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ORIGINAL PAPER

Antioxidant and binding properties of methanol extracts from indigo plant leaves

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This study was conducted to clarify the effect of ultra-pressure treatment on the extraction of bioactive compounds from indigo plant leaves (*Polygonum tinctorium* Lour.) and on their properties. Leaves were harvested the in mid-August, 2013, from Naju City (Korea), and treated using two methods: ultra-pressure (550 MPa, 3 min) and hot-air (70°C, 24 h). Then, the leaves were ultrasonically extracted with methanol. The content of indirubin in leaves treated with ultra pressure and hot air was (535.55 ± 26.14) mg kg^{-1} and (52.63 ± 6.45) mg kg^{-1}, respectively, and that of tryptanthrin was (165.55 ± 8.74) mg kg^{-1} and (153.00 ± 7.62) mg kg^{-1}, respectively. Polyphenolic content in the leaves extract was (127.24 ± 13.67) mg kg^{-1} after the ultra-pressure and (88.22 ± 5.33) mg kg^{-1} after the hot-air treatment. The content of flavonoids was (2298.67 ± 83.27) mg kg^{-1} after the ultra-pressure and (3224.00 ± 21.45) mg kg^{-1} after the hot-air treatment. Di(phenyl)-(2,4,6-trinitrophenyl)iminoazanium (DPPH) radical scavenging activities of the indigo extract obtained by ultra-pressure and hot-air treatment methods at the concentration of 1 mg mL^{-1} were (80.25 ± 0.73) % and (66.54 ± 2.35) %, respectively, and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) radical scavenging activities were estimated as (90.14 ± 0.79) % and (64.45 ± 8.97) %, respectively. The methanol leaf extract after ultra-pressure treatment exhibited higher binding properties to human serum albumin in comparison with catechin and conventional treatments. Consequently, it was assumed that the ultra-pressure treatment is an effective method for the extraction of bioactive compounds from indigo leaves.

Keywords: indigo leaves, bioactive compounds, antioxidant activity, binding properties

Introduction

There are many herbs used for medicinal purposes (Abdel-Sattar et al., 2011; Kim & Song, 2011) and one of them is indigo (*Polygonum tinctorium* Lour., an annual herbaceous plant of *Polygonaceae*), which originates from Central Asia and China (Kim et al., 2012). Traditional people in Korea, China and Japan had made good use of indigo plants as blue dyeing and drug materials thousand years ago (Heo et al., 2012). In traditional oriental medicine, indigo leaves were used as an anodyne, anti-febrile, detoxicant and

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anti-inflammatory agent (Heo et al., 2012, 2013). The main compounds present in the leaves are indigo, indirubin, tryptanthrin and kaempferol (Iwaki et al., 2011). Indirubin is a crimson anti-tumor agent with anti-microbial and anti-cancer activities against osteomyelitis and leukosis (Kim et al., 2013a, 2013b). Tryptanthrin is well known as an effective material for its atopic allergy and anti-inflammatory activity (Recio et al., 2006). The research on the indigo plant extracts was mainly accomplished in the field of antifungal activity (Iwaki et al., 2006), extracting solvents and bioactivity (Heo et al., 2013). Bioactivity of indigo plants varies using different extracting solvents. Consequently, extracting methods are very important when increasing the recovery rate of useful materials present in the indigo plants (Fernández-Agulló et al., 2013; Monrad et al., 2012; Sánchez-Burgos et al., 2013). Therefore, this study was conducted to examine and to compare the effect of ultra-pressure and hot-air treatment methods on the amounts of bioactive compounds, and on their antioxidant and binding properties.

Experimental

General

Indigo leaves (*Polygonum tinctorium*) were grown and harvested in Naju City (Korea) in mid-August, 2013. They were dried and treated by the ultra-pressure and hot-air method. Pressure of 550 MPa was applied for 3 min using ultra pressure machinery (QFP 35L, Avure technologies, Sweden) to fresh leaves. Or, fresh indigo leaves were spread out to dry at 70 ℃ for 24 h following their harvest.

