characterized by similar pathophysiological and pathobiochemical changes as rheumatoid arthritis (RA) in humans. In this study the antirheumatic activity of coenzyme Q_{10} supplementation was tested not only as to its capability to suppress the inflammation edema of the hind paw and to improve the body weight of the arthritic animals, but also to improve so important biochemical parameters as markers of inflammation and oxidative stress, and of mitochondrial bioenergetics. Despite the unfavorable effects on the rheumatic processes observed by monitoring biometric parameters (hind paw volume, relative body weight, relative weight of spleen), a significant protective effect was observed on the level of mitochondrial energetic and antioxidant disbalance. This finding speaks in favor of CoQ_{10} supplementation in rheumatic patients, presumably as combinatory therapy with classical antirheumatics, e.g. NSAIDs.

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SIMULTANEOUS DETERMINATION OF 1,4-NAPHTOQUINONE, LAWSONE, JUGLONE AND PLUMBAGIN BY LIQUID CHROMATOGRAPHY WITH UV DETECTION

Petr Babula[a], Radka Mikelová[b], David Potěšil[b,c], Vojtěch Adam[b,c], René Kizek[b], Ladislav Havel[d], Zdeněk Sladký[a]

[a] *Department of Natural Drugs, University of Veterinary and Pharmaceutical Sciences, Palackého 1-3, CZ-612 42 Brno, Czech Republic*

[b] *Department of Chemistry and Biochemistry and* [d]*Department of Botany and Plant Physiology, Faculty of Agronomy, Mendel University of Agriculture and Forestry, Zemědělská 1, CZ-613 00 Brno, Czech Republic*

[c] *Department of Analytical Chemistry, Faculty of Science, Kotlářská 2, CZ-611 37 Brno, Czech Republic*

e-mail: kizek@sci.muni.cz

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ABSTRACT

Naphthoquinones, compounds of natural origin, mostly appeared as chromatic pigments. They are deposited in cells vacuoles, where they are dissolved (in glycoside form). Naphthoquinones are very toxic, antimicrobial, antifungal, antiviral and antiparasitic effects were observed. The aim of our work was to optimize the high performance liquid chromatography coupled with diode array detector for the determination of naphtoquinones (1,4-naphtoquinone, lawsone; juglone and plumbagin). Detection limits (3 S/N) of naphtoquinones were – lawson 65 $ng.ml^{-1}$, 1,4-naftochinon 50 $ng.ml^{-1}$, juglon 75 $ng.ml^{-1}$ and plumbagin 39 $ng.ml^{-1}$. The optimized method was consequently used for the determination of the mentioned naphtoquinones in plant sample (*Dionaea muscipula*).

INTRODUCTION

Naphthoquinones, compounds of natural origin, mostly appeared as chromatic pigments. They are deposited in cell vacuoles, where are dissolved in form of glycosides[1]. Naphtoquinones as a group of secondary metabolites occur in number of plant families (*Plumbaginaceae, Juglandaceae, Ebenaceae, Boraginaceae, Dioncophyllaceae, Ancistrocladaceae, Iridaceae, Verbenaceae, Scrophulariaceae, Avicenniaceae, Balsaminceae, Bignoniaceae, Gentianaceae, Droseraceae, Nepenthaceae, Lythraceae, Euphorbiaceae*[2-5], fungi and microorganisms (*Streptomyces, Fusarium*)[6]. Biosynthesis of naphtoquinones proceed by way of six biosynthetic pathways[6-9]. It was found that the basic precursor of biosynthesis of majority part of naphtoquinones is shikimic acid.

An anticancer effect of naphthoquinones awakes an interest in determination and characterization of single derivates of 1,2 and 1,4 quinone in biological samples. High performance liquid chromatography with UV detection and electrochemical techniques are the most commonly used method for these purposes[10-13]. In addition de Paiva et. al. used mass spectrometry as a tool for study of plumbagin[14]. On the other hand a just a few scientists occupy themselves with the determination of naphthoquinones in the biological samples[10-14].

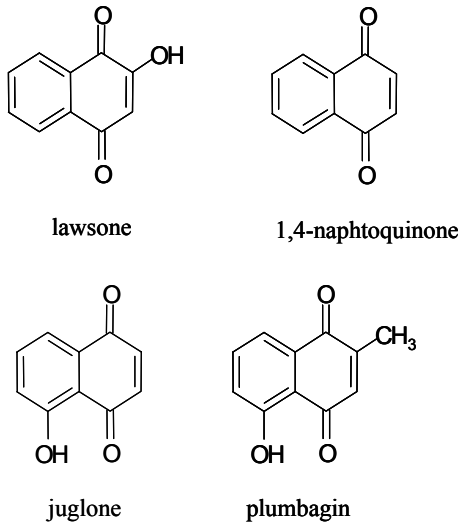

Fig. 1. Chemical structures of 1,4-naphtoquinone, lawsone; juglone and plumbagin.

