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Evaluation of extraction factors influence on total phenolic content and antioxidant activity of *Melissa officinalis* L. leaves extracts

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Extraction factors influence the quantity and quality of extracts. It is important to choose suitable conditions for production of valuable products. The aim of this study was to evaluate the influence of extraction factors on the quality of lemon balm extracts. Dry leaves of *Melissa officinalis* L. were extracted by dynamic maceration using ultrasound. Extraction factors were solvents – water and 50% ethanol; the extraction time was 5, 10, 15 and 30 min, the raw material and solvent ratio was 1:10, 1:20 and 1:30, and the particle size of dry leaves. Qualitative parameters – the amount of the total phenolic content and antiradical activity using the 2,2-diphenyl-2-picrylhydrazyl (DPPH) radical inactivation method – were determined spectrophotometrically. The total quantity of phenolic compounds of all determinations ranged between 40.13 ± 2.0 and 78.49 ± 3.9 mg of rosmarinic acid equivalents per weight of dry leaves of lemon balm. The higher amount of phenolics was extracted with 50% ethanol than with water. 1:10 ratio of leaves and solvent was most effective using ethanol as a solvent. 1:30 ratio was most effective using water as a solvent. The reduction of particle size increased the yield of phenolics over 20%. The antioxidant activity of all measurements ranged between 37.60 ± 1.9–89.48 ± 4.5% inactivated DPPH radicals. The highest antiradical activity was achieved with ethanolic extracts at 1:10 ratio after 15 minutes of sonication using a reduced particle size of leaves. The relationship between the total content of phenolics and antiradical activity was evaluated and the correlation was not determined.

Keywords: *Melissa officinalis*, phenolics, antiradical activity, extracts

INTRODUCTION

Melissa officinalis L. is a traditional herbal material widely used in the world. It is well known for treatment of nervous system diseases and as a source of natural antioxidants, and has radical scavenging, antibacterial and antiproliferative activity [1, 2]. Medicinal plants produce a variety of chemical constituents with the potential to inhibit viral replication therefore they are interesting as possible sources to control viral infection [3]. Recently lemon balm was investigated for the antiviral properties: against herpes viruses and HIV in experimental models *in vitro*; against Herpes simplex virus type 1 and type 2 *in vitro* [4, 5].

Lemon balm has a broad chemical composition. It contains hydroxycinnamic acids (up to 6% of rosmarinic acid, p-coumaric and caffeic acids) and up to 0.37% of an essential oil composed of more than 40% of monoterpenes and more than 35% of sesquiterpenes [6]. Among the most significant terpenoids there are citral, citronellal, geraniol, nerol, linalool, farnesyl acetate, humulene, β-caryophyllene and eremophilene. There are also other constituents, such as flavonoids, e. g. glycosides of luteolin, quercetin, apigenin and kaempferol, as well as tannins and acidic triterpenes (ursulic and oleanolic acids). Important bioactive constituents of *Melissa officinalis* L. are the phenolic acids: rosmarinic, ferulic, caffeic, syringic, chlorogenic, galic acids [7].

Botanical extracts are a suitable form of bioactive compounds for the modelling pharmaceuticals or cosmetic

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preparations. The main health promoting activities are linked to phenolic compounds, which are secondary metabolites of plants. There are various methods for the extraction of phenolics in practice: maceration, percolation, soxhlet extraction, microwave or ultrasound assisted. All methods have some advantages and disadvantages. The solvent extraction may remain a toxic substance or have a poor extraction efficiency; microwave-assistance extraction provides a very rapid and efficient extraction [8], but equipment is expensive; the soxhlet extraction solvent used can be reduced but requires a long extraction time [9]. These disadvantages could cause the loss of active ingredients because of hydrolysis, oxidation and thermal decomposition during the high temperature extraction [10]. Ultrasound assisted extraction is widely used because it can improve the extraction efficiency and extraction rate, reduce the extraction temperature and increase the selection ranges of the solvents compared with traditional methods. Ultrasound extraction has 2 main principles that constitute its advantage over other leaching techniques. These are cavitation phenomena and the mechanical mixing effect, both of which increase the extraction efficiency and reduce the extraction time. In addition, since ultrasound is a nonthermal process, thermal decomposition of heat-sensitive compounds is avoided [10].