Determination of indirubin and tryptanthrin contents

Samples (20 g) were put in 100 mL of methanol (70 %), extracted in an ultrasonic equipment (UC-20; Jeio Tech, Korea) for 1 h and concentrated in a water bath at 50 ℃. Samples were dissolved in 100 mL of distilled water and lyophilized (Bondiro, Iishin, Korea). Experimental solutions prepared by mixing of acetonitrile and DMSO ($\varphi_r = 1 : 2$), and an addition of 0.3–0.5 g of lyophilized indigo extracts, were kept in a dark room for five days and then filtered at room temperature. HPLC was used for the determination of indirubin and tryptanthrin contents (1,200 Series from Waters, UK; Waters Spherisorb 5μm ODS2 (4.6 mm × 250 mm)). Indirubin was determined in a methanol (70 %; 1 mL min^{-1}) solution at 540 nm and tryptanthrin in a methanol (47 %; 1 mL min^{-1}) solution at 251 nm using a UV detector (Zhou et al., 2013). Standard sample used in this experiment to analyze the indirubin and tryptanthrin was purchased from Sigma (USA; 98 % purification).

Determination of polyphenol compounds

Samples (25 μL) were put in 48 wells after dilution with 5 mg mL^{-1}, and 50 μL of the Folin–Ciocalteu reagent (FCR; Sigma) were added. Samples were prepared standing for 3 min and mixed with 2 mass % sodium carbonate (1 mL). Composition of the samples was analyzed on an enzyme immunity equipment at 759 nm. Standard material was chlorogenic acid (CHA) at 5 μL mL^{-1}, 10 μL mL^{-1}, 25 μL mL^{-1} and 50 μL mL^{-1} (Filipiak-Szok et al., 2012).

Determination of flavonoids content

The volume of 25 μL of each indigo sample was put in 48 wells at 37 ℃ for 1 h after dilution with 5 mg mL^{-1}, and addition of 500 μL of diethylene glycol (Sigma) and 50 μL of 1M NaOH (Sigma), the enzyme immunity equipment at 420 nm was used for the measurement. Quercetin (QUE) at 10 μL mL^{-1}, 25 μL mL^{-1}, 50 μL mL^{-1} and 100 μL mL^{-1} was used as the standard material.

Determination of antioxidant activities

To 10 μL of a sample, 500 μL of di(phenyl)-(2,4,6-trinitrophenyl)iminoazanium (DPPH) solution (8 mg 100 mL^{-1}) were added and it was kept at room temperature for 30 min. DPPH radical scavenging activity was measured at the absorbance of 517 nm using a microplate spectrophotometer.

The same amount of 2.45 mM potassium persulfate (Sigma) was added to 7 mM, 2′-azinobis(3-ethylbenzthiazoline-6-sulphonic acid) radical ($ABTS^{\bullet+}$; Sigma) and kept it in a dark room from 12 h to 16 h. Samples were diluted 17 times until the absorbance was reached 0.7 μL, and 10 μL of the samples were mixed with 500 μL of the $ABTS^{\bullet+}$ solution. ABTS radical scavenging activity was measured at the absorbance of 734 nm using the microplate spectrophotometer.

Reducing activity

To the samples (880 μL) of 0.2 M potassium phosphate buffer (Sigma) at pH 6.6 and 100 μL of 1 mass % $K_3Fe(CN)_6$ (Sigma) were added and the solution was kept in a 1.5 mL tube for 20 min at 50 ℃. Then, 100 μL of 10 vol. % trichloroacetic acid (TCA) were added. The mixture was centrifuged at 3000 min^{-1} for 10 min. To 250 μL of supernatant, 250 μL of diluted solvent (3 × 10 mL) reacted with 5 μL of 10 mass % $FeCl_3$ (Sigma) were added and the reducing activities were measured at the absorbance of 700 nm using the microplate spectrophotometer.

Fluorometric measurements

Fluorometric measurements (FL) were used for the evaluation of the binding properties of indigo plant extracts to human serum albumin. Two dimensional (2D-FL) and three dimensional (3D-FL) fluorescence measurements for indigo extracts at the concentration of 0.01 mg mL^{-1} were recorded on a model FP-6500, Jasco spectrofluorometer (serial N261332, Japan), equipped with 1.0 cm quartz cells and a thermostat bath. The 2D-FL measurement was done at the emission wavelengths from 310 nm to 500 nm and the excitation of 295 nm. The 3D-FL spectra were collected with subsequent scanning emission spectra from 250 nm to 500 nm at 1.0 nm increments by varying the excitation wavelength from 200 nm to 350 nm at 10 nm increments. Catechin (Sigma) was used as the standard. All solutions for protein interaction were prepared in 0.05 mol L^{-1} Tris-HCl buffer (Sigma; pH 7.4) containing 0.1 mol L^{-1} NaCl. The final concentration of human serum albumin (HSA) was 2.0×10^{-6} mol L^{-1}. HSA was mixed with catechin in the proportions of HSA/extract (φ_r = 1 : 1).

