# 2,2'-DIPHENYL-1-PICRYLHYDRAZYL RADICAL-SCAVENGING ACTIVITY OF DIFFERENT *Teucrium montanum* L. EXTRACTS

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***Abstract:***

*The sequential extraction of Teucrium montanum L. was realized with five solvents of different polarities (70% methanol, petroleum ether, chloroform, ethyl acetate, n-butanol). The scavenging activity (SA) of obtained extracts was tested by measuring their ability to scavenge stable 2,2`-diphenyl-1-picrylhydrazyl (DPPH) free radical using electron spin resonance (ESR) spectroscopy. The results demonstrated that the SA depended on the type and concentration of applied extracts. In investigated range of concentrations (0.10-0.15 mg/mL) petroleum ether and chloroform extracts did not show any SA. Other extracts exhibited SA in the following order: n-butanol > methanol > ethyl acetate > water extracts. The antioxidant properties were in correlation with the contents of total phenolic compounds (0-296 mg/g) in investigated extracts.*

*The investigated Teucrium montanum L. extracts probably had the SA due to the hydrogen donor ability of the flavonoids (quercetin, rutin) and phenolic acids (chlorogenic, caffeic, gallic, ellagic) which were identified by TLC. Another mechanism is "scavenging" activity (one DPPH$^{\bullet}$ molecule forms complex with one aryl radical formed from phenolic compounds).*

***Keywords:***

*Teucrium montanum L. extracts; polyphenols; scavenging activity; DPPH; ESR; TLC.*

## 1. INTRODUCTION

Nowadays there is an increasing interest in antioxidant activity of phytochemicals present in the diet. Antioxidants are believed to play a very important role in the body defence system against reactive free radical species, which are harmful by products generated during normal cell aerobic respiration [1].

Among the recognized antioxidants (vitamins C and E, carotenoids, tocopherols, etc.) there is an extensive family of diverse components that are found in all foodstuffs of plant origin, known generally as polyphenolic compounds [2]. Herbals and especially herbal extracts, which contain different classes of polyphenols, such as

flavonoids and phenolic acids, are very attractive not only for the modern phytotherapy but also for food industry.

*Teucrium montanum* L. (mountain germander) is a grass crop that has long been consumed both as an herbal medicine and as a nourishing food. It is widely used as diuretic, stomachic, analgesic and antispasmodic agent and also possesses antibacterial, antifungal, antiinflammatory and antioxidative activity. From the aerial parts of *Teucrium montanum* L. diterpenoids 19-acetylgnaphalin, montanin B, D, E, teubotrin (teulamifin B), flavone cirsiliol, *neo*-clerodane diterpenoid, montanin H (4$\alpha$, 18:15,16-diepoxy-6$\alpha$-hydroxy-7-keto-*neo*-cleroda-13(16),14-diene,-12$\xi$-acetoxy,20*R*, 19-hemiacetal), have been isolated [3].

The task of this study is to investigate the free-radical scavenging activity of methanol, petroleum ether, chloroform, ethyl acetate, *n*-butanol and water extracts of *Teucrium montanum* L. on stable 2,2`-diphenyl-1-picrylhydrazyl (DPPH) free radical by ESR spectroscopy.

## 2. EXPERIMENTAL

Methanol, ethyl acetate, petroleum ether, chloroform, n-butanol, formic acid and acetic acid were purchased from "Zorka" Šabac. 2,2`-Diphenyl-1-picrylhydrazyl, DPPH, quercetin, rutin, chlorogenic acid, caffeic acid, ellagic acid and gallic acid were from Sigma Chemicals Co., USA. Folin-Ciocalteu reagent was from Fluka, USA.

*Teucrium montanum* L. was collected from the region of Zlatibor.

**Extraction.** Dried plant of *Teucrium montanum* L. (20 g) was extracted with 70 % methanol (2 x 500 mL) at room temperature for 2 x 24 h. 20% v/v of obtained extract was evaporated to dryness under reduced pressure and used further as methanol extract. The rest (80 % v/v) of the extract was concentrated under reduced pressure. After removing methanol, the extract was successively treated with petroleum ether (2 x 20 mL), chloroform (2 x 20 mL), ethyl acetate (2 x 20 mL) and n-butanol (2 x 20 mL). The petroleum ether, chloroform, ethyl acetate, n-butanol and remained water extract were evaporated to dryness under reduced pressure. The yields of extracts were: methanol, m = 0.8488 g; petroleum ether, m = 0.1195 g; chloroform, m = 0.1554 g; ethyl acetate, m = 0.1065 g; n-butanol, m = 1.1132 g and water, m = 2.2759 g.

