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SHORT COMMUNICATION

OPTIMIZATION OF TEMPERATURE AFFECTED EXTRACTION OF INDIGO DYE IN THE LEAF EXTRACTS OF *POLYGONUM TINCTORIUM* Ait. CULTIVATED IN POLAND – PRELIMINARY STUDIES

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Polygonum tinctorium Ait. (Polygonaceae) known as dyer's knotweed is an annual plant growing in the sandy and loamy soils of China, Japan and Eastern Russia, where it is cultivated for both industrial and pharmacological usage (1).

Indigo - one of its main constituents - has been widely used in traditional chinese medicine, and also, as a natural dye, in the production of denim. Although its synthesis is already known, significant amounts of indigo are still being recovered from naturally occurring plant material for medicinal use, by means of water extraction and alkaline precipitation (2).

Currently, indigo-related compounds are of significant interest both as natural colorants and pharmacologically active compounds. Numerous references treating on their detoxifying, antipyretic, antiviral, antidermatophytic or antinociceptive properties confirm the importance of their recovery (3-5).

The aim of presented study was to analyze the indigo dye content in the leaf extracts of *Polygonum tinctorium* Ait. collected in the late summertime (beginning of September) and late fall (mid October after ground-frost) from the garden of Chair and Department of Pharmacognosy (Medical University of Lublin) located in Poland, to check, whether the production of indigo by plant cells differs depending on the climatic conditions. Up to date, dyer's knotweed has been cultivated for medicinal use mainly in Asia. Satisfactory content of indigo in *P. tinctorium* cultivated in Poland from seedlings could be meaningful and spread the cultivation area of this plant to cooler climatic zones. Seasonal variations in the production of indigo in the Polish specimen were monitored and compared to its content in dried leaf of dyer's knotweed of Chinese origin. Furthermore, the influence of different extraction pattern on indigo content in the leaf extracts was measured.

EXPERIMENTAL

Chemicals and reagents

The standard of indigo CRS was purchased from Sigma Aldrich (St. Louis, MO, USA). Methanol of HPLC grade was from J.T. Baker (Gross-Gerau, Germany), whereas water for HPLC purposes was purified on Millipore water purification system.

Methanol, chloroform, chloral hydrate and dichloromethane (reagent grade) came from Polish Reagents (POCH, Gliwice).

Discovery HS RP 18 (250 mm × 4.6 mm, dp = 5 μm) (Supelco, Sigma Aldrich) HPLC column was employed in HPLC chromatographical separation of extracts.

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Agilent Technologies Series 1100 HPLC/PDA system was applied in the qualitative and quantitative analysis of the extracts, as well as in the preparation of indigo calibration curve.

Plant material

Seedlings of *Polygonum tinctorium* Ait. (in their vegetative form) were brought from Japan, from Tokushima Bunri University garden, authenticated by the authors and subsequently planted in the garden of Chair and Department of Pharmacognosy with Medicinal Plant Unit, Medical University of Lublin, Poland. The study on seasonal variation of indigo was performed after leaf collection in the late summer (beginning of September 2011 – and called a "PT A" sample) and early autumn (mid October 2011 – named as "PT B" sample) – one day after the first ground-frost. The leaves were dried in air oven at 35°C until constant weight to determine the mean dry weight and pulverized into a coarse-grained powder.

Voucher specimens (WK0911001 for PT A and WK1011003 for PT B) were deposited in the same department.

Dried and powdered leaves of *Polygonum tinctorium* Ait. were additionally purchased in Taiwan (Taipei) from Sun Ten Pharmaceutical to constitute the reference for Polish crops and were named a "PT C" sample.

Extraction

Investigated plant material was dried and ground after its collection and extracted using dichloromethane or 2% solution of chloral hydrate in chloroform. The extraction potential of extrahents was confronted based on the indigo peak area obtained in the HPLC analysis. Subsequent HPLC extracts' profiling showed the sample related differences in the indigo content.

The results of extraction and HPLC optimization of the obtained extracts were confronted with those presented in the monograph of *Polygonum tinctorium* Ait. in the Chinese Pharmacopoeia (2005) [6].

Test solution

Forty milligrams of powdered plant material was suspended in 8 mL of extrahent and ultrasonicated in a water bath (power 250 W, frequency 33 kHz) at 30°C for 90 min in a 10 mL volumetric flask. The extract was allowed to cool, then was diluted to volume and mixed well. The obtained bright blue extract was filtered through a membrane filter in the end (nominal pore size 0.45 μm, Waters) and subjected to HPLC analysis.

Described extraction procedure was performed for PT A, PT B and PT C samples of leaves of *Polygonum tinctorium* Ait. and repeated three times using fresh plant material each time.

Two percent solution of chloral hydrate (previously dried over silica gel in a dessicator) in chloroform and dichloromethane were used as extrahents. Their extraction potential was confronted based on intensities of indigo peaks obtained in the HPLC analysis.

Subsequent HPLC extracts' profiling showed the sample related differences in the indigo content.