Statistical analyses

To verify the statistical significance, mean ± SD of five independent measurements were calculated. Differences between the groups were tested by two ways ANOVA. In the assessment of the antioxidant activity, Spearman correlation coefficients (R) were used. Linear regressions were also calculated and p-values of < 0.05 were considered significant.

Results and discussion

Indirubin content

Fresh indigo leaves were naturally dried and freeze dried after the ultra-pressure treatment. The content of indirubin extracted from the indigo leaves by ultrasonic methods after natural drying and freeze drying were (535.55 ± 26.14) mg kg^{-1} and 1170.46 mg kg^{-1}, respectively (Fig. 1).

The content of indirubin in the indigo leaves dried naturally and freeze dried after hot water treatment was (52.63 ± 6.45) mg kg^{-1} and (87.54 ± 4.25) mg kg^{-1}, respectively (Fig. 1). Indirubin has been well known in traditional medicine for treating leucosis (Kim et al., 2013b). Derivatives of indirubin, indirubin-3′-oxime (IO), are effective in the treatment of regressive brain diseases (Xie et al., 2004). Consequently, the increase in the recovery rate of indirubin is very important. In this study it was found that the amount of indirubin increased after the ultra-pressure pre-treatment. And it is necessary to further determine the optimum treatment pressure and time to achieve the increase in the amount of the indirubin extracted.

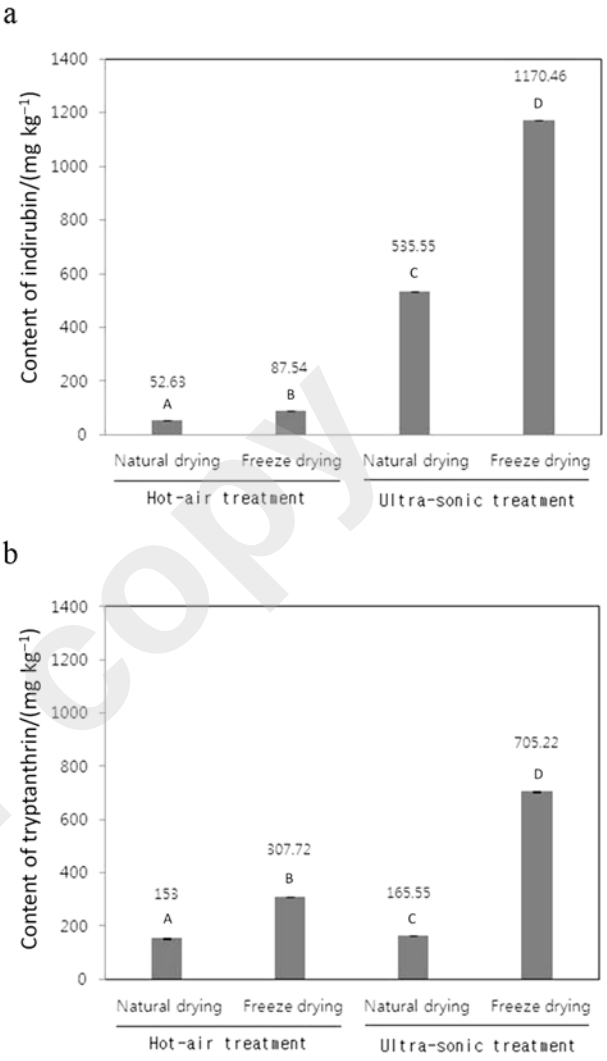

Fig. 1. Effects of pre-treatment and drying methods on the content of indirubin (a) and tryptanthrin (b) in the ultrasonically treated indigo extract. All values are mean ± SD. A–D – means with different letters in the bars significantly differ at $p < 0.05$ as obtained by the Duncan's multiple range test.

Tryptanthrin content

The content of tryptanthrin extracted from fresh indigo leaves by ultrasonic methods after the ultra-pressure treatment at 550 MPa and natural or freeze drying was (165.55 ± 8.74) mg kg^{-1} and (705.22 ± 35.32) mg kg^{-1}, respectively (Fig. 1).