**Total Phenolic Content (TPh)**. Total phenolic compounds in extracts were determined spectrophotometrically using the Folin-Ciocalteu reagent. The results are expressed as chlorogenic acid equivalents per g dry weight [4].

**Thin-layer chromatography (TLC).** Thin-layer chromatography was performed on 20 x 20 cm plates precoated with microcrystalline cellulose (Camag, Muttanez, Switzerland). A volume of 1 µL of 1% of methanolic solutions of standards and investigated extracts was spotted on the plates. Analysis was performed with ethyl acetate : formic acid : acetic acid : water in volume ratio 100:11:11:26 as mobile phase. Spots were observed by spraying with 1% $FeCl_3$.

**DPPH radical assay.** A volume of x µL of 1% of the methanolic solutions of investigated extracts was added to (200-x) µL methanol and 600 µL 0.4 mM methanolic solution of DPPH. The final concentrations of the investigated extracts were: 0.1, 0.125 and 0.15 mg/mL. After that the mixture was stirred for 2 min., transferred to a quartz flat cell ER-160FT and analysed by ESR spectroscopy. Blank probe was obtained by mixing the 600 µL 0.4 mM methanolic solution of DPPH and 200 µL of methanol.

The scavenging activity (SA) of extracts was defined as:

$$SA = \frac{100\%(h_0 - h_x)}{h_0} \quad (1)$$

where:
$h_0$ - the height of the second peak in the ESR spectrum of DPPH radical of the blank
$h_x$ - the height of the second peak in the ESR spectrum of DPPH radical in reaction mixture with the addition of the extracts

The ESR spectra were recorded on a Bruker 300E ESR spectrometer (Rheinstetten, Germany) under the following conditions: field modulation 100.00 kHz, modulation amplitude 0.226 G, time constant 40.96 ms, conversion time 671.089 ms, centre field 3440.00 G, sweep width 100.00 G, x-band frequency 9.64 GHz, power 20 mW, temperature 23°C.

## 3. RESULTS AND DISCUSSION

**Total Phenolic Content.** The TPh values of different *Teucrium montanum* L. extracts are shown in Table 1. n-Butanol extract had the highest phenolic content (296 mg/g). Petroleum ether did not extract any of the phenolic compounds. This fact is in correlation with polarity of the solvents used for extraction and solubility of phenolic compounds in them.

***Table 1.*** *Total Phenolic Content in Teucrium montanum L. extracts*

| Extract | Total Phenolic Content ( mg/g ) |
|---|---|
| Methanol | 154 |
| Petroleum ether | 0 |
| Chloroform | 0.0956 |
| Ethyl acetate | 20.4 |
| n-Butanol | 296 |
| Water | 59.8 |

**Thin-layer chromatography (TLC).** Qualitative characterization of TLC chromatogram was performed comparing the $R_f$ values and colour (green/grey) of separated components of the examined extracts and the standard compounds (Fig. 1.). Methanol, n-butanol and ethyl acetate extract were very rich in phenolic compounds. n-Butanol extract had chlorogenic, gallic acid, rutin and ellagic acid. Ethyl acetate extract contained quercetin, caffeic, chlorogenic, gallic acid, rutin and ellagic acid. In methanol extract the same substances were detected. Water extract contained rutin and ellagic acid. The smallest amount of phenolic compounds was detected in petroleum ether (quercetin) and chloroform (quercetin and caffeic acid) extract. Also, some other unidentified substances were spotted.

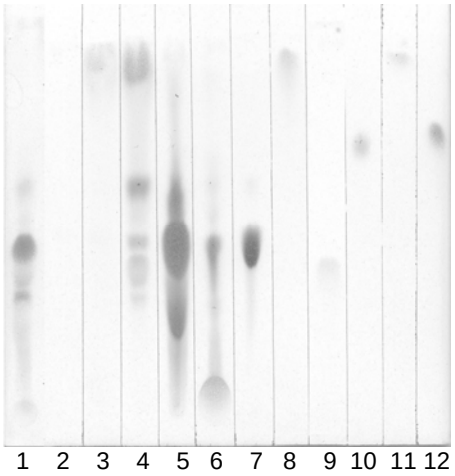


***Fig. 1.*** *TLC chromatogram of Teucrium montanum L. extracts*
*1 - methanol extract, 2 - petroleum ether extract, 3 - chloroform extract, 4 - ethyl acetate extract, 5 - n-butanol extract, 6 - water extract, 7 - rutin, 8 - quercetin, 9 - ellagic acid, 10 - chlorogenic acid, 11 - caffeic acid, 12 - gallic acid*

**DPPH radical scavenging activity of extracts.** Most of the methods described in the bibliography for determining antioxidant activity are based on the study of a reaction in which a free radical is generated and how this reaction is inhibited by the addition of the sample that is object of the measurement of antioxidant power. In this paper we used direct, rapid, simple and reliable method - DPPH method in combination with ESR spectroscopy.