Most of the reported results associated functional properties of lemon balm with the composition of phenolic compounds. The extracts of lemon balm due to the antiviral effect can be used for topical therapeutic application against recurrent herpes infections. Therefore, the objective of this study was to evaluate the solvent extraction time, raw material and solvent ratio and the influence of dry leaves particle size on the extract quantity and quality. *Melissa officinalis* L. leaves were extracted by dynamic maceration assisted by ultrasound. The influence of extraction factors on the total phenolic content and antiradical activity was evaluated.

EXPERIMENTAL

Materials and instruments

The dried lemon balm leaves were obtained from Acorus Calamus, Lithuania. The initial size of dry leaves particles was 2.5 mm – 710 μm, the additionally reduced size was 710–125 μm. 2,2-diphenyl-1-picrylhidrazyl (DPPH$^{\bullet}$) reagent and sodium carbonate were purchased from Sigma-Aldrich, Germany; ethanol 96% from Vilniaus Degtinė, Lithuania; Folin–Ciocalteu reagent from Darmstadt, Germany. Extraction was performed in the ultrasonic bath Sonorex Digitec (Bandelin Electronic GmbH & Co. KG, Berlin, Germany). Solid phase separation was performed with the centrifuge Sigma 3-18 KS, Germany. Spectrophotometric measurements were performed with the spectrophotometer UV-1800 SHIMADZU, Japan.

Extraction

Ultrasound-assisted extraction was performed in an ultrasonic bath with internal dimensions 500 × 140 × 100 mm and a tank capacity of 6 L approximately, with an ultrasonic peak output of 200 W, equipped with a digital temperature controller/indicator. The experiment was performed at 25 ± 2 °C temperature and 35 kHz ultrasound frequency. The extraction time was 5, 10, 15 and 30 min, the raw material and solvent ratio was 1:10, 1:20 and 1:30 g/mL. 10 mL of extract was produced. The extracts were centrifuged at 10000 rpm for 5 min and kept in 50 mL dark glass bottles.

Total phenolic content

The method is based on the colorimetric oxidation/reduction reaction using the Folin–Ciocalteu reagent with modifications [11]. Because rosmarinic acid is the main phenolic acid in *Melissa officinalis* L., the total phenolic content of extracts was expressed as rosemary acid equivalents. The standard calibration curve was plotted ($R^2 = 0.9989$) using rosmarinic acid 0.0625–1.0 mg/ml dissolved in distilled water. The reaction mixture consisted of 0.5 mL of the test solution, 2.5 mL of Folin–Ciocalteu reagent 1:10 and 2 mL of sodium carbonate (75 g/L). The samples were left for 30 min and the absorbance at 765 nm was measured. The results were expressed in milligrammes of rosmarinic acid equivalent per gram of dry material (mg/g RAE).

Antiradical activity

Antiradical activity was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) free radical scavenging method [12]. The analysed extracts of lemon balm (0.1 mL) were mixed with a 0.1 mM ethanolic (96%) DPPH$^{\bullet}$ solution (2.90 mL) in a 1 cm path length disposable cuvette. The measurements at 515 nm were recorded after the incubation period of 30 min at room temperature in the dark. The standard calibration curve was plotted ($R^2 = 0.9985$) using trolox 5–30 μg/0.1 ml dissolved in ethanol. The results are expressed as μg/mL trolox equivalent (TE).

Statistical analysis

Five independent determinations were performed (n = 5). The data were presented as means ± standard deviation (SD). Statistical analysis was performed using the Student's *t*-test and the correlation matrixes test, and $P < 0.05$ was used as the level of significance.

RESULTS AND DISCUSSION

Total phenolic content

The results of water extracts in Fig. 1 show that the total phenolic content (TPC) ranged from 42.1 ± 2.1 mg/g RAE to 54.2 ± 2.7 mg/g RAE using 2.5 mm–710 μm size of dry leaves. TPC was higher and ranged from 49.2 ± 2.4 mg/g RAE to 69.6 ± 3.4 mg/g RAE in the extracts using additionally reduced particles to 710–152 μm. The highest extraction yield from bigger size particles was after 15 min of sonication using the 1:10 ratio of solid to solvent and 30 min using 1:20 and 1:30 ratios. The total phenolic content in water extracts from smaller size particles was significantly higher ($p < 0.05$)