Reference solution

Twenty five milligrams of *indigo CRS* was diluted in 250 mL of dichloromethane R and ultrasonicated in a water bath (power 250 W, frequency 33 kHz) in 30°C for 90 min in a volumetric flask. Afterwards, it was allowed to cool, diluted in dichloromethane to volume and mixed well. The obtained solution was filtered through a membrane filter (nominal pore size 0.45 μm) and subjected to further HPLC assay.

HPLC/DAD profiling of extracts

All derived extracts were redissolved in methanol (HPLC grade) and subjected for RP-HPLC-DAD analysis. More specifically, an HPLC system (Agilent Series 1100) was employed for the profiling of the extract and consisted of a pump, a degasser, autosampler, and a PDA detector. ChemWin software was used for the management of data. For phytochemical profiling an RP 18 (250.0 mm × 4.0 mm, 5.0 μm) Discovery-Sigma Aldrich column was used and the injection volume was 10 μL. The mobile phase consisted of water (solvent A) and methanol (solvent B). Optimization of HPLC separation of PT A, PT B and PT C extracts was performed using different isocratic runs and methods' run time. The elution conditions employed in the search for the best separation system were: B/A (6 : 4), (7 : 3), (8 : 2) with flow rate of 1 mL/min, as well as B/A (7 : 3) with flow rate of 1.5 mL/min. The method run time was set at 40 min at first, the post time at 3 min and the chromatograms were recorded at 260 and 290 nm by monitoring spectra within the wavelength range of 200-500 nm, at room temperature (25°C). Qualitative and quantitative analysis of indigo content in the obtained extracts was performed after triplicate injections of each sample.

Calibration curve

Basing on Chinese Pharmacopoeia monograph, the calibration curve of indigo reference solu-

tion was developed by subsequent injections of different volumes (1, 4, 8, 12, 16 and 20 μL) [6]. The calibration curve was established based on triple injections data. The HPLC method was set as for test solutions.

Statistical analyses

The data of the experimental observations (the mean of values (x), standard deviation (SD) and Student *t*-test) were computed using STATISTICA version 6 software of StatSoft Inc., Tulusa, OK, USA and Microsoft Office Excel 2003. The p-value of 0.05 was set as a limit for statistically significant difference in the studies.

RESULTS

RP-HPLC conditions adjustment

Methanol : water (7 : 3) isocratic separation within 20 min, with 1 mL/min flow rate, three-

Table 1. Averaged values of indigo peak areas obtained after chloroform and dichloromethane extractions for PT A, PT B, and PT C samples.

Extrahent	Peak area		
	PT A	PT B	PT C
Dichloromethane 1	409.68	201.30	294.33
Dichloromethane 2	400.44	196.57	293.97
Dichloromethane 3	374.89	181.61	283.80
Dichloromethane 4	376.30	189.35	283.54
Average	390.33	192.21	288.91
Standard deviation	17.44	8.61	6.06
2% sol. of chloral hydrate in chloroform 1	718.13	468.33	659.87
2% sol. of chloral hydrate in chloroform 2	724.28	468.25	656.56
2% sol. of chloral hydrate in chloroform 3	744.66	504.39	714.10
2% sol. of chloral hydrate in chloroform 4	739.85	505.83	718.72
Average	731.73	486.70	687.32
Standard deviation	12.57	21.27	33.68

Table 2. Indigo content in the investigated samples (PT A, PT B and PT C). DCM – dichloromethane extraction, CP – extraction with 2% solution of chloral hydrate in chloroform.

Extrahent	PTA		PTB		PTC	
	Sample weight [mg]	% indigo content	Sample weight [mg]	% indigo content	Sample weight [mg]	% indigo content
DCM 1	39.25	0.96	39.92	0.57	39.18	0.75
DCM 2	39.12	0.94	39.87	0.57	39.21	0.75
DCM 3	40.30	0.87	41.10	0.52	43.20	0.66
DCM 4	38.70	0.91	38.80	0.57	43.30	0.66
Average	39.34	0.92	39.92	0.56	41.22	0.71
CP 1	40.73	1.46	39.29	1.06	38.07	1.45
CP 2	40.92	1.46	39.25	1.06	39.02	1.41
CP 3	40.31	1.52	39.99	1.11	36.68	1.61
CP 4	41.10	1.48	40.04	1.11	36.63	1.62
Average	40.77	1.48	39.64	1.09	37.60	1.52

minute-long postrun and the analysis detection at 290 nm was found to be the most satisfactory one. Under the above conditions, the peak of indigo was observed with the retention time of 9.4 min. An increase in the flow rate up to 1.5 mL/min moved the retention time of indigo to 8.0 min. Further decrease in system's polarity (B/A: 8 : 2) changed the retention time of this alkaloid to 6.2 min, which remains in contrast to the pharmacopoeial result of 14.0 min for the B/A ratio of 6 : 4.

Similar conditions were applied in the preparation of indigo standard calibration curve. The identification of indigo dye in the extracts was performed using the comparison of both retention time (R_t = 9.4 min) and UV spectrum with a standard (λ_{max} = 245, 296, 335).