The aim of this work was to optimize the simultaneous analysis of naphthoquinones (1,4-naphthoquinone, lawsone, juglone and plumbagin; see in Fig. 1) by high performance liquid chromatography coupled with diode array detector (HPLC-DAD). The optimized technique was consequently used for detection and quantification of naphthoquinones in plant material (*Dionaea muscipula*).

MATERIAL AND METHODS

Chemicals

Naphtoquinones (1,4-naphtoquinone, lawsone, juglone and plumbagin) were purchased from Sigma Aldrich Chemical Corp. (St. Louis, USA). Methanol for HPLC and other analytical reagents of ACS purity were purchased from Sigma Aldrich too. Solutions were prepared using deionised ACS water (Sigma). The stock standard solutions of naphtoquinones at 100 μg.ml^{-1} concentration were prepared in ACS methanol and stored in the dark at 4 °C. The working standard solutions were prepared daily by dilution of the stock solutions. All solutions were filtered through a 0.45 μm Teflon membrane filters (MetaChem, Torrance, USA) prior to HPLC separations.

Plant material

Dionaea muscipula Ell. plants were originally established at the Department of Natural Medicinals of the University of Veterinary and Pharmaceutical Sciences in Brno. The plants were cultivated on a 20 ml of Murashige Skoog Medium (MS)(ref.[15]) with addition of sucrose (30 g/l) and of activated carbon (1.5 g/l) in Erlenmayer flasks (100 ml).

The cultivation was performed in a cultivation boxes for 7 days, 14 hours long daylight per a day (maximal light intensity was about 100 μEm^{-2}s^{-1}), at a temperature 26.5 °C and humidity 60–70 %.

HPLC analysis

An HP 1100 liquid chromatographic system (Hewlett Packard, Waldbronn, Germany) was equipped with a vacuum degasser (G1322A), a binary pump (G1312A), an auto sampler (G1313A), a column thermostat (G1316A), and a UV-VIS diode array detector (model G1315B) working at 190–690 nm. The ChemStation software (Rev. A 08.01) controlled the whole liquid chromatographic system. Spectra were registered in the range of 190-400 nm (SBW 100 nm). Chromatograms were registered at 280 nm. Naphtoquinones were separated on a reversed-phase Zorbax C18-AAA chromatographic column (150 mm × 4.6 mm, 3.5 μm particle size, Agilent Technologies, USA) in an isocratic mode. The most effective chromatographic

