

ROSMARINIC ACID – AN IMPORTANT PHENOLIC ACTIVE COMPOUND OF LEMON BALM (*MELISSA OFFICINALIS* L.)

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Rosmarinic acid is known as the antiviral and antioxidative principle of lemon balm (*Melissa officinalis* L.). A simple HPLC method was developed to determine the content of this compound. The influence of the plant development phase at harvest time on the content of rosmarinic acid was studied in *Melissa* leaf samples of Slovak origin. Only slight variability of rosmarinic acid content was observed. Maximal values in the respective leaf drug samples were found in the plant development phase of full flowering (3.91 %).

Key words: lemon balm – *Melissa officinalis* L. cv. Citra – rosmarinic acid – HPLC

INTRODUCTION

Lemon balm (*Melissa officinalis* L.) is one of the oldest and still most popular medicinal plants. It is a representative of the Lamiaceae family that is known for many aromatic and medicinal plants commonly used in Europe's traditional medicine and gastronomy. Originally growing in the Mediterranean area, lemon balm is now spread in the flora of South Slovakia, and Moravia as well [1]. For pharmaceutical use the plant is being cultivated. The domestic cultivar Citra belongs to the subspecies *officinalis*, one of at least three subspecies of lemon balm in Europe [2-4].

The most commonly known therapeutic properties of lemon balm are sedative, carminative, antispasmodic, antibacterial, antiviral, anti-inflammatory and antioxidative [5-13]. Essential oil is considered to be the therapeutic principle mainly responsible for most of the activities mentioned, but plant phenolics, especially rosmarinic acid, are involved too.

The essential oil is the most thoroughly studied active complex of lemon balm. Although its total content in the herbal drug is relatively low (usually 0.05 – 0.2 % V/m), compared to other Lamiaceae plants, the characteristic lemon smell is still considered a main criterion of the commercial quality of the drug. The main chemical components of

the essential oil are citrals A and B (geranial and neral), citronellal, and β -caryophyllene. A constant relation of the content of geranial and neral (4:3) is, together with the presence of 6-methylhept-5-en-2-one – a product of citral oxidation, considered to be the main identifier of authenticity of the essential oil. Several experimental studies analysed the total oil content in plants of different origin and its composition. A remarkable variability in both these parameters can be expected under the influence of intrinsic and extrinsic factors, affecting the drug quality as well [14-18].

Rosmarinic acid, together with similar compounds, has been known as „Labi-atengerbstoff“ even before its chemical structure was elucidated [19]. Indeed, it is a tannin-like compound, sometimes described as a depside of caffeic acid. Originally identified in rosemary (*Rosmarinus officinalis* L.), the structure was elucidated as an ester of caffeic acid and 3-(3,4-dihydroxyphenyl)lactic acid (Fig. 1) [20]. The compound has been reported to occur in several taxonomically non-related families of the plant kingdom [21,22]. It has attracted much attention since it was identified to be the main compound responsible for the antiviral activity of lemon balm in treating *Herpes simplex* [6,11,12].

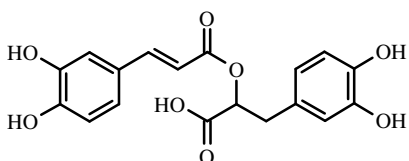


Fig. 1. Chemical structure of rosmarinic acid

Both *Melissa* leaf and herb are used as herbal drugs in Slovak and Czech Republics, western pharmacopoeias prefer the leaf that is the main source of therapeutic principles [23-29]. Gradually, the pharmacopoeias also reflected the results of phytochemical research on lemon balm, and the main criterion of drug quality was changed from the traditional one, total essential oil content, to a new one, total hydroxycinnamic derivatives content. Essential oil content is determined by hydrodistillation, hydroxycinnamic derivatives are determined by a spectrophotometric assay and expressed as rosmarinic acid.

The mentioned officinal spectrophotometric assay does not determine the amount of rosmarinic acid in the herbal drug selectively. Therefore, a simple HPLC method was developed to determine the content of rosmarinic acid. This method was used to analyse samples of *Melissa* leaf of Slovak origin. Plant material was harvested in the course of a vegetation period and changes in rosmarinic acid content were followed.

