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Optimization and validation of post-column assay for screening of radical scavengers in herbal raw materials and herbal preparations

Raimondas Raudonis[a,*], Lina Bumblauskiene[a,1], Valdas Jakstas[a,2], Audrius Pukalskas[b], Valdimaras Janulis[a,3]

[a] Kaunas University of Medicine, Department of Pharmacognosy, Mickeviciaus 9, Kaunas, Lithuania
[b] Kaunas University of Technology, Department of Food Technology, Radvilenu pl. 19, Kaunas, Lithuania

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ABSTRACT

On-line method, which combines HPLC distribution and post-column reaction, was designed for the search of individual antioxidants. Optimization of the assay was performed evaluating optimal $ABTS^{\bullet+}$ radical cation concentration in the reactor, reaction time, impact of flow rate, reaction coil length. HPLC-ABTS assay validation in this work was performed by assessing reference antioxidant negative peak areas in radical scavenging chromatogram. Sample free radical scavenging activity is expressed as trolox equivalent antioxidant capacity (TEAC). Optimized and validated method was applied in detection of compounds possessing free radical scavenging ability in complex mixtures. Antioxidant compounds were studied in perilla (*Perilla frutescens* (L.) Britton var. *crispa* f. *viridis*) herbal raw material and its preparations. The HPLC-separated antioxidant compounds were identified using HPLC-photodiode array coupled to mass spectrometer, using a reference mass for determining accurate masses. Radical scavenging characteristics of rosmarinic acid, which is the dominant phenolic compound in medicinal herbal raw material of perilla and its preparations, were confirmed by the calculated TEAC values. Compounds responsible for antioxidant effect in herbal raw materials and herbal preparations were identified, evaluated and compared.

1. Introduction

Large quantities of free radicals are produced by exogenous sources, such as ionizing radiation, tobacco smoke, pesticides, air pollutants, pharmaceuticals; also quite a big part of free radicals is continuously produced as by-products in cells of numerous intracellular systems: cytoplasm molecules, membranes ferment systems, peroxisomes, mitochondria electron transmission systems, and microsome electron transmission systems [1–3]. When the protective antioxidant systems of the organism are insufficient, or if there is a lack of intrinsic anti-oxidants, free radicals may cause oxidative stress [4]. Oxidative stress in a human body causes initiation and development of most of the neuro-degenerative, cancerous diseases.

Research of raw herbal materials is the first step in the search of natural antioxidants [5]. Biologically active compounds of the herbal raw materials are distinct for different strength of the effect and different activity mechanisms [6–8]. Phenols of herbal origin are known for their radical scavenging activity [9–13].

Perilla frutescens (L.) Britton var. *crispa* f. *viridis* is an annual medicinal herbal plant of deadnettle (*Lamiaceae* Lindl.) family, originated from East Asia [14]. Rosmarinic acid, caffeic acid, luteolin, apigenin, scutellarein glycosides, triterpenic acids, which are main biologically active compounds accumulated by perilla herb, determine the most important pharmacological affects of the perilla raw material [8]. Many scientific studies have been carried out, and antimicrobial, immunostimulating, antiallergic, antitumor effects of *Perilla frutescens* L. preparations have been determined [15].

The initial selection of herbal compounds possessing the antioxidant effects is carried out by *in vitro* methods. Spectrophotometric *in vitro* methods are widely applied [6,12,16–22]. They determine total amount of antioxidants and evaluate the total antioxidant activity in the researched complex samples. Most frequently these methods use stable free radicals, thus, 1,1′-diphenyl-2-picrylhydrazyl radical ($DPPH^{\bullet}$) and 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$) are the most common ones. After the reaction with an antioxidant these stable free radicals convert to colorless compounds and the

* Corresponding author. Tel.: +370 67843678.
E-mail addresses: raimondas.raudonis@gmail.com (R. Raudonis), lina3b@gmail.com (L. Bumblauskiene), Valdas.jakstas@med.kmu.lt (V. Jakstas), audrius.pukalskas@ktu.lt (A. Pukalskas), farmakog@kmu.lt (V. Janulis).
[1] Tel.: +370 68241377.
[2] Tel.: +370 67200844.
[3] Tel.: +370 69802919.

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decrease of absorption is recorded in the visible light wave spectrum [23,24]. Radical scavenging ability is evaluated according to alteration in absorption and evaluated by standard estimates [24].

The main problem of natural antioxidants is that they are not pure substances, and there is a lack of data on the safety use [25]. The original nature of active compounds does not guarantee safe effect on organism's systems [26]. It is necessary to perform efficacy and safety researches of natural antioxidants [27]. Here arises the need for the systems that would help to distinguish, evaluate and, if possible, to identify individual compounds possessing antioxidant activity in complex samples. Once the antioxidant active substance is determined and the scavenging power of radicals is assessed according to standard estimates, the performance of efficacy and safety researches inside *in vivo* systems becomes possible.

Currently the on-line methods, which combine HPLC distribution and post-column reaction, are designed for the search of individual antioxidants. The greatest advantages of on-line methods are their selectivity, informatory capability and high sensitivity for precise determination of antioxidant-active compound and evaluation of its activity in complex compounds. These systems could also be applied to assess the quality of herbal raw materials, food supplements and herbal preparations. Manufacturers straightforwardly present numerous food supplements and herbal preparations as a source of antioxidants; however, there is no reliable methodology for assessing the antioxidant activity, safety and stability control during the release.

The objective of this work is (1) to develop and validate the on-line HPLC-ABTS assay; (2) to apply the latter in detection of compounds possessing the radical scavenging ability in complex mixtures; (3) to evaluate and compare the antioxidant activity of separate compounds; (4) to identify the compounds responsible for antioxidant effect in herbal raw materials and herbal preparations.