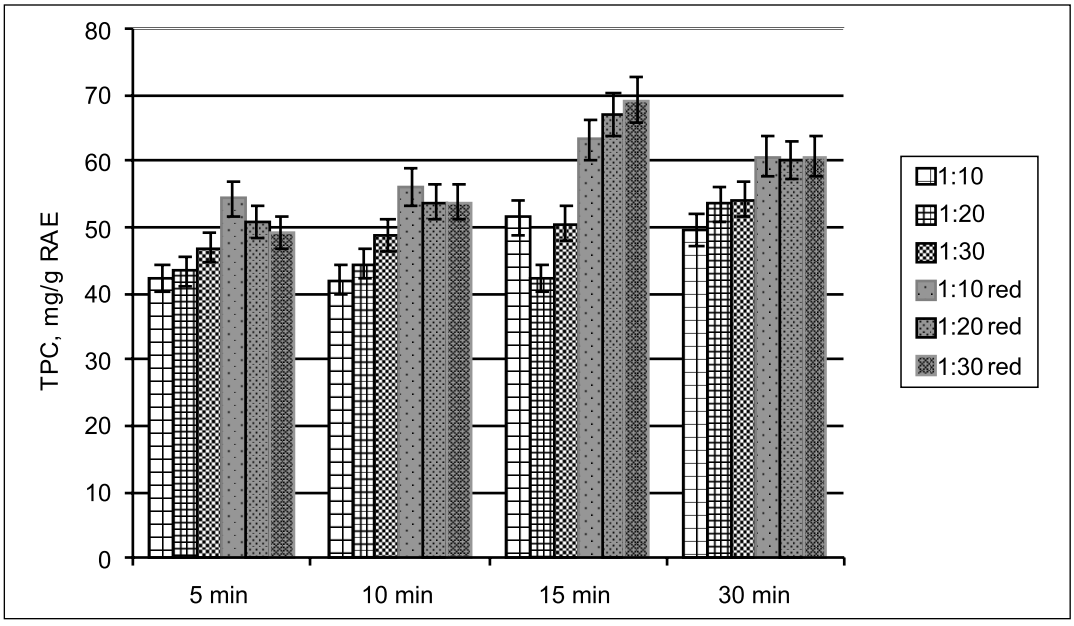

Fig. 1. Total phenolic content of water extracts of different size dry lemon balm leaves

* Solid and solvent ratio 1:10, 1:20 and 1:30 of the 2.5–710 µm size of dry leaves particles, red – reduced to 710–125 µm size.

after 15 min of sonication using the 1:30 ratio of solid to solvent. TPC extracted after 5 min was similar ($p > 0.05$), just the 1:10 ratio of solid to solvent using additionally reduced particles was significantly higher ($p < 0.05$). The 10 min extraction showed a small increase of the phenolic amount ($p > 0.05$), just the 1:10 ratio of solid to solvent using additionally reduced particles was significantly higher ($p < 0.05$) as after the 5 min extraction. Concluding the results of water extracts the highest amount was obtained after 15–30 min of sonication using an additionally reduced size of particles of lemon balm dry leaves.

Other solvent used for the extraction was ethanol. Concentration of 50% (v/v) as suggested by other researches was used as most effective [13, 14]. As shown in Fig. 2, the total phenolic content of ethanolic extracts ranged from 40.13 ± 2.1 mg/g RAE to 78.49 ± 3.9 mg/g RAE and was higher than in water extracts ($p < 0.05$). Ethanol of various concentrations is used more frequently than water for