The ESR spectra of DPPH radicals in the blank and in probes with investigated extracts were characterized by their five lines of relative intensities 1:2:3:2:1 and hyperfine splitting constant $a_N$=9.03 G (Fig. 2).

The scavenging activity (SA) of different concentrations of methanol, petroleum ether, chloroform, ethyl acetate, *n*-butanol and water extracts of *Teucrium montanum* L. on DPPH radicals is evident from Fig. 2.

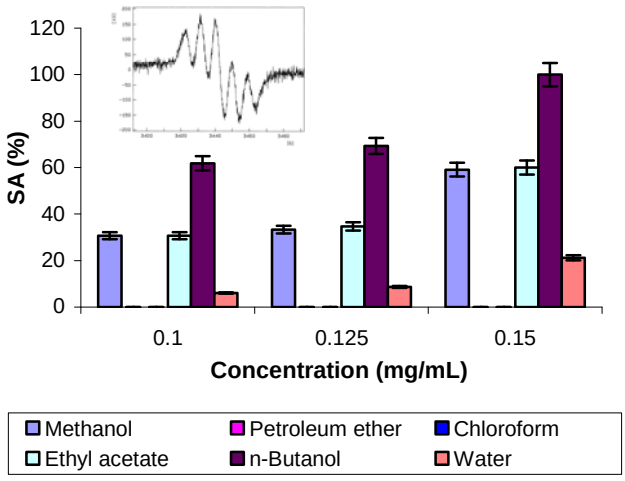


***Fig. 2.*** *The scavenging activity (SA) of different concentrations of methanol, petroleum ether, chloroform, ethyl acetate, n-butanol and water extracts of Teucrium montanum L. on DPPH radicals. Values are means ±SD of three independent experiments.*

On the basis of the obtained results it is evident that the SA on DPPH radicals depended on the type and concentrations of the investigated extracts.

n-Butanol extract had the strongest effect and also exhibit very rapid increase of SA in a narrow range of concentrations. n-Butanol extract scavenged 61.85%, 69.36% and 100% DPPH radicals at concentrations 0.10 mg/mL, 0.125 mg/mL and 0.15 mg/mL, respectively. The same concentrations of ethyl acetate and methanol extract had similar activities, but lower than n-butanol extract, ranging from 30.64% to 60% and 30.64% to 59.09%, respectively. The water extract exhibited much lower SA, from 6.06% to 21.21%. SA of petroleum ether and chloroform extract was not evident at any of tested concentrations..

The antioxidant properties (Fig.2) were in correlation with the contents of total phenolic compounds (Table 1).

Natural phytochemicals such as phenolic compounds found in numerous herbs, commonly involve an aromatic ring as a part of the molecular structure, with one or more hydroxyl groups. They can act as antioxidants as their extensive conjugated $\pi$-electron systems allow ready donation of electrons or hydrogen atoms from the hydroxyl moieties to free radicals. The less reactive aroxyl radicals were obtained during this reaction, and stabilized their structure by electron delocalization (forming aryl radicals) [5,6].

The antiradical efficiencies based on this mechanism are typical for different phenolic acids (chlorogenic, syringic, gallic and ellagic acid) and flavonoids (possessing two or three hydroxyl groups on the carbon in the B or C ring of the molecules), whose presence was proved in *Teucrium montanum* L extracts. Another mechanism proposed for SA of extracts on DPPH radicals is “scavenging” activity (one $DPPH^{\bullet}$ molecule forms complex with one aryl radical).

## 4. CONCLUSION

The antioxidant properties of *Teucrium montanum* L. extracts were assessed by electron spin resonance (ESR) spectroscopy.

SA depended on the type and concentration of applied extracts.

In investigated range of concentrations (0.10-0.15 mg/mL) petroleum ether and chloroform extracts did not show any SA.

Other extracts exhibited SA in the following order: n-butanol > methanol > ethyl acetate > water extracts.

Antioxidant properties were in correlation with the contents of total phenolic compounds (0-296 mg/g) in investigated extracts.

The investigated *Teucrium montanum* L. extracts probably had the SA due to the hydrogen donor ability of the flavonoids (quercetin, rutin) and phenolic acids (chlorogenic, caffeic, gallic, ellagic) which were identified by TLC. Another mechanism was “scavenging” activity (one $DPPH^{\bullet}$ molecule forms complex with one aryl radical formed from phenolic compounds).

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