Phytochemistry Letters 20 (2017) 415–424

Contents lists available at ScienceDirect

Phytochemistry Letters

journal homepage: www.elsevier.com/locate/phytol

Optimisation of preparative HPLC separation of four isomeric kaempferol diglycosides from *Prunus spinosa* L. by application of the response surface methodology

Aleksandra Owczarek[a,*], Anna Magiera[a], Magdalena Matczak[a], Dorota Gabriela Piotrowska[b], Monika Anna Olszewska[a], Anna Marchelak[a]

[a] Department of Pharmacognosy, Faculty of Pharmacy, Medical University of Lodz, 1 Muszynskiego St., Lodz 90-151, Poland
[b] Department of Bioorganic Chemistry, Faculty of Pharmacy, Medical University of Lodz, 1 Muszynskiego St., Lodz 90-151, Poland

ARTICLE INFO

Article history:
Received 24 November 2016
Received in revised form 9 January 2017
Accepted 24 January 2017
Available online 5 February 2017

Keywords:
Prunus spinosa
Preparative HPLC
Kaempferol 3-O-β-D-xylopyranoside-7-O-α-L-rhamnopyranoside
Central composite design
CCD

ABSTRACT

A fast and efficient preparative HPLC-PDA method was developed for the separation and isolation of four rare isomeric kaempferol diglycosides from leaves of *Prunus spinosa* L. The separation procedure of the enriched diglycoside fraction of the 70% (*v/v*) aqueous methanolic leaf extract was first optimised on analytical XBridge C18 column (100 mm × 4.6 mm i.d., 5 μm) and central composite design combined with response surface methodology was utilized to establish the optimal separation conditions. The developed method was directly transferred to preparative XBridge Prep C18 column (100 mm × 19 mm i.d., 5 μm) and the final separation was accomplished by isocratic elution with 0.5% acetic acid-methanol-tetrahydrofuran (75.2:16.6:8.2, *v/v/v*) as the mobile phase, at a flow rate of 13.6 mL/min, in less than 12 min for a single run. Under these conditions, four flavonoid diglycosides: kaempferol 3-*O*-α-L-arabinofuranoside-7-*O*-α-L-rhamnopyranoside, kaempferol 3,7-di-*O*-α-L-rhamnopyranoside (kaempferitrin), and reported for the first time for *P. spinosa* kaempferol 3-*O*-β-D-xylopyranoside-7-*O*-α-L-rhamnopyranoside (lepidoside) and kaempferol 3-*O*-α-L-arabinopyranoside-7-*O*-α-L-rhamnopyranoside, were isolated in high separation yield (84.8–94.5%) and purity (92.45–99.79%). Their structures were confirmed by extensive 1D and 2D NMR studies. Additionally, the UHPLC-PDA-ESI–MS^3 qualitative profiling led to the identification of twenty-one phenolic compounds and confirmed that the isolates were the major components of the leaf material.

1. Introduction

Prunus spinosa L. (blackthorn or sloe) is a large thorny shrub or small tree widespread in the temperate regions of the northern hemisphere. It has been known since ancient times as medicinal and dietary plant. Its leaves has been used traditionally for various purposes, *e.g.* for the treatment of urinary tract inflammations and in blood cleansing therapies (Berger, 1950). The plant material is a rich source of flavonoid glycosides (up to 4.27% dry weight), comparable to other widely applied flavonoid herbal remedies such as birch leaves (*Betulae folium*) and elder flower (*Sambuci flos*) (Olszewska et al., 2001). Nine different flavonoids have been isolated to date from the leaves of *P. spinosa* including kaempferol, quercetin, their 3-*O*-α-L-arabinofuranosides, kaempferol 3- and 7-*O*-α-L-rhamnopyranosides, kaempferol 3,7-di-*O*-α-L-rhamnopyranoside, kaempferol 3-*O*-α-L-arabinofuranoside-7-*O*-α-L-rhamnopyranoside, quercetin 3-*O*-(2″-*O*-β-D-glucopyranoside)-α-L-arabinofuranoside and kaempferol 3-*O*-(2″-*E*-*p*-coumaroyl)-α-L-arabinofuranoside-7-*O*-α-L-rhamnopyranoside (Olszewska and Wolbiś, 2002a). Some of these compounds, especially mono- and dipentosides, which are characteristic of *P. spinosa*, and relatively rare in nature, could probably be applied as analytical markers in standardisation of plant materials derived from the taxon (Olszewska and Wolbiś, 2001, 2002a,b). However, the typical flavonoid pattern of the leaves still requires full characterisation, as no comprehensive studies on their phenolic profile has been published so far.

