



Assessment of Indigo (*Polygonum tinctorium* Ait.) water extracts' bioactive compounds, and their antioxidant and antiproliferative activities

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ABSTRACT

There are a few investigations on Indigo (*Polygonum tinctorium* Ait.), as a medicinal plant. Therefore, the aim of this study was to assess the contents of bioactive compounds, antioxidant and antiproliferative activities and to compare with another medicinal plant Prolipid. It was found that the bioactive compounds from different parts of Indigo water extracts varied ($P < 0.05$): polyphenols and flavonoids were significantly higher in Prolipid, flavanols in Indigo seeds, and vitamin C similar in Indigo leaves and Prolipid. The antioxidant activity by ABTS, FRAP, CUPRAC and DPPH assays was significantly different ($P < 0.05$): the highest in Prolipid, following by Indigo mature leaves. DPPH kinetic measurements compared, distinguished and discriminated the antiradical activity among Indigo and Prolipid water extracts by multivariate analysis. The interaction between polyphenol extracts of Indigo mature leaves and BSA showed that Indigo has a strong ability, as other medicinal plants, to quench the intrinsic fluorescence of BSA by forming complexes. In conclusion, for the first time Indigo mature leaves were analyzed and compared with widely consumed Prolipid. The relatively high content of bioactive compounds, positive antioxidant, fluorescence and antiproliferative properties of Indigo justify the use of this plant as a new source of valuable antioxidants.

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1. Introduction

Indigo (*Polygonum tinctorium* Ait.) was known as natural dye for thousands of years (Cooksey, 2007; Pawlak, Puchalska, Miszczak, Rosloniec, & Jarosz, 2006). Indigo plant is a general name of various plants: Ban-Lan-Gen is the dry root of *Isatis indigotica*, Qing-Dai (*Indigo naturalis*) is a dark blue powder prepared from the leaves of *Baphicacanthus cusia* (Acanthaceae), *P. tinctorium* (Polygonaceae), *I. indigotica* (Brassicaceae), *Indigofera suffruticosa* (Fabaceae) and *Indigofera tinctoria* (Fabaceae). All these plants contain different amounts of the blue dye indigo, red isomer indirubin, colorless precursor conjugates, indican (from *Indigofera* and *Polygonum* species amongst others) or isatan B (from *Isatis*

tinctoria). *P. tinctorium* Ait. is a herbaceous subtropical annual plant, belonging to the family Polygonaceae. Within the cells of its leaves *P. tinctorium* accumulates large amounts of a colorless glycoside, indican (indoxyl beta-D-glucoside), from which the blue dye indigo is synthesized. *P. tinctorium* is well-known in Japan, where it had been cultivated to produce natural indigo for textile dyeing (Angelini, Tozzi, & Di Nasso, 2004). The antibacterial, antiviral, anti-inflammation, cytotoxic and apoptosis actions of *P. tinctorium* were also reported (Ishihara et al., 2000; Iwaki, Koya-Miyata, Kohno, Ushio, & Fukuda, 2006; Kimoto et al., 2001; Zhong et al., 2005). Less is known about indigo as a natural healing and medicine (Hamburger, 2002; Ho & Chang, 2002; Wang et al., 2009). However, there is a scientific basis for the successful use of indigo in traditional medicine (Wei et al., 2005). These authors isolated from the ethanol extract of the Chinese medicinal herb a new indigoid derivative, bisindigotin (1), with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-antagonistic activity. Lin, Leu, Huang, et al. (2009) and Lin, Leu, Yang, et al. (2009) reported about the anti-

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¹ This article was written in memory of my dear brother Prof. Simon Trakhtenberg, who died in November 2011, who encouraged me during all his life.