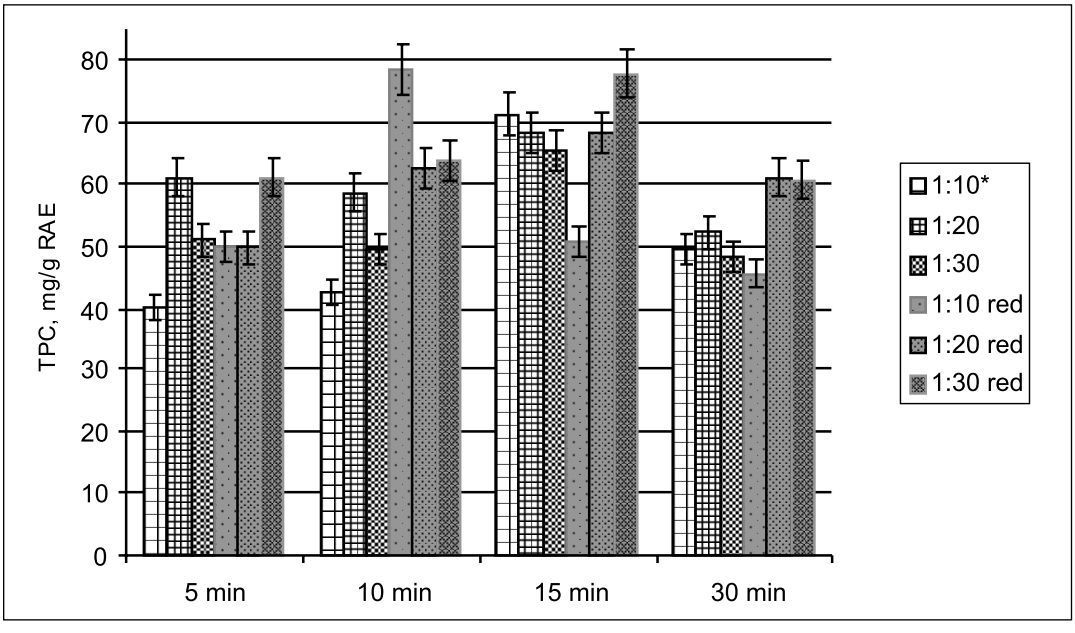

Fig. 2. Total phenolic content of ethanolic extracts of different size dry lemon balm leaves

* Solid and solvent ratio 1:10, 1:20 and 1:30 of the 2.5–710 µm size of dry leaves particles, red – reduced to 710–125 µm size.

extraction of phenolics and antioxidant. These results agree with previous reports, which suggest that a binary solvent system was superior to a mono-solvent system for extraction of phenolic antioxidants and preserves their relative polarity [15, 16]. Lemon balm extract could be used as an additive, food supplement or be destined for the preparation of cosmetic formulations where ethanol can be an unacceptable ingredient; therefore extraction with water was used. Stability of water extracts is insufficient compared with that of ethanolic ones, but in some cases it can be a more efficient solvent than ethanol [17].

A significantly higher amount of TPC was extracted from an additionally reduced particle size of dry leaves after 10 min using a 1:10 ratio of solid to solvent and after 15 min using a 1:30 ratio of solid to solvent. A reduced size of particles of dry herbal material commonly enhances the yield of active substances, because a limiting step of the extraction is often the diffusion of substances out of the plant matrix and a larger surface area provides a better permeability between the plant matrix and the solvent [18, 19]. The total phenolic content extracted with ethanol at all ratios of solid to solvent was significantly higher ($p < 0.05$) after 15 min of sonication from a bigger size of particles compared with other duration of sonication. The efficient extraction time for TPC extraction was 10 min using an additionally reduced material at a 1:10 ratio of solid to solvent and 15 min at the 1:30 ratio. It was determined that ethanol enhanced the yield of extracted TPC during a shorter time using the 1:10 ratio and a reduced size of particles compared with water. The extraction time of 20 min was chosen as optimal in the other ultrasound extraction of lemon balm assay [20]. Reviewing plenty researches about extraction of phenolic compounds the extraction time to 30 min is mostly used and analysed, concluding that a longer duration decreases the total amount of phenolics what may be caused by degradation of phenolic compounds.

A solid to solvent ratio of 1:30 provided a higher concentration of phenolics in water extracts. These results coincide with those obtained by other authors that have extracted dry leaves of lemon balm. Ince et al. demonstrated that the solid-to-solvent ratio of 1:30 provided a significantly higher TPC than 1:10 or 1:20 using ultrasound and microwave extraction [20]. Herodez et al. reported that a positive effect on TPC was shown by increasing the amount of the solvent for the conventional extraction of balm leaves [21].

Antiradical activity

Phenolic compounds of *Melissa officinalis* are a potential source of natural antioxidants.

The results of DPPH scavenging activity expressed by trolox equivalents are presented in Figs. 3 and 4. Analyzed extraction factors affected TPC significantly, but the antioxidant capability did not vary visibly. Using ethanol as a solvent, more phenolic compounds were extracted, however, their antioxidant activity increased just about 17%.