Tryptanthrin content in the leaves dried naturally after hot-air treatment was (52.63 ± 6.45) mg kg^{-1}; however, freeze dried leaves after hot-air treatment contained (307.72 ± 23.65) mg kg^{-1} of tryptanthrin. Overall results suggest that the ultra-pressure treatment and freeze drying method increase the recov-

Table 1. Effect of ultra high-pressure treatment on physiological activities of the ultrasonic indigo extract

Concentration	Pretreatment					
	Hot air	Ultra high pressure	Hot air	Ultra high pressure	Hot air	Ultra high pressure
(mg mL^{-1})	DPPH radical scavenging activity/%		ABTS radical scavenging activity/%		Reducing power activity/%	
125	$(20.56 \pm 1.51)^a$	$(30.35 \pm 2.94)^a$	$(16.08 \pm 1.96)^a$	$(20.38 \pm 1.85)^a$	$(0.04 \pm 0.01)^a$	$(0.05 \pm 0.00)^a$
250	$(27.67 \pm 1.59)^b$	$(43.59 \pm 1.84)^b$	$(24.60 \pm 5.08)^b$	$(36.38 \pm 1.93)^b$	$(0.05 \pm 0.01)^b$	$(0.09 \pm 0.01)^b$
500	$(44.83 \pm 1.28)^c$	$(65.17 \pm 2.17)^c$	$(47.50 \pm 2.27)^c$	$(61.89 \pm 1.76)^c$	$(0.16 \pm 0.01)^c$	$(0.19 \pm 0.02)^c$
1000	$(66.54 \pm 2.35)^d$	$(80.25 \pm 0.73)^d$	$(64.45 \pm 8.97)^d$	$(90.14 \pm 0.79)^d$	$(0.21 \pm 0.02)^d$	$(0.33 \pm 0.02)^d$

a–d) Values within different superscripts are different within the same column at $p < 0.05$ as obtained by the Duncan's multiple range test. All values are mean ± SD

ery rate of tryptanthrin. Tryptanthrin has strong anti-microbial and anti-cancer activities (Iwaki et al., 2006) and it also kills leukemia cells (Kimoto et al., 2001; Iwaki et al., 2011). The tryptanthrin content was 4.6 times higher after the ultra-pressure treatment and freeze drying method than when prepared by the conventional methods: drying under natural conditions after the hot-air treatment.

Polyphenol compounds content

Polyphenol content in the extracts from the indigo leaves treated ultrasonically using methanol extraction after ultra-pressure and hot-air treatments was (127.24 ± 13.67) mg of CHA per kg and (88.22 ± 5.33) mg of CHA per kg, respectively. It is well known that phenols with protein possess various physiological activities such as anti-microbial, anti-cancer, hypertensive, preservation of liver, anti-spasmodic and anti-oxidation (Chon et al., 2008; Lee et al., 2006). The increase of the polyphenol content in the indigo plants in this study was significantly higher compared with that extracted from white lotus (Im et al., 2012). In this study, the polyphenol content in the extracts from indigo plants pre-treated by ultra-pressure increased more than when other methods were used for the pre-treatment. It can be suggested that the pre-treatment method is the most important factor for the increase of the recovery rate of polyphenolic compounds.

Flavonoids content

Flavonoids content of the indigo plants extracted ultrasonically with methanol after ultra-pressure and hot-air pre-treatment was (3224.00 ± 21.45) mg of QUE per kg and (2298.67 ± 83.27) mg of QUE per kg, respectively. Flavonoids have many useful functions such as the anti-oxidation activity, prevention of blood circulation system diseases, anti-allergic activity, anti-microbial and anti-virus activities, decrease in the lipid content, increase in immunity, and the reinforcing activity of capillary vessels (Nalini et al., 2012; Zeng et al., 2013). The content of flavonoids after the ultra-pressure pre-treatment was higher than those achieved by other methods.

DPPH radical scavenging activity

At the concentration of 250 μg mL^{-1}, the DPPH radical scavenging activity of the extract from indigo plants extracted ultrasonically with methanol after the ultra-pressure and hot-air pre-treatment was (43.59 ± 1.84) % and (27.67 ± 1.59) %, respectively (Table 1). Antioxidant activity after the ultra-pressure treatment was (80.25 ± 0.73) %, and after the hot-air treatment it decreased to (66.54 ± 2.35) % at the concentration of 1000 μg mL^{-1}. These data induce the active excess of the glutamate acceptor. Sensitive inductions of amino acids led to cell toxicity (Lu & Xu, 2013). DPPH radical scavenging activity can provide free radicals for electrons and suppress oxidation of lipids in food. And so, it can inhibit aging due to free radicals (Lee et al., 2006). The DPPH radical scavenging activity of the extract of indigo plants dried under hot-air conditions was (66.54 ± 2.35) % at the concentration of 1000 μg mL^{-1}. However, it increased to (80.25 ± 0.73) % in the plot of ultra-pressure pre-treatment.

