled at 800 W for 30 min using an adapted microwave distillation apparatus which consists of a microwave oven (Mars, CEM) connected to a Clevenger-type apparatus and a cooler circulator (WiseCircu, fuzzy control system).

GC/MS Analytical Condition: The volatile oil analysis was carried out using gas chromatography-mass spectrometry instrument stands at the Department of Medicinal and Aromatic Plants Research, National Research Center with the following specifications. Instrument: a TRACE GC Ultra Gas Chromatographs (THERMO Scientific Corp., USA), coupled with a THERMO mass spectrometer detector (ISQ Single Quadrupole Mass Spectrometer). The GC/MS system was equipped with a TG-WAX MS column (30 m x 0.25 mm i.d., 0.25 μ m film thickness). Analyses were carried out using helium as carrier gas at a flow rate of 1.0 mL/min and a split ratio of 1:10 using the following temperature program: 40 C for 1 min; rising at 4.0 C/min to 160 C and held for 6 min; rising at 6 C/min to 210 C and held for 1min. The injector and detector were held at 210 °C. Diluted samples (1:10 hexane, v/v) of 0.2 μ L of the mixtures were always injected. Mass spectra were obtained by electron ionization (EI) at 70 eV, using a spectral range of m/z 40-450. Most of the compounds were identified using two different analytical methods: relative retention time to Carvacrol and mass spectra (authentic chemicals, Wiley spectral library collection and NSIT library).

Extraction, fractionation and isolation

1 kg of the air dried powdered herb of *S. montana* L. was extracted by maceration in aqueous ethanol (80%) three successive times. The combined extracts were evaporated under reduced pressure at 45°C to yield a residue 150 g. 100 g were dissolved in aqueous methanol, left overnight, then filtered. The filtrate was subjected to fractionation by shaking first with methylene chloride (1Lx3) then with ethyl acetate (1Lx3) and finally with *n*-butanol (1Lx3). The ethyl acetate fraction, of the highest antioxidant effect, was subjected to silica gel CC, eluted with methylene chloride 100%, then with gradient increase of methanol (10% each) till 100% methanol. 100 fractions (100 ml each) were collected, and monitored on TLC silica gel plates. The spots were detected under UV before and after spraying. Similar fractions were pooled together into subfractions. The subfractions 4, 5 were further subjected to CC on sephadex to give luteolin (**1**) (50 mg), caffeic acid (**2**) (60 mg) and rosmarinic acid (**3**) (90 mg). The isolated compounds were further purified by CC on sephadex and preparative PC.

DPPH Assay

For the DPPH radical scavenging assay, 20 μ L of extract diluted appropriately in DMSO was mixed with 180 μ L of DPPH in methanol (4 mg/mL) in wells of a 96-well plate. The plate was kept in the dark for 15 min, after which the absorbance of the solution was measured at 540 nm in a Multiskan automatic kinetic microplate reader ((Labsystems Multiskan RC reader). Appropriate blanks (DMSO) and standard (trolox solutions in DMSO) were run simultaneously. Extracts were first tested at a single concentration of 4 mg/mL, and those showing good evidence of antioxidant activity were tested over a range of concentrations to establish the EC₅₀ (extract concentration providing 50% inhibition) [18]. Tests were carried out in triplicate.

TLC scanner: Densitometric Analysis

The CAMAG HPTLC system (Muttentz, Switzerland) consisted of TLC scanner 3 connected to a computer running WinCATS version 1.2.3; and Linomat V sample applicator connected to a nitrogen tank. Samples and standard solutions were prepared by dissolving 2 mg in 1 ml HPLC grade methanol. 25 μ L sample solutions were applied in the form of 10 mm bands with CAMAG microlitre syringe on pre-coated silica gel glass plate 60 F254 (20x10 cm with 0.2mm thickness; Merck, Germany). The TLC procedure was optimized with a view to quantify the extract of total alcohol of *S. montana* L. Initially two phenolic standards were used (Rosmarinic and Caffeic acids). The mobile phase methylene chloride/methanol (70:30 v/v) gave good resolution for the sample. The bands of rosmarinic and caffeic acids from sample solution were confirmed by comparing the R_f and spectra of the bands with that of standards. The peak purity of phenolic acids was assessed by comparing the spectra at three different levels, i.e., peak start, peak apex and peak end positions of the bands [19,20].