The flavonoid fraction of blackthorn leaves was proven in *in vivo* studies to significantly reduce capillary permeability, exhibit anti-inflammatory effects in the animal skin and internal organs, normalize the blood cholesterol and a cholesterol/phospholipid ratio in atherogenic rabbits, display spasmolytic effects on isolated

* Corresponding author.
E-mail address: aleksandra.owczarek@umed.lodz.pl (A. Owczarek).

http://dx.doi.org/10.1016/j.phytol.2017.01.010
1874-3900/

uterine and intestinal segments from different animals, increase the amplitude of heart contractions in perfusion of isolated frog hearts, and demonstrate significant diuretic and natriuretic activity in rats (Lisevitskaya et al., 1970; Makarov, 1972, 1970; Makarov and Khadzhai, 1969). These effects were especially pronounced for kaempferol 3-*O*-α-L-arabinofuranoside-7-*O*-α-L-rhamnopyranoside and kaempferol 3,7-di-*O*-α-L-rhamnopyranoside – activity of which was higher than that of rutin – suggesting that the 3,7-disubstituted kaempferol derivatives might be primary determinants of the activity of *P. spinosa* leaves (Makarov and Khadzhai, 1969). However, despite these promising results, published in the early 70's of the 20th century, further *in vivo* studies on the target flavonoids were hindered by their commercial unavailability and lack of effective methods for isolation from natural sources.

According to our previous studies on *P. spinosa*, the blackthorn leaves appear rich in kaempferol dipentosides, the fraction of which is, on one hand, easy to isolate from the plant due to unique solubility, but extremely difficult to separate into components (due to their considerable structural similarities) on the other. The target fraction was composed of at least four analytes, two of which remain structurally unidentified to date. Moreover, two major constituents of the fraction (kaempferol 3-*O*-α-L-arabinofuranoside-7-*O*-α-L-rhamnopyranoside and kaempferol 3,7-di-*O*-α-L-rhamnopyranoside) were isolated from the leaves but with unsatisfactory yield resulting from low resolution of the applied conventional open column chromatography techniques (Olszewska and Wolbiś, 2002a).

Therefore, the aim of the present study was to develop and optimise the first preparative HPLC procedure for fast and efficient isolation of four isomeric kaempferol dipentosides from the leaves of blackthorn. Since the optimisation of HPLC separations by a trial-and-error method can be inefficient and time-consuming, and does not guarantee the optimal results, it was facilitated by the application of the response surface methodology (RSM) – a systematic experimental design and statistically-assisted construction of a response function describing relationships between the process variables and performance parameters of the chromatographic separation. The optimal conditions, based on isocratic elution with ternary mobile phase, established in an analytical scale, were directly transferred to a preparative one, and successful isolation was then achieved with a run time of 12 min. The purity of isolates and their structures were thoroughly analysed by the spectroscopic (ESI–MS, ^{1}H NMR, ^{13}C NMR, COSY, HMQC, HMBC) studies and hydrolytic experiments. Finally, the qualitative composition of the raw leaf extracts and the target dipentoside fraction was fully characterised by an UHPLC-PDA-ESI–MS^{3} assay with comparison to the currently and previously isolated standards.