Extraction efficacy

Calibration curve

The calibration curve for indigo was prepared by an external standard method. It was linear and reproductible, which is confirmed by its correlation coefficient (R) of 0.9978. The coefficient of variability (standard deviation/mean × 100%) [7] values for indigo after 4 repetitive measurements were ranging between 1.8 and 4.9% for both extrahents and chosen samples, which shows good repeatability of the presentented HPLC assay. The calibration curve equation is as follows:

$$y = 141.64x - 122.91$$

Test solutions

The extraction process was performed on three plant samples: PT A, PT B and PT C in quadruple. Each extract was injected on HPLC column in triplicate. The indigo content in the obtained extracts was different. Table 1 shows the averaged values of peak areas obtained for all the prepared extracts.

Indigo peak areas in the sample PT A extracted with dichloromethane (DCM) range around 390.3, whereas those extracted with 2% chloroform solution of chloral hydrate (CP) – around 731.7. Higher quantity of indigo in the chloroform extracts may be observed in the PT B and PT C samples as well, and they average to 192.2 (DCM), 486.7 (CP) for PT B and 288.9 (DCM) and 687.3 (CP) for PT C.

Percentage content of indigo dye in the tested dyer's knotweed's leaf extracts is shown in Table 2. It presents the final results, which state, that the indigo content in the investigated samples varies significantly. Sample PT A extracted with DCM contained 0.92% of indigo, whereas extracted with 2% chloroform solution of chloral hydrate: 1.48% of this coloring agent. Indigo % (0.56 and 1.09%) for DCM and CP extraction, respectively, were calculated for PT B, and 0.71 and 1.52% for PT C.

DISCUSSION AND CONCLUSIONS

Optimization of HPLC conditions

This study shows the efforts which were focused on the development of a modification of pharmacopoeial HPLC method with better resolution and precision, which could be applicable to the analysis of secondary metabolites of *Polygonum tinctorium*. According to Chinese Pharmacopoeia, standard HPLC separation should be performed in the isocratic system: methanol : water (6 : 4). No additional data concerning the run's time were proposed. Based on the chromatograms obtained according to Chinese Pharmacopoeia suggestions, it was concluded that the conditions were characterized by poor efficacy. It resulted in both: higher analysis costs and time consumption. For this purpose, the optimization of HPLC method based on isocratic elution was carried out by changing runs' time and methanol concentrations. Best separation conditions were obtained for the gradient of methanol : water (B/A) 7 : 3 and flow rate of 1 mL/min. At this optimized conditions, the indigo peak resolution was found satisfactory compared to other trials. Furthermore the analysis time was shortened up to 20 min.

Although the application of a mixture B/A (8 : 2) led to shorter runs (the retention time of indigo dye was 6.2 min), the peaks' purity was not satisfactory. Similar observation was made in case of higher flow rate (1.5 mL/min). The selectivity of these systems decreased significantly.

The modification of the pharmacopoeial HPLC method presented above seemed to be an improvement in the isocratic separation of secondary metabolites present in the leaf extracts of *Polygonum tinctorium* in terms of both analysis time and resolution.

Extraction efficacy

According to statistical analysis, the differences between PT A and PT B samples are statistically significant. Similar results were obtained for relations of PT A and PT C, as well as PT B and PT C extracts with $p < 0.05$.

On the basis of the above data, indigo content in the investigated samples varied. Chloroform occurred to be a stronger extrahent than dichloromethane. The amounts of indigo in chloroform extracts were twice as high as in dichloromethane ones. The latter, even if less toxic,

remained a weaker extrahent. Furthermore, the differences between extrahents were statistically significant with $p < 0.05$.

PT A extracts coming from the leaves of *Polygonum tinctorium* Ait. grown in Poland were found to deliver the most rich in indigo, among tested samples, dichloromethane extracts. Their chloroform solutions contained a comparable amount of this coloring agent to a Chinese sample (PT C).

The received data are of significant value, as they may lead to a conclusion, that Polish climatic zone as well as soil conditions allow to obtain similarly rich plant material and may turn into advisory *Polygonum tinctorium* Ait. cultivation areas.

Interesting remarks may be done considering the PT B extracts. Ground frost and possibly lower amount of sunlight may decrease the indigo content of 39% in dichloromethane extracts and 17% in chloroform extracts.

Presented preliminary results shed new light on *Polygonum tinctorium* Ait. cultivation. According to the obtained observations, the plant may be cultivated in colder climatic zones – in Poland – successfully. According to HPLC chromatograms, the concentration of indigo – the main active metabolite from *Polygonum tinctorium* Ait. – collected in Poland was similar to the content in Chinese samples. It is of high importance to collect the leaves before first ground frost, because the indigo content diminished in lower temperatures significantly.

Finally, current paper modified the suggested by Chinese Pharmacopoeia quantitative analysis of *Polygonum tinctorium* Ait. metabolites employing the HPLC assay and compared the extraction efficiency of chloroform and dichloromethane. The latter was found to be a weaker extrahent for indigo dye.

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