ABTS radical scavenging activity

ABTS radical scavenging activity of the extracts from indigo plants extracted ultrasonically with methanol after the ultra-pressure and hot-air pre-treatment was (36.38 ± 1.93) % and (24.60 ± 5.08) %, respectively, at the concentration of 250 μg mL^{-1}. These values obtained at the concentration of 1000 μg mL^{-1} after the ultra-pressure and hot-air pre-treatment were (90.14 ± 0.79) % and (64.45 ± 8.97) %, respectively (Table 1).

Reducing activity

At the concentration of 250 μg mL^{-1}, reducing activity of the extract from indigo plants extracted ultrasonically with methanol after the ultra-pressure and hot-air pre-treatment was (0.09 ± 0.01) % and

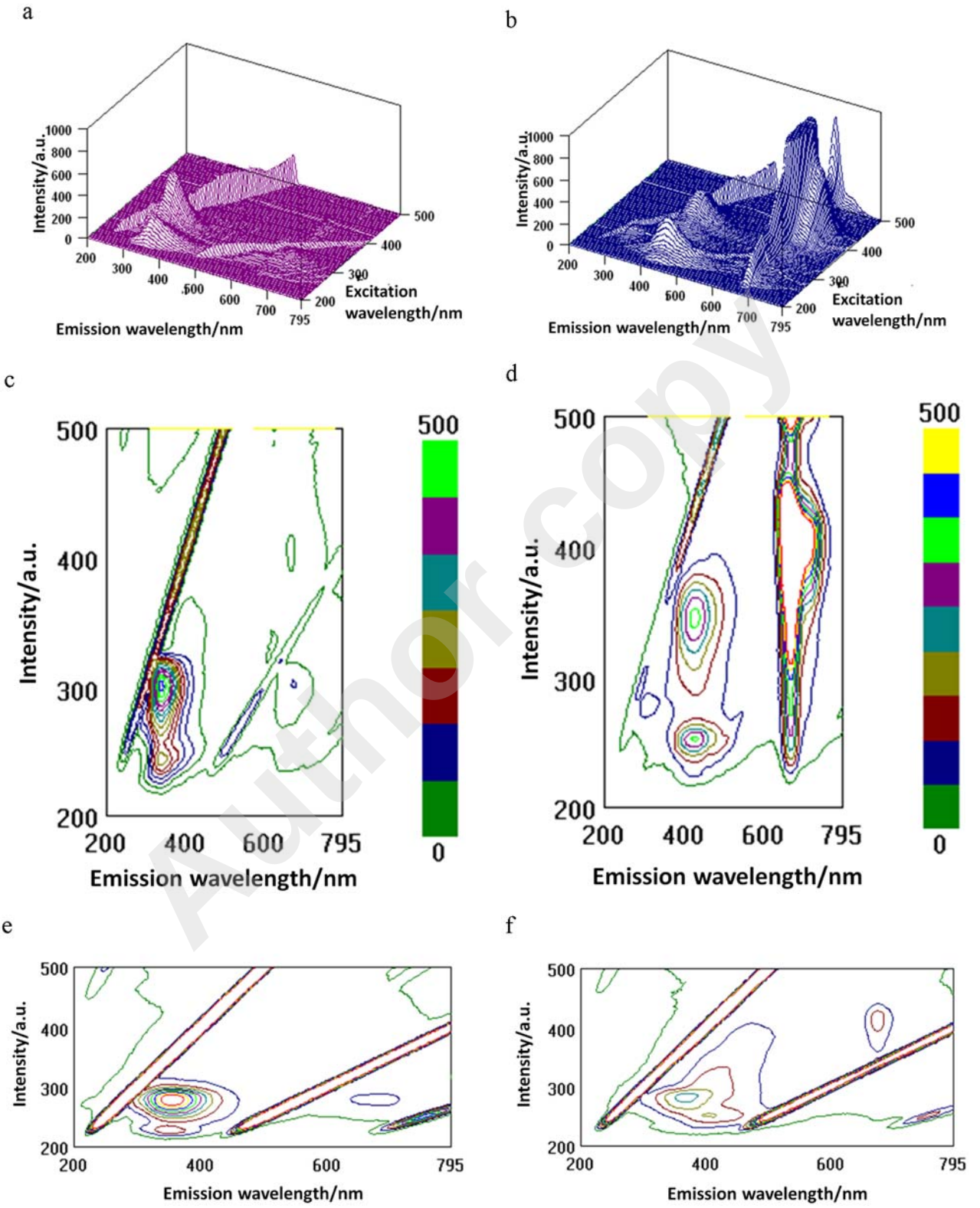

Fig. 2. Three-dimensional fluorescence (3D-FL) spectra of methanol extracts of indigo leaves treated with: hot air (70 °C, 24 h) (a); ultra pressure (550 MPa, 3 min) (b); cross maps of extracts treated with: hot air (70 °C, 24 h) (c); ultra pressure (550 MPa, 3 min) (d); contour maps of extracts treated with: hot air (70 °C, 24 h) (e); and interaction with HSA (human serum albumin); ultra pressure (550 MPa, 3 min) with HAS (f). In all reactions: HSA (2.0×10^{-6} mol L^{-1}); catechin (1.7×10^{-6} mol L^{-1}); extracts (50 μg mL^{-1}). Binding activity at 25 °C for 1 h.

(0.05 ± 0.01) %, respectively (Table 1). However, at the concentration of 1000 μg mL^{-1}, these values were (0.33 ± 0.02) % and (0.21 ± 0.02) % for the ultra-pressure and hot-air pre-treatment, respectively.

Overall results suggest that the ultra-pressure (550 MPa) pre-treatment method is more effective in the useful extraction from indigo plants. Consequently, it was assumed that in further research it is important to determine the optimum pre-treatment pressure and time of the ultra-pressure method. These conditions could be applied also to other plants, not only to indigo leaves.

Fluorometric data

3D-FL spectra of methanol extracts of indigo leaves treated by two methods differ in the wavelength of the main peak and its fluorescence intensity (FI). The following data were registered: indigo extract of leaves treated with hot air at 70 ℃ for 24 h (Figs. 2a and 2c; excitation wavelength (λ_{ex}) is 220 nm and emission wavelength (λ_{em}) is 333 nm, and 611 nm with FI of 168.89 and 34.47); treated with ultra pressure of 550 