RESULTS AND DISCUSSIONS

GC/MS of essential oil

The essential oil of *S. montana* growing in Egypt was subjected to detailed GC/MS analysis. The yield of the oil was about 3%. Exactly 15 compounds, mostly aromatic, were identified, representing 94.56% of the total oil. The major compound was carvacrol 79.75%. Other important compounds were thymol (2.26%), o-Cymene (4.26%) and 1-Octen-3-ol (2.33%), most findings were in agreement with the results presented in this study but with variable concentrations, as indicated that the essential oils obtained from *Satureja* species showed significant variability in their chemical composition depending on location and stages of development [21]. The percentage of carvacrol proved that savory essential oil clearly belongs to the phenolic chemotype with high percentages of monoterpene phenols (Table 1).

Table1: Principal constituents of *Satureja montana* essential oil and its relative percentage of total chromatogram area

Compound	RRT ^a	RA ^b %
α -pinene	0.102	0.08
α -Thujene	0.104	0.11
α -Myrcene	0.173	0.20
α -Terpinene	0.178	0.32
Limonene	0.192	0.06
Eucalyptol	0.199	0.41
γ -Terpinene	0.224	1.05
o-Cymene	0.244	4.26
1-Octen-3-ol	0.403	2.33
trans-Sabinene hydrate	0.406	0.64
Linalol	0.474	1.47
trans-caryophyllene	0.495	0.52
4-terpineol	0.513	1.10
Thymol	0.738	2.26
Carvacrol	1.000	79.75

^aRRT: Relative retention time to Carvacrol 38.33min. ^bRA: Relative area

Isolated compounds

By correlating with spectral data (TLC, UV, ¹H-NMR) of literature values, compounds 1-3 were identified as luteolin **1**, caffeic **2** and rosmarinic acid **3** (Fig. 1).

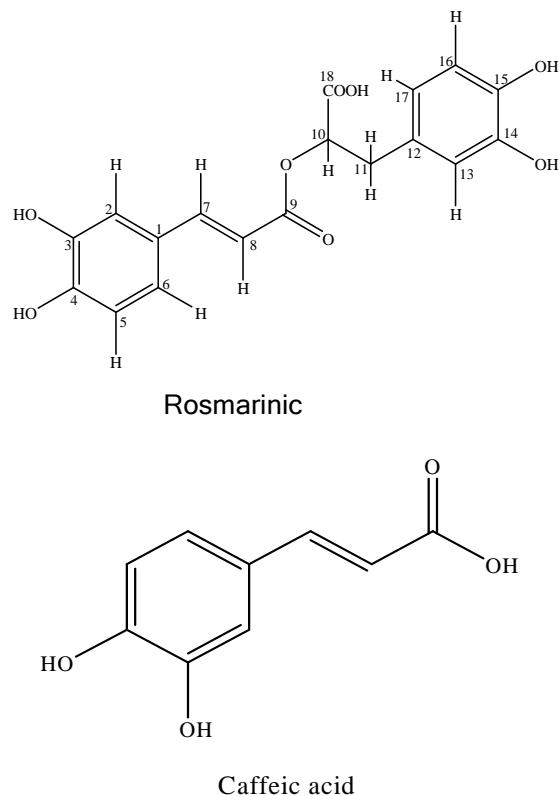


Fig. 1: Isolated Compounds (Rosmarinic and Caffeic acids)

Luteolin (1)

(50 mg) was obtained, R_f (PC) = 0.77 (BAW), gives purple and yellow spot appearance (UV) before and after spraying with spray reagent. UV λ max in MeOH: 271, 349; NaOMe: 267, 393; NaOAc: 260, 330; (NaOAc/H₃Bo₃) 271, 373; AlCl₃: 277, 441; AlCl₃/HCl: 277, 393. ¹H-NMR (methanol-D₃) δ (ppm): 7.39 (d, 1H, H-2'), 6.88 (d, 1H, H-5'), 7.38 (dd, 1H, H-6'), 6.88, 6.95 (d, H-6, H-8) appearing separately, while H-3 appears as singlet at 6.59 [22].

Caffeic acid (2)

(60 mg) was obtained, R_f (PC) = 0.84 (BAW), gives fluorescent blue spot appearance (UV) compared to standard. UV λ max in MeOH: 290, 330. ¹H-NMR (methanol-D₃) δ (ppm): 7.02 (1H, s, H-2), 6.77 (1H, d, H-5), 7.06 (1H, d, H-6), 7.48 (1H, d, H-7), 6.26 (1H, d, H-8) which is correlated to published data [23].