Water extracts after 5 min at 1:10, 1:20 and 1:30 ratios from the unreduced material showed a significantly lower activity while from the reduced one it was very high at the same conditions. The reduced size of material demonstrated a higher result after 5 and 10 min, later the influence of this factor was not significant (Fig. 3). The antiradical activity of ethanolic extracts varied from 249.58 to 300.87 µg/mL TE and no significant difference was observed. This study

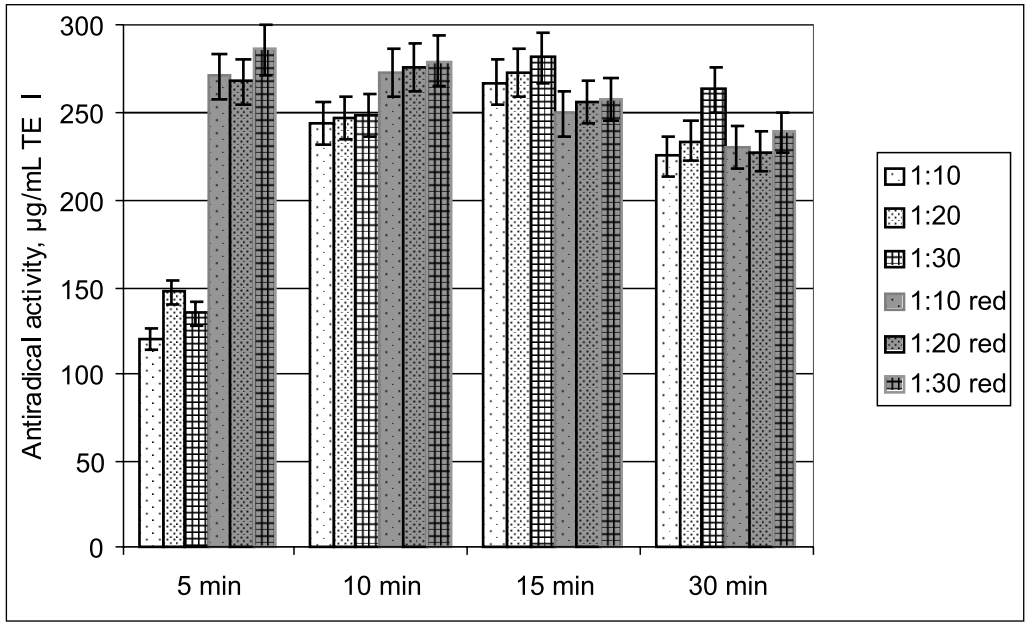

Fig. 3. Antiradical activity of water extracts of different size dry lemon balm leaves

* Solid and solvent ratio 1:10, 1:20 and 1:30 of the 2.5–710 µm size of dry leaves particles, red – reduced to 710–125 µm size.

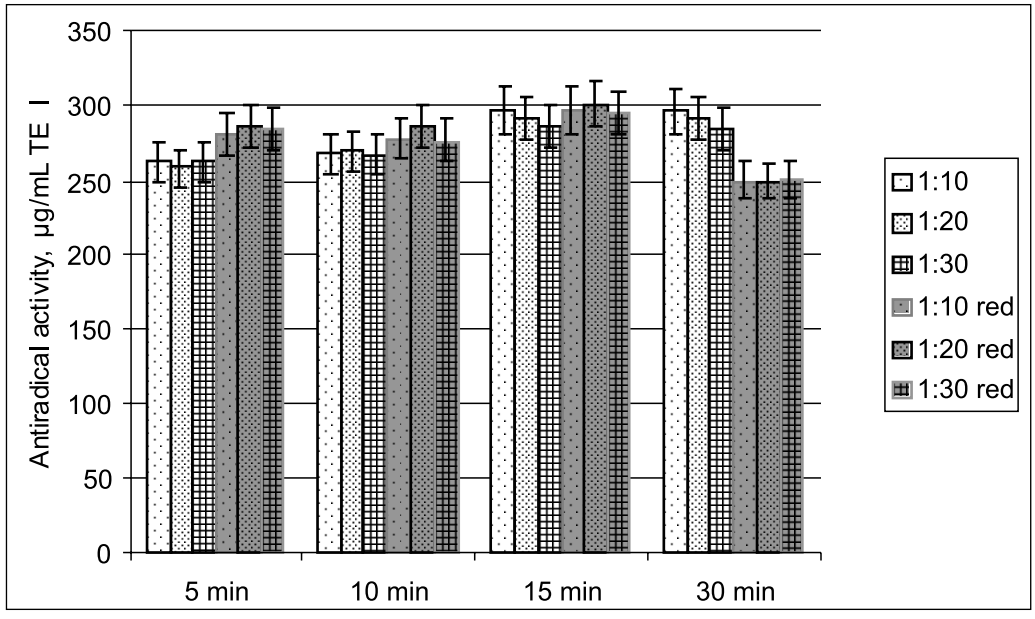

Fig. 4. Antiradical activity of ethanolic extracts of different size dry lemon balm leaves

