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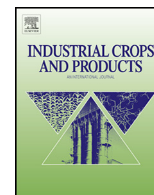

Short communication

Quantitative standarization and CPC-based recovery of pharmacologically active components from *Polygonum tinctorium* Ait. leaf extracts

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ABSTRACT

Polygonum tinctorium Ait. (dyer's knotweed) is a commonly known Asian perennial containing indole alkaloids – indigo and indirubin. Its leaves constitute a pharmacopoeial material (Chinese Pharmacopoeia) known for its antineoplastic, antipsoriatic, antiviral and antibacterial actions. This article presents the results of a detailed study into the optimization of the extraction and separation (CPC-based purification) process, and also the quantitative analysis of the extracts. The applied extraction parameters considered the impact of the extraction temperature, its duration and the solvent selection on the quantitative analysis of both indigo and indirubin. The study concludes that a 30 min long extraction of the leaves, in a 2% solution of chloral hydrate in dichloromethane, was found to be most beneficial and reproductive. The compounds' poor solubility, low stability and high toxicity of the so far used solvents, were some of the challenges encountered. CPC purification by heptane:ethyl acetate:methanol:water (6:1:6:1) in ascending mode led to the most satisfactory results in terms of purity, solvent consumption and duration. The elaborated method can be used as a suitable tool for the detailed quantitative indigo and indirubin standardization in the leaves of the *P. tinctorium*. The indirubin recovery model may serve for further scale-up for industrial applications.

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1. Introduction

Polygonum tinctorium Ait. (dyer's knotweed) is a perennial plant from the Polygonaceae family, which can be found across Southern and Eastern Asia. The leaves are used as colouring agents in the cotton and textile industries due to high content of indigo – and indole alkaloid characterized by a deeply blue color (Hunger, 2007).

Only naturally dyed textiles exhibit antipsoriatic, antifungal and antibacterial properties, therefore they are growing in popularity, in contrast to those textiles colored with synthetic indigo (Islam et al., 2013).

The dying process is performed in the presence of composted leaves with the addition of wheat bran, wood-ash lye and limestone at a temperature of ca. 20 °C and pH exceeding the value of 10, which result in a lasting colorization of fabrics of different kind (Meijer, 2006; Stasiak et al., 2014).

Moreover, *P. tinctorium* leaves constitute a pharmacopoeial material (Chinese Pharmacopoeia (Pharmacopoeia of the People's Republic of China, 2005)) and are described as components of various phytomedical formulations.

Former qualitative and quantitative studies on the composition of *P. tinctorium* leaves indicated the presence of another indigo-like component – indirubin – in the extracts (see Fig. 1S) (Stasiak et al., 2014; Heo et al., 2013). The latter compound was found to exhibit significant pharmacological activities, such as anti-HIV-1 (Zhong et al., 2005), antiproliferative (Kim et al., 2012), anti-inflammatory (Toshio et al., 2000), antileukemic (Iwaki et al., 2011) and insects repelling (Islam et al., 2013) properties. The potential benefits and broad applications of this natural product, justifies the importance of this study and other related research.

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In this context the recovery of pharmacologically active indirubin from the *P. tinctorium* leaves or waste products is crucial.

Current survey resulted in the preparation of a rapid analytical method suitable for the quantity assessment of both indigo and indirubin in the *P. tinctorium* leaf samples. Furthermore, an optimized extraction procedure to control and gain maximum benefit from the content of pharmacologically active metabolites in indigoferous plants, as well as time-saving recovery process of indirubin from the crops were elaborated.

Presented outcomes of a detailed qualitative and quantitative study aimed towards the preparation of a monograph, to the European Pharmacopoeia, but also towards the application of elaborated models in the industrial scale.

2. Materials and methods

2.1. Plant material

The content of indigo and indirubin was assessed in three different *P. tinctorium* leaf samples. Sample A was collected in November 2012 after the first frost from the botanical garden of the Chair and Department of Pharmacognosy within the Medicinal Plant Unit of the Medical University of Lublin, Poland. This sample was cultivated over two years from the seedlings brought from the Tokushima Bunri University Botanical Garden, Japan. Sample B was purchased from Sun Ten Pharmaceutical in Taiwan and Sample C was collected in April 2013, from the vicinity of the Museum of Indigo in Tokushima, Japan. The dried and powdered leaves of all three samples were given respective specimen numbers WK0912002, WK1012002 and WK0513001.

2.2. Loss on drying

The content of water in each sample was determined by drying each sample (1.0 g) in an oven at 105 °C, for 2 h.