MATERIAL AND METHODS

Plant material

Plants of *Melissa officinalis* L. cv. Citra were cultivated in the Garden of Medicinal Plants of the Faculty of Pharmacy, Comenius University in Bratislava. Cultures on plots

of 10 m² were planted on a light sand-loam soil in a sunny locality; plants' spacing 40 cm × 40 cm; fertilisation with (kg/ha): 90 N, 60 P₂O₅, 80 K₂O; irrigation once a week in the dry period; no pesticides were used. The herb was harvested from a 3-year-old culture (overwintering without damage). The samples were obtained by cutting the herb manually in a height of about 20 cm above ground. Samples were collected in the course of one vegetation period, in five phases of flower ontogenesis: flower calix formation, flower calix development, just before flowering, begin of flowering, full flowering. Harvests were always carried out on sunny days, at about 11 a.m. The herb was dried at 32 °C and stored in paper sacks in a dark, cool and dry depository. Leaves were separated manually from the stems prior to analysis. Voucher specimens (№ Mo-29/4-Citra) are deposited in the Herbarium of the Faculty of Pharmacy, Comenius University in Bratislava.

Chemicals

Methanol (HPLC-grade, Merck, Slovakia), tetrachloromethane (p.a., Loba, Austria), phosphoric acid (p.a., Lachema, Czech Republic), rosmarinic acid (Aldrich, USA).

Equipment

The HPLC system (Ecom, Czech Republic) consisted of a gradient programmer (GP3), a micropump (LCP3001) and a variable UV-VIS detector (LCD 2082). Data were analysed with the integrator CSW 1.7 (DataApex, Czech Republic). The analytical column was a reversed phase SGX C18, 4 mm × 250 mm, 5 µm (Tessek, Czech Republic).

For sample preparation, an ultrasonic bath (Tesla, Slovakia), a pH meter (Knick, Germany) and a centrifuge (Mechanika Precyzyjna, Poland) were used.

Sample preparation

Melissa leaf was ground to a fine powder (400) and 1.000 g of the powdered drug was refluxed with 50 ml of methanol in a Soxhlet extractor for 3 h and evaporated to dryness. The residue was dissolved in methanol, filtered and diluted to 25.0 ml with methanol prior to analysis (solution A).

For sample pre-separation, 0.1 ml of solution A was diluted with 0.4 ml of methanol in an Eppendorf tube and 0.2 ml of tetrachloromethane was added to give a clear solution. Adding further 0.3 ml of distilled water to the solution causes the formation of a two-phase system. The tube was shaken, and centrifugation (10 000 rpm, 10 min) was used to obtain a sharp interface. The upper, water-methanolic layer was separated and injected into the HPLC system.

Chromatographic analysis

The mobile phase consisted of methanol and water (pH 2, adjusted with phosphoric acid). The linear gradient used for chromatographic separation is shown in Table 1. The

flow rate was 0.5 ml/min and injection volume was 20 µl. All analyses were carried out at ambient temperature. Spectra were recorded at 320 nm. The method of external standard was used for quantification. Retention time of rosmarinic acid was 19.15 min. The content of rosmarinic acid was calculated with reference to the drug dried at 105 °C and expressed in % m/m.

Table 1. Timetable of the linear HPLC solvent gradient system

t [min]	Methanol [%]	Water (pH 2) [%]
0	40	60
2	40	60
12	50	50
17	80	20
25	80	20

RESULTS AND DISCUSSION

Rosmarinic acid has become a compound attracting much interest amongst the active principles of lemon balm in last years. It was proven to be the main substance responsible for the healing activity of lemon balm extracts in *Herpes simplex* treatment [6,11,12]. Its antioxidative properties are known as well [9,10].

The content of the other important active principle of the plant, essential oil, is known to depend considerably upon extrinsic and intrinsic factors, including soil and climatic conditions, plant ontogenesis phases, harvest and drug storing conditions [14-18]. Optimal harvest time to guarantee high content of essential oil and consecutively a drug of high quality was reported to be at the plant development phase just before flowering [30,31]. No similar data concerning the seasonal variability of rosmarinic acid are known yet, however.

Rosmarinic acid is a simple phenolic compound and there are several different analytical methods described in the literature to determine its content in Lamiaceae species, including UV-VIS spectrophotometry, HPLC and GC [4,9,10,21,22,32-36].