2. Materials and methods

2.1. Materials

All the used solvents are of HPLC grade. Methanol (99.9%) was purchased from Sigma–Aldrich GmbH (Buchs, Switzerland), acetic acid (99.8%) from Fluka Chemie (Buchs, Switzerland). Ultrapure water (18.2 $m\Omega\,cm^{-1}$) was prepared by Millipore (USA) water purification system.

The following reagents were used: 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, 99%), potassium persulfate (99%) from Fluka (Buchs, Switzerland); potassium permanganate (99%), potassium chloride (99.5%), potassium phosphate (99.5%), chlorogenic acid (98%), rosmarinic acid (96%) and caffeic acid (98%) which were delivered from Sigma–Aldrich Chemie GmbH (Steinheim, Germany); (R)-6-methoxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox®, 97%) from Acros Organics (New Jersey, USA); ethanol (96.3%) from Stumbras (Kaunas, Lithuania); sodium citrate (p.a.) and citric acid (p.a.) from Roth (Karlsruhe, Germany).

2.2. Sample materials

Perilla frutescens (L.) Britton var. *crispa* f. *viridis* medicinal herbal raw material was prepared in the collection of medicinal plants trial area in Kaunas Botanical Garden of Vytautas Magnus University in August. The raw material was air dried at room temperature (20–25 °C) in well ventilated room, protected from direct sun rays. Loss on drying was determined by the method indicated by European pharmacopoeia. Data of the study was recalculated for absolute dry herbal raw material by assessing the received data obtained from loss on drying samples.

The dry extract (10:1) of *Perilla frutescens* (L.) Britton var. *crispa* f. *viridis* was produced by extracting the perilla medicinal raw material with water according to percolation method. One centimeter thickness layer of extract frozen on the surfaces of the sublimation vessels in the rotating freezing module of the liophilisator Freeze Dryer FD8512S (ilShin® Europe, Ede, The Netherlands), and then dried in this liophilisator at 5 milithorium pressure (condenser temperature −85 °C).

The following herbal preparations were obtained commercially: *Perilla frutescens* (L.) Britton concentrated leaf extract (5:1) (CLE) and capsulated herbal preparation (CHP). One capsule of capsulated extract contains 150 mg standardized *Perilla frutescens* (L.) Britton leaf extract, which is equivalent to 8 g of fresh perilla leaves.

2.3. Preparation of sample solutions

Reference compounds (trolox 80 μM, rosmarinic acid 110 μM, caffeic acid 60 μM, chlorogenic acid 150 μM) were dissolved in 96.3% ethanol.

Air-dried *Perilla frutescens* (L.) Britton var. *crispa* f. *viridis* medicinal raw material was crushed to particles passing through the 355 μm sieve. About 0.5 g (precise weight) of crushed perilla medicinal raw material was weighed and extracted with 50 ml 60% (V/V) ethanol.

The samples of *Perilla frutescens* (L.) Britton var. *crispa* f. *viridis* dry extract (10:1) and of concentrated *Perilla frutescens* (L.) Britton leaf extract (CLE) (5:1) were prepared by dissolving 0.1 g (precise weight) of dry extract powder in 25 ml of water. Herbal preparation capsules were taken apart, 3 g of powder was weighted and dissolved in 25 ml of water. The concentrations of herbal preparations samples were selected according to minimum detectible concentrations (MDC) and minimum detectible amounts (MDA) of standard compounds. All samples were filtered through 0.22 μm pore size membrane filters (Carl Roth Gmbh, Germany) and 20 μl of the sample solution was injected for HPLC-ABTS analysis.

2.4. HPLC-ABTS radical scavenging system

The principle diagram of on-line HPLC-ABTS assay is shown in Fig. 1. Linear binary gradient is formed by chromatograph Beckman Programmable Solvent Module 126 (Fullerton, USA) at constant flow rate of 1 ml/min. Solvent A is 0.5% acetic acid, solvent B is methanol. Changes in gradient: 0 min, 90% A and 10% B; 40 min, 20% A and 80% B; 50 min, 10% A and 90% B; 52 min, 0% A and 100% B; 53 min, 90% A and 10% B; 60 min, 90% A and 10% B. Samples are injected by Rheodyne 7125 manual injector (Rheodyne, RohnertPark, CA) with 20 μl injection loop. Analytes are distributed by ACE C18 analytic column (5 μm, 150 mm × 4.6 mm) fitted with guard column ACE 5 μm C18 (Aberdeen, Scotland). The distributed compounds are detected by UV absorption detector Beckman System Gold 166 Programmable Detector Module (Fullerton, USA) at 290 nm wavelength. After the detection the eluent is directly mixed with $ABTS^{\bullet+}$ reagent solution for the assessing the scavenging of free radicals. 110 μM (A_{650} = 0.90 AU) of $ABTS^{\bullet+}$ working solution is supplied to the post-column at constant 0.5 ml/min flow rate by continuously working pump Waters Reagent Manager (Milford, USA). Reaction between $ABTS^{\bullet+}$ radical cation and analyte happens in the reaction coil. The following reaction coils were used: 3 and 15 m (0.3 mm ID and 1.58 mm OD) made of TFE (Teflon®) tube, 15 and 30 m (0.25 mm ID and 1.58 mm OD) made of PEEK (polyetheretherketone) tube. The decrease of reaction mixture absorption after reaction with antioxidant is directly recorded at 650 nm wavelength by UV/Vis type detector Gilson 118 (Middleton, USA). The compounds possessing antioxidant activity are recorded as negative peaks in radical scavenging (RS) chromatogram. Data received from experimental research is processed