2.3. Ash determination

Total ash and ash insoluble in 10% HCl were determined by ashing in a muffle furnace at 550 °C according to the protocol described by Falodun and Irabor (2008), six times for each sample.

2.4. Reagents

Indigo and indirubin standards were purchased from Sigma–Aldrich (St. Louis, USA). Reagent grade solvents used in the extraction process were obtained from Polish Reagents (Gdynia, Poland), HPLC grade methanol was acquired from Merck (Darmstadt, Germany) and a Millipore purification system was used to obtain HPLC water.

2.5. Extraction conditions

Ultrasound-assisted extraction in different solvents (2% solution of chloral hydrate in chloroform, 2% solution of chloral hydrate in dichloromethane, dichloromethane and 2% solution of acetone in dichloromethane) was performed on dried and powdered plant material in an ultrasonic bath (100 kHz) cooled with ice for 10, 20, 30 or 40 min. Extracts from the plant material were subsequently cooled, diluted to volume, mixed, filtered through nylon membrane filters with a diameter of 0.45 μm and subjected to HPLC analysis. Similar dissolution conditions were applied for standard solutions of both indigo and indirubin.

2.6. HPLC/DAD analysis

The quantitative analysis of the obtained extracts was performed on an Agilent 1100 Series (Agilent Technologies, USA) HPLC system equipped with a PDA detector (G1315B), autosampler (G1329A), quaternary pump (G1311A) and a degasser (G1322A). The HPLC elution conditions applied for all of the conducted measurements were: an isocratic run of; 65:35, 70:30, 75:25 or 80:20 (v/v) of methanol in water, developed on a RP-18 silica gel column (Supelco, Sigma–Aldrich 250 mm × 4.6 mm, 5 μm) and a UV-DAD detection in the 260 nm and 290 nm ranges. The spectra were recorded within a wavelength range of 200–500 nm at room temperature.

All injections were performed at a constant interval between the extraction and chromatographic analysis due to the weak stability of indigo dye in the extracts. The identification of peaks was based on standard addition, UV spectra and the retention time of both indigo and indirubin. Preliminary linearity range studies were performed for indigo and indirubin in the concentration range of 20–100 mg/50 mL, in the extracts from three parallel sets of determinations. The limit of detection (LOD) (S/N = 3) was 0.11 μg/mL for indigo and 0.50 μg/mL for indirubin. The limit of quantification (LOQ) (S/N = 10) was 0.37 μg/mL and 1.67 μg/mL, respectively. The reproducibility of the HPLC analysis was assessed by six separate measurements. The relative standard deviation (RSD) of all of the measured samples was calculated to be 6.25%. Furthermore, blank samples were injected to check whether no peaks were eluted in the same retention time.

2.6.1. Standard solution

3.3 mg of indigo and 1.7 mg of indirubin were transferred to a 50 mL volumetric flask, dissolved in 40 mL of a 2% solution of chloral hydrate in dichloromethane and underwent ultrasonication for 30 min.. The solution was then filled to volume using the same solvent, filtered through a 0.45 μm nylon membrane filter (Cronus), transferred into amber glass HPLC vials and subjected to HPLC analysis. Calibration curves for the standard solution were prepared using a serial dilutions method. The obtained solution was diluted from 5 mg to 2.5, 1, 0.5 and 0.25 mg in 50 mL of a 2% solution of chloral hydrate in dichloromethane. 10 μL of each diluted solution were injected, in triplicate to perform HPLC analysis. The obtained calibration curve equations were as follows: $y = 505.8x + 87.057$ with correlation coefficient (R^2) of 0.9946 for indigo and $y = 229.01x - 138.74$ with R^2 of 0.9802 for indirubin over the range applied.

2.6.2. Test solution

The dried and powdered leaves of *P. tinctorium* were weighed and transferred to a 50 mL volumetric flask, dissolved in 40 mL of solvent and underwent ultrasonication for 30 min. The extracts were then filled to volume using the same solvent, stirred, filtered through a 0.45 μm nylon membrane filter (Cronus), transferred into amber HPLC vials and subjected to HPLC analysis. 10 μL from each sample were then injected into a column to facilitate the analysis of each sample. Various sample weights were used for the preparation of extracts (20, 25, 50, 75, 100, and 150 mg in 50 mL of extracting solvent) to find the linearity area and check the influence of concentration on sample solubility.

2.7. Centrifugal partition chromatography (CPC)

The choice of a separation mixture was based on the existing research data (Arizona system) and Sorensen diagrams (Berthod et al., 2005) and was validated by an HPLC analysis (method described above). Separation of extract's constituents was performed in several solvent systems. The partition coefficient values

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