Rosmarinic acid (3)

(90mg) was obtained, R_f (PC) = 0.77 (BAW), with fluorescent blue spot (UV) referred to standard. UV λ max in MeOH: 291, 333. ¹H-NMR (methanol-D₃) δ (ppm): 7.49 (1H, d, H-7), 7.00 (1H, s, H-2), 7.02 (1H, d, H-6), 6.74 (1H, d, H-5), 6.69 (1H, s, H-13), 6.64 (1H, d, H-16), 6.60 (1H, d, H-17), 6.25 (1H, d, H-8), 5.07 (1H, dd, H-10), 3.05 (1H, dd, H-11), 2.91 (1H, dd, H-11), this data confirming the structure of rosmarinic acid [24].

DPPH Assay

From the DPPH assay, all the extracts and essential oil exhibited antioxidant activity. When compared with the oxidative potential of the standard compound (Trolox, 98.2%) used in this study, both the essential oil and the ethyl acetate fraction studied, exerted remarkable activity (84.9 and 94.4 % respectively). The EC₅₀ values

of both were 44±1.63 and 128±2.26 respectively (figure 2). This could be attributed to phenotype nature of the oil, and the rosmarinic acid and caffeic acid content in the ethyl acetate fraction

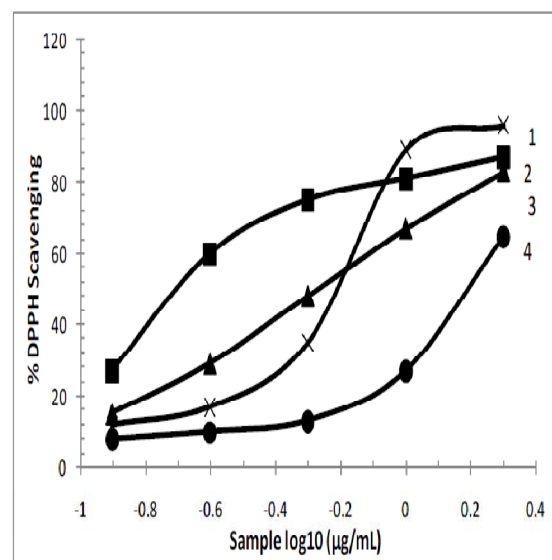


Fig. 2: Concentration-response curves for the DPPH radical scavenging activity of trolox (positive control, **1**), dichloromethane fraction (**2**), volatile oil (**3**) and ethyl acetate fraction (**4**) of *S. montana*

Densitometric Analysis

The phenolic acids were detected by UV scanning at 365 nm and sprayed with Neu's reagent to compare the band colour with that of the standard (figure 3). Quantitative analysis was done to check the percentage of major phenolic acids. The content of rosmarinic and caffeic acids were calculated with the use of two calibration curves obtained from the correspondent standard solutions. Calculation results show that rosmarinic and caffeic acid concentrations are about (0.1±0.27 and 0.17±0.65) % w/w in the dry herb of *S. montana* growing in Egypt. This means that the local conditions of cultivation of the herbal plant have no significant effects on its medical marker.

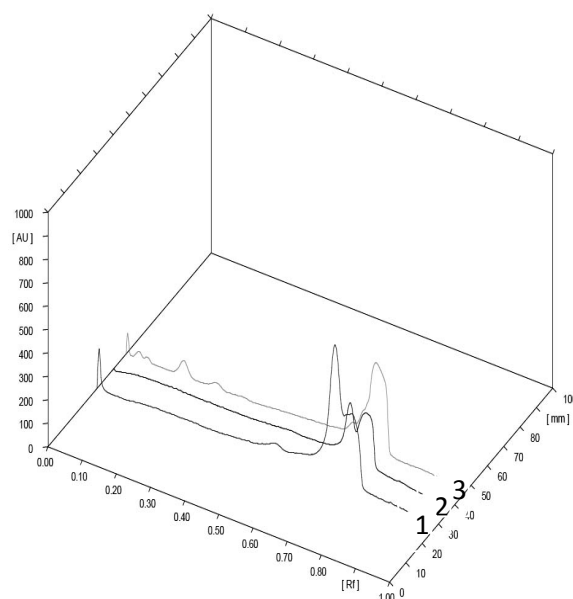


Fig. 3: UV absorbance spectra of rosmarinic acid (**1**), total alcoholic extract of *S. montana* (**2**) and caffeic acid (**3**)

DISCUSSION

Previous studies showed the potent antioxidant efficacy of *Satureja* essential oil and extracts [10]. Our study, allowed evidencing that *S. montana* growing in Egypt contains a high content of phenolic and flavonoid compounds which could be responsible for a remarkable radical scavenging and antioxidant properties observed. This promising efficacy suggests its possible role as antioxidant agent, in order to improve antioxidant status and counteract the risk of diseases associated with oxidative stress. Thus, bioactive study will be conducted on the hydroalcoholic extract of *S. montana* L. to evaluate its effect.

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