The simple isocratic method [32] was modified in our work to fit our chromatographic conditions, and the linear gradient method was used to better separate the desired compound.

Attention has to be paid to extraction and pre-separation conditions in plant material extraction [35-37]. Several pre-separation techniques were also tested in order to eliminate non-polar constituents of the drug extracts, including plant pigments (e.g. chlorophyll) and so to prolong the chromatographic column's lifetime. Comparing a number of liquid-liquid and liquid-solid extraction systems, best results were obtained using a modified method of Römisch [38], that gave satisfying results in respect to both the requirements, i.e. firstly to remove undesired non-polar compounds from the methanolic extracts, and secondly not to affect the content of rosmarinic acid. Tetrachloromethane was the solvent of choice amongst other chlorinated and non-chlorinated solvents, convincing with its analytical properties as well as its non-flammability, low chemical interaction with the material of the plastic Eppendorf tubes (polyethylene and polypro-

pylene) and its physico-chemical properties (e.g. high density) resulting in better handling of the two-phase liquid system. Certain care has to be paid to health security in manipulation with this organic solvent due to its known potential health risks.

The results of the herbal samples' analyses show only slight, statistically non-significant, variability of rosmarinic acid content. However, maximal values in the respective leaf drug samples were observed in the plant development phase of full flowering (3.91 %) and minimal values in the phase just before flowering (3.50 %), Fig. 2.

It has to be noted that tannin-like plant phenolics are considered to be plant defense metabolites and their production in the plant increases under stress conditions. This fact is used to stimulate the production of rosmarinic acid in cell cultures with considerable results [21,22,39]. The influence of long-time climatic conditions, as well as drug drying and storing conditions are also of interest in the study of factors influencing rosmarinic acid content in plant material and corresponding further research is due in our laboratory.

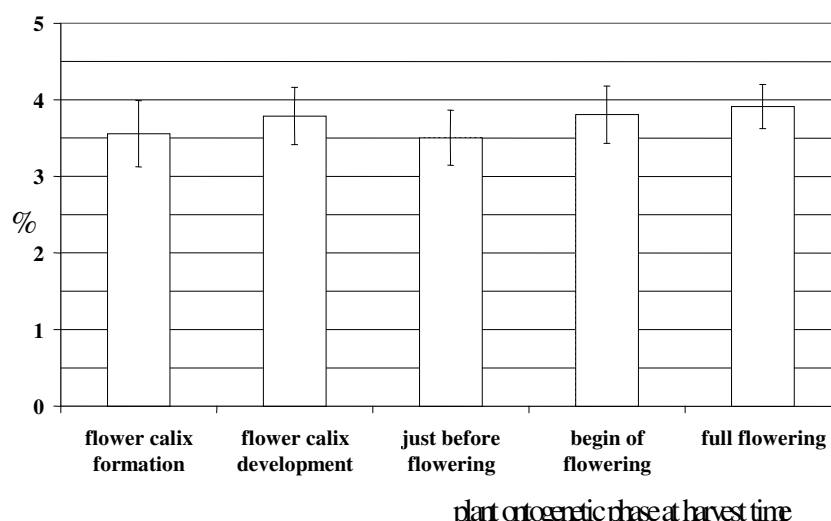


Fig. 2. Content of rosmarinic acid [%] in samples of *Melissa* leaf in dependence of the plant ontogenetic phase at harvest time. (n=4)

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ÚČINNÁ LÁTKA MEDOVKY LEKÁRSKEJ (*MELISSA OFFICINALIS* L.)**

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Kyselina rozmarínová je známa obsahová látka medovky lekárskej (*Melissa officinalis* L.) s antivirálnymi a antioxidantnými vlastnosťami. Na stanovenie obsahu tejto látky bola vyvinutá jednoduchá HPLC metóda. Vo vzorkách medovkových listov slovenského pôvodu bol sledovaný vplyv vývojovej fázy rastliny v čase zberu na obsah kyseliny rozmarínovej v droge. Boli pozorované iba mierne rozdiely v obsahu kyseliny rozmarínovej. Maximálny obsah bol v listovej droge získanej zberom vo fáze plného kvitnutia (3,91 %).

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