inflammatory effects of *Indigo naturalis* extracts in human neutrophils. Lin, Leu, Huang, et al. (2009) and Lin, Leu, Yang, et al. (2009) described anti-psoriatic effects of *Indigo naturalis* on the proliferation and differentiation of keratinocytes with indirubin as the active component. Farias-Silva et al. (2008) have shown that indigo is effective on rat gastric mucosa submitted to ethanol-induced gastric ulcer. There are still few data on indigo plant, therefore it is possible to compare this plant with other medicinal plants. Xia, Wu, Shi, Yang, and Zhang (2011) described that *Prunus mume* seeds have been used as a healthy food and traditional drug in China. Research was done on guava leaf extract (Nantitanon, Yotsawimonwat, & Okonogi, 2010). Aqueous extract, proanthocyanidin rich extract, and organic extracts of *Cymbopogon schoenanthus* L. Spreng (lemon grass) shoots from three different locations in South Tunisia were screened for their antioxidant, acetylcholinesterase and antimicrobial activities (Khadri et al., 2010). In order to find out new sources of safe and inexpensive antioxidants, the antioxidant capacities of 45 selected medicinal plants were evaluated using ferric reducing antioxidant power (FRAP) and Trolox equivalent antioxidant capacity (TEAC) assays, respectively, and the total phenolic contents of these plants were measured by the Folin–Ciocalteu method (Li, Wong, Cheng, & Chen, 2008). The interaction between drugs and bovine serum albumin (BSA) is important in the metabolism of drugs (Ni, Zhang, & Kokot, 2009). Such interaction between the extracted polyphenols and BSA can provide knowledge for the use of indigo as a medicinal plant. Therefore the functional properties of indigo and prolipid extracts was studied by the interaction of water polyphenol extracts with a small protein such as BSA, using 3D-FL and FTIR. The data concerning the proliferative activity of indigo are very limited (Iwaki & Kurimoto, 2002). It was of interest to know if also water extracts of indigo have the same properties as some other medicinal plants. Therefore, in addition to study of the contents of the bioactive compounds and antioxidant activity of indigo, also its anti-proliferative properties were analyzed. As far as we know, there are no published results of such investigations.

2. Material and methods

2.1. Chemicals

6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox); catechin; quercetin; Tris, tris (hydroxymethyl) aminomethane; bovine serum albumin, 2,2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS); 1,1-diphenyl-2-picrylhydrazyl (DPPH); Folin–Ciocalteu reagent (FCR); lanthanum (III) chloride heptahydrate; $\text{FeCl}_3 \times 6\text{H}_2\text{O}$; $\text{CuCl}_2 \times 2\text{H}_2\text{O}$; 2,9-dimethyl-1,10-phenanthroline (neocuproine) and bovine serum albumin (BSA) were purchased from Sigma Chemical Co., St Louis, MO, USA. 2, 4, 6-Tripyridyl-s-triazine (TPTZ) was purchased from Fluka Chemie, Buchs, Switzerland. All reagents were of analytical grade. Deionised and distilled water was used throughout.

2.2. Samples and preparation

There are three probes of indigo plant (*P. tinctorium*): seeds, and two probes of leaves. The leaves with slight green color (immature leaves) were harvested on April 10, 2010; and the leaves with green brown color (mature leaves) were harvested on July 20, 2010, from the same place. The leaves were dried for 5 days under sunlight. The leaves were pulverized in the laboratory conditions. The particle size was 200 mesh. For comparison Prolipid was used (Jastrzebski, Medina, Moreno, & Gorinstein, 2007). Prolipid is a mixture of the following plants: *Sonchus 532 Z. Arvensis* L. from the Compositae (Asteraceae) family, *Guazuma ulmifolia* L. from the Sterculiaceae

family and *Murraya paniculata* L. from the Rutaceae family. Prolipid contains extracts of *G. ulmifolia* [20 g/100 g dry weight (dw)], *M. paniculata* (10 g/100 g dw) and *Sonchus arvensis* (10 g/100 g dw). Prolipid capsules were obtained as a gift from the drug importer COWIK (Warsaw, Poland).