* Solid and solvent ratio 1:10, 1:20 and 1:30 of the 2.5–710 µm size of dry leaves particles, red – reduced to 710–125 µm size.

presents that the antioxidant capacity was sensitive to solvent polarity, slightly to the particle size and did not depend on time and the solid to solvent ratio for the extraction of antioxidant plant compounds. The results did not show a correlation between the total amount of phenolics and antiradical activity. Similarly, no significant differences between the DPPH scavenging capacity were observed in the recent ultrasound extraction of TPC studies using water [20] and using ethanol [22]. Supposedly, this may be due to the difference in concentrations of individual phenolic compounds when the assay consists of the total phenolic or flavonoid content. Other researches maintain that a high yield of individual phenolic compounds does not necessarily indicate the maximal antioxidant capacity [23] and presents a significant correlation between TPC and DPPH results.

CONCLUSIONS

The aim of the present work was to evaluate the effects of extraction factors on the ultrasound extraction of antioxidants from lemon balm leaves. The highest amounts of phenolics (78.49 mg/g RAE) were determined in the ethanolic extracts at 1:10 solid to solvent ratio after 10 min of sonication and 1:30 ratio after 15 min of sonication (77.9 mg/g RAE). 10–15 min was efficient time for the TPC extraction and the decreasing particle size of dry leaves increased the extraction yield. Antiradical activity was comparable of all extracts produced at different extraction time and solid to solvent ratios. The results present that the antioxidant capacity of ethanolic extracts was higher than that of water. The results did not show any correlation between the total phenolic content and antiradical activity by the DPPH method.

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EKSTRAKCIJOS ĮTAKOS *Melissa officinalis* L. LAPŲ EKSTRAKTO BENDRAM FENOLINIŲ JUNGINIŲ KIEKIUI IR ANTIOKSIDACINIAM AKTYVUMUI ĮVERTINIMAS

S a n t r a u k a

Ekstrakcija veikia ekstraktų kiekybinę sudėtį ir kokybę. Svarbu parinkti tinkamas sąlygas vertingam produktui pagaminti. Tyrimo tikslas – įvertinti ekstrakcijos įtaką vaistinių melisų ekstraktų kokybei. Sausi *Melissa officinalis* L. lapai ekstrahuoti dinaminės maceracijos būdu naudojant ultragarsą. Ekstrakcijai naudoti tirpikliai – vanduo ir 50 % etanolis; ekstrakcijos laikas – 5, 10, 15 ir 30 min.; žaliavos ir tirpiklio santykis – 1:10, 1:20 ir 1:30 g/ml, ir sausų lapų dalelių dydis. Kokybiniai rodikliai – bendras fenolinių junginių kiekis ir antiradikalinis aktyvumas naudojant 2,2-difenil-2-pikrilhidrazilo (DPPH) radikalo inaktyvinimo metodą – nustatyti spektrofotometriškai. Bendras fenolinių junginių kiekis visuose ekstraktuose svyravo nuo 40,13 ± 2,0 iki 78,49 ± 3,9 mg rozmarino rūgšties ekvivalento sausų melisos lapų masei. Didesnis fenolių kiekis ekstrahuotas su 50 % etanoliu nei su vandeniu. Lapų ir tirpiklio santykis 1:10 buvo efektyviausias, kai tirpikliu naudotas etanolis. Kai tirpikliu naudotas vanduo, efektyviausias santykis – 1:30. Dalelių dydžio sumažinimas padidino fenolinių junginių išeigą 20 %. Antiradikalinis aktyvumas svyravo nuo 37,60 ± 1,9 % iki 89,48 ± 4,5 % inaktyvintų DPPH radikalų. Didžiausias antiradikalinis aktyvumas nustatytas etanoliniame ekstrakte po 15 min. ekstrakcijos, naudojant smulkintą žaliavą ir 1:10 santykį. Vertintas ryšys tarp bendro fenolinių junginių kiekio ir antiradikalinio aktyvumo, koreliacija nustatyta nebuvo.