MPa for 3 min (Figs. 2b, and 2d; $\lambda_{ex/em}$ of 220/310 nm, 436 nm and 670 nm with FI of 67.71, 108.15 and 385.43). The change in the fluorescence intensity as a result of the binding affinity with methanol extracts provided the following data: HSA at the beginning of the binding with FI = 883.24; HSA + indigo leaves extract treated with hot air at 70 ℃ for 24 h decreased the FI value to 673.73 (Fig. 2e), indigo leaves extract treated with ultra pressure of 550 MPa for 3 min decreased the FI value to 243.90 (Fig. 2f). One of the main HSA peaks was found at $\lambda_{ex/em}$ of 220/355 nm. The interaction of HSA and the methanol extracts of two samples of indigo (Fig. 2e) showed a slight change in the position of the main peak at the wavelengths of 356 nm and 365 nm and the decrease in the FI value. The following decrease in the FI (%) value occurred during the interaction of methanol extracts with HSA: to 27.6 % for HAS + indigo leaves extract treated with hot air at 70 ℃ for 24 h; to 72.4 % for HSA+ indigo leaves extract treated with ultra pressure of 550 MPa for 3 min. These results are in direct relationship with the antioxidant properties of the extracts. Our very recent results showed that fluorescence is significantly quenched because of the conformation of proteins, phenolic acids and flavonoids (Namiesnik et al., 2013). This interaction was investigated using tryptophan fluorescence quenching. Our results confirm that quercetin, as an aglycon, is more hydrophobic and demonstrates stronger affinity toward HSA than other flavonoids. Differences of other results (Xiao et al., 2011) can be explained by possible variety of antioxidant abilities of pure flavonoids and different ranges of fluorometry scanning ranges used in similar studies.

The strong binding properties of phenolics prove their effectiveness in the prevention of atherosclerosis under physiological conditions. Quercetin can suppress HSA. Bioactivities of citrus flavanones seem to significantly affect blood and microvascular endothelial cells, therefore it is essential to investigate the interaction between kiwi fruit polyphenols and serum albumin. 3-D fluorescence can be used as an additional tool for the characterization of the polyphenol extracts of indigo plants and their binding properties.

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