2. Results and discussion

2.1. Optimisation of the separation procedure

The target kaempferol diglycosidic fraction was isolated from the leaves of *P. spinosa* according to the simplified procedure described previously (Olszewska and Wolbiś, 2002a), *i.e.* from *n*-butanolic fraction of the defatted 70% (*v*/*v*) aqueous methanolic extract, separated from accompanying polymeric proanthocyanidins and quercetin diglycosides by gel permeation chromatography on Sephadex LH-20. Thus, 2.1 g of the dipentoside fraction was obtained from 100 g of the dried plant material (2.1% yield, *w*/*w*). Before optimisation of the HPLC separation, the qualitative profiles of the flavonoid fraction and mother 70% methanolic extract were evaluated by UHPLC-PDA-ESI–MS^{3} (Fig. 1, Table 1; for detailed description see Section 2.4), which confirmed purity of the fraction and predominant character of its constituents in the plant material.

Although the development of a chromatographic separation procedure can be carried out in any scale, in order to minimize the solvent use, it is often beneficial to optimise the separation on an analytical column and then transfer the established conditions to a preparative one. In such cases, the crucial factor is to use columns with identical chemistry, particle size and, preferably, of the same length. This approach ensures the similar separation power of the columns and allows for maintaining similar resolution between crucial peak pairs (Aubin and Cleary, 2009; Huber and Majors, 2007). In this study, two matching Waters XBrigde C18 columns (10 cm × 4.6 mm i.d. and 10 cm × 19 mm i.d., 5 μm particle size each) were used. Octadecyl silica is a clear choice, as a vast majority of polyphenol separations has been performed using this column chemistry (Stalikas, 2007; Stefova et al., 2003). The selection of column length of 100 mm allowed to shorten the analysis time.

Apart from the column chemistry, the most important factor influencing the effectiveness of the separation is the composition of the mobile phase. In flavonoid separations, binary mobile phases consisting of water with acetic or formic acid and methanol or

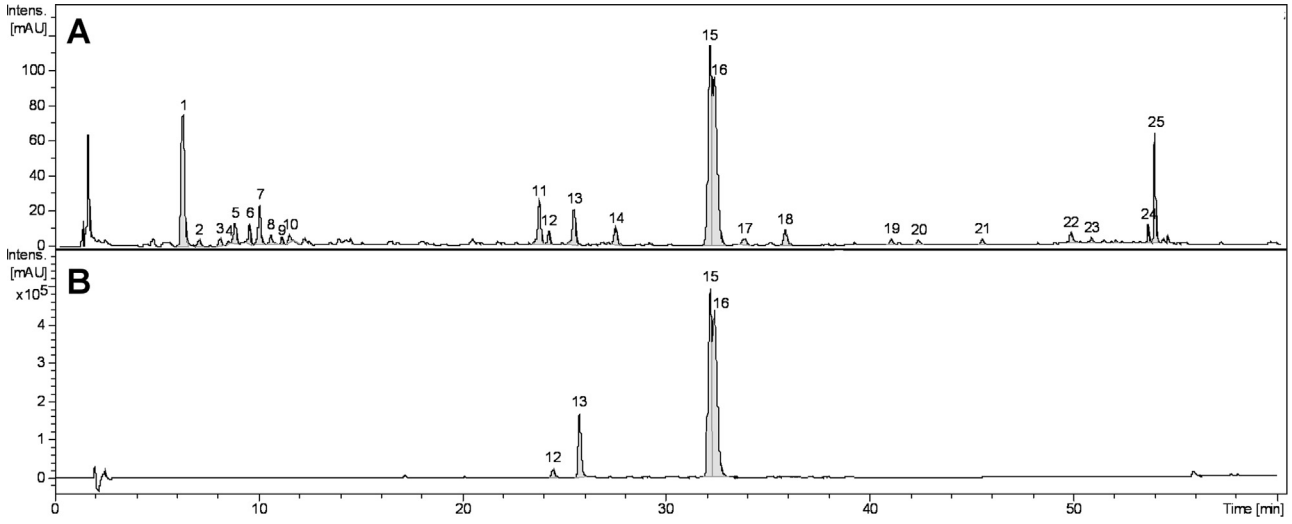

Fig. 1. Representative UHPLC chromatograms at λ = 350 nm of: (**A**) dry 70% (*v*/*v*) aqueous methanolic extract from the leaves of *P. spinosa* (5 mg/mL); (**B**) fraction of kaempferol dipentosides from the leaves of *P. spinosa* (1 mg/mL).