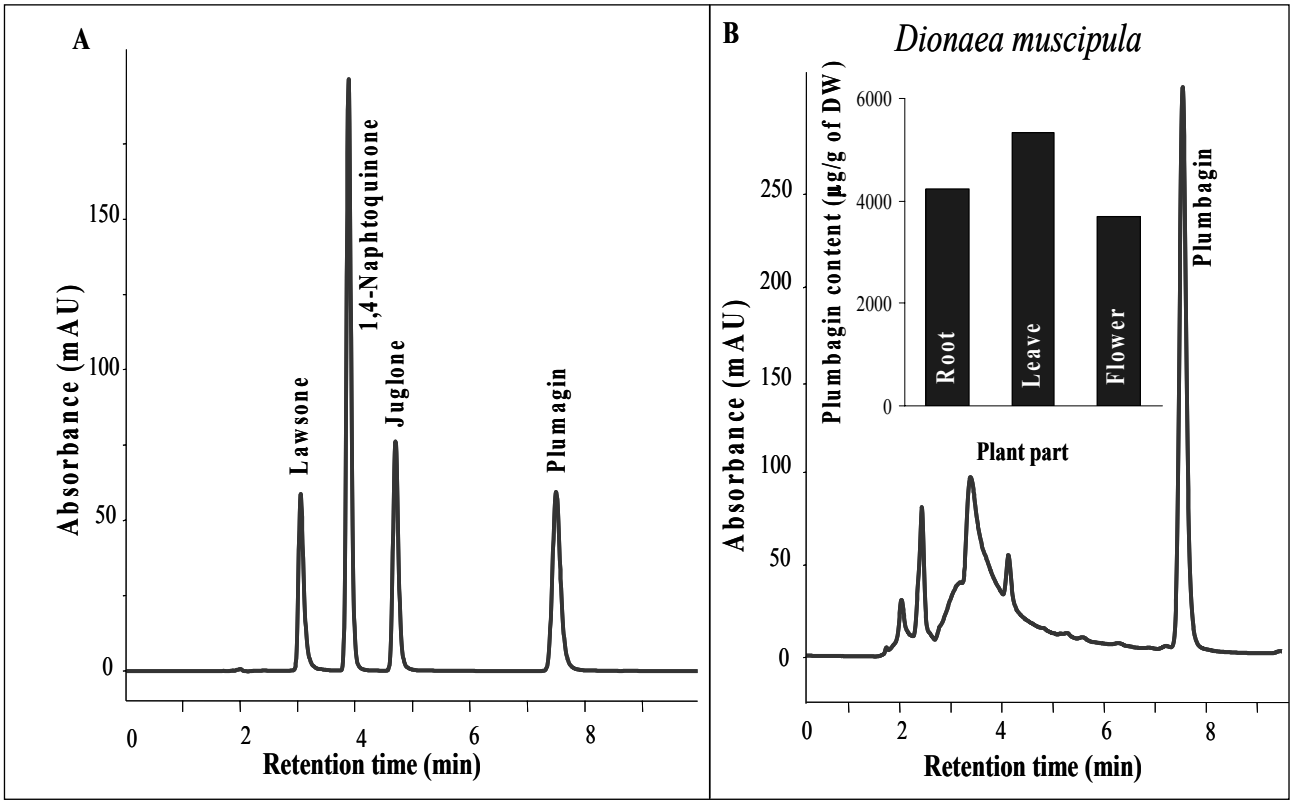

Fig. 2. Simultaneous determination of the naphthoquinones. HPLC-DAD chromatogram of 1,4-naphtoquinone, lawsone; juglone and plumbagin (**A**) and of *Dionaea muscipula* leave (**B**); in inset: content of plumbagin in leave, root and flower of *Dionaea muscipula*. HPLC-DAD conditions were as follows: mobile phase: 0.1 mol.l^{-1} acetic acid:methanol in ratio 35 : 65; flow rate: 0.8 ml.min^{-1}; column and detector temperature: 40 °C; auto sampler injection was 5 μl.

Tab. 1. HPLC-DAD characteristics of analysed naphthoquinones

Naphthoquinone	t_R (min)	Equation[a]	R^2	LOD ($ng.ml^{-1}$)	LOQ ($ng.ml^{-1}$)	R.S.D. (%)
1,4-naftochinone	3.8	y = 47.733x – 9.0435	0.9999	50.5	168.3	1.91
Juglone	4.7	y = 20.756x – 5.6828	0.9998	75.6	252.0	2.25
Lawsone	3.0	y = 15.951x – 5.2675	0.9997	65.3	217.7	2.86
Plumbagin	7.4	y = 24.416x – 6.0176	0.9998	38.5	128.3	1.11

[a] ... The concentration range was from 1.7 to 25.0 $\mu g.ml^{-1}$.

conditions were – mobile phase: 0.1 $mol.l^{-1}$ acetic acid: methanol in ratio 35 : 65; flow rate: 0.8 $ml.min^{-1}$; column and detector temperature: 40 °C. Auto sampler injection was 5 µl.

Sample preparation

Plant tissues were lyophilized at –51°C for 48 h (CHRIST-Alpha 1-2). Then the lyophilized samples were homogenized by an Ika A11 basic grinder (IKA Werke GmbH and Co., Staufen, KG, Germany). The homogenized samples (0.02–0.10 g) were dissolved in 99.999 % methanol and sonicated at the laboratory temperature for 30 min on K5 Sonicator (Czech Republic) at 150 W, 38 kHz. The samples were filtered through a 0.45 µm Teflon membrane filters (MetaChem, Torrance, CA, USA) prior to injection into the HPLC system.

RESULTS AND DISCUSSION

As we mentioned in the Introduction section, naphthoquinones have a broad range of biological action. That is why we aimed on simultaneous determination of naphthoquinones (1,4-naphthoquinone, lawsone, juglone and plumbagin; see in Fig. 1). The chromatogram of the mentioned naphthoquinones obtained at the most effective chromatographic conditions (see Materials and Methods section) is shown in Fig. 2A. The dependence of height and area of DAD signal (254 nm) of naphthoquinones on their concentrations were strictly linear in the range from 1.7 to 25.0 $\mu g.ml^{-1}$, R^2 0.9995 – 0.9999 (Tab. 1). The limit of detection and quantification of naphthoquinones are shown in Tab. 1.

In addition we applied the optimised technique for the determination of naphthoquinones in biological sample – *Dionaea muscipula* (Fig. 2B). To be specific, we analysed the content of naphthoquinones in leaves, root and flower of the mentioned plant specie. We found out that the *Dionaea muscipula* contained only one naphthoquinone – plumbagin. The highest amounts of plumbagin were determined in leaves (5338 $\mu g.g^{-1}$), followed by root (4230 $\mu g.g^{-1}$) and flower (3698 $\mu g.g^{-1}$), see in inset in Fig. 2B. The HPLC-DAD chromatogram of the analysis of *Dionaea muscipula* leave is shown in Fig. 2B.

As we mentioned above, we did not determine any other naphthoquinone, which we studied. In addition the presence of other quinones derivates such as 3-chloroplumbagin, hydroplumbagin 4-O-β-glukopyranoside, diomuscipulone, diomuscinone and 8,8`-biplumbagin in *Dionaea muscipula* has been described[16–20]. The suggested technique could be used for studying of naphthoquinones not only in plant material but also in cell, embryos and food samples.

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