2.3. Determination of the contents of the main bioactive compounds

The extracts from seeds and leaves were prepared by the same way for all tests (bioactive compounds, antioxidant and anti-proliferative assays). The probes are phenols extracted with water from either the indigo powder, seeds or the prolipid (concentration 25 mg/mL) at room temperature twice during 3 h (Im et al., 2011). The prolipid capsules were opened, and the content was dissolved in water at the same conditions. The polyphenols were determined by Folin–Ciocalteu method (Singleton, Orthofer, & Lamuela-Raventos, 1999) with measurement at 750 nm with spectrophotometer (Hewlett–Packard, model 8452A, Rockville, USA). The results were expressed as mg of gallic acid equivalents (GAE) per g dw. Total flavonoid content was determined by an aluminum chloride colorimetric method (Zhishen, Mengcheng, & Jianming, 1999) with some modifications (Liu et al., 2002). Briefly, 0.25 mL of the indigo or prolipid sample extract was diluted with 1.25 mL of distilled water. Then 75 μL of a 5 g/100 g NaNO_2 solution was added to the mixture. After 6 min, 150 μL of a 10 g/100 g $\text{AlCl}_3 \times 6\text{H}_2\text{O}$ solution was added, and the mixture was allowed to stand for another 5 min. Half of a milliliter of 1 mol/L NaOH was added, and the total was made up to 2.5 mL with distilled water. The solution was well mixed. The absorbance was measured immediately against the blank at 510 nm in comparison with the standards prepared similarly with known (+)-catechin concentrations. The total flavanols amount was estimated using the p-dimethylaminocinnamaldehyde (DMACA) method, and then the absorbance at 640 nm was read. To ensure the presence of flavanols on the nuclei subsequent staining with the DMACA reagent resulted in an intense blue coloration in plant extract (Feucht & Polster, 2001). As it was mentioned previously, (+)-catechin served as a standard for flavonoids, and flavanols, and the results were expressed as catechin equivalents (CE). The absorbances for total anthocyanins were measured for two pH's (1.0 and 4.5) in a Beckman spectrophotometer at 510 nm, using the pH differential method (Lo Scalzo, Genna, Branca, Chedin, & Chassaigne, 2008). Results were expressed as mg of cyanidin-3-glucoside equivalent (CGE). Total ascorbic acid was determined by CUPRAC assay. The water extract was prepared from 100 mg of the sample and 5 mL of water, then mixed, stirred for 24 h and centrifuged. The extract (1 mL) was mixed with 2 mL of 3.0×10^{-3} mol/L of lanthanum (III) chloride heptahydrate. Ethylacetate (EtAc) was used for extraction in order to avoid the interference of flavonoids. For determination of ascorbic acid by CUPRAC assay the aqueous phase was examined. One mL of Cu (II)-neocuproine (Nc), in ammonium acetate-containing medium at pH 7, was mixed with 1 mL of the obtained extract, where the absorbance of the formed bis (Nc)-copper (I) chelate was measured at 450 nm (Ozyurek, Guclu, Bektasoglu, & Apak, 2007).

2.4. Determination of antioxidant activity (AA), $\mu\text{mol TE/g dw}$

The AA was determined by four complementary assays:

- (1) 2,2-Azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt (ABTS^+) method for the screening of antioxidant activity is reported as a decolorization assay applicable to both lipophilic and hydrophilic antioxidants, including

flavonoids, hydroxycinnamates, carotenoids, and plasma antioxidants. The pre-formed radical monocation ABTS was generated by oxidation of ABTS with potassium persulfate and was reduced in the presence of such hydrogen-donating antioxidants. The influences of both the concentration of antioxidant and duration of reaction on the inhibition of the radical cation absorption were taken into account when determining the antioxidant activity. ABTS⁺ radical cation was generated by the interaction of ABTS (7 mmol/L) and K₂S₂O₈ (2.45 mmol/L). This solution was diluted with water until the absorbance in the samples reached 0.7 at 734 nm (Re et al., 1999).

- (2) Ferric-reducing/antioxidant power (FRAP) assay measures the ability of the antioxidants in the investigated samples to reduce ferric-tripiridyltriazine (Fe³⁺-TPTZ) to a ferrous form (Fe²⁺). FRAP reagent (2.5 mL of a 10 mmol ferric-tripiridyltriazine solution in 40 mmol HCl plus 2.5 mL of 20 mmol FeCl₃·xH₂O and 25 mL of 0.3 mol/L acetate buffer, pH 3.6) of 900 μL was mixed with 90 μL of distilled water and 30 μL of plant samples as the appropriate reagent blank. The absorbance was measured at 595 nm (Benzie & Strain, 1996).
- (3) Cupric reducing antioxidant capacity (CUPRAC): This assay is based on utilizing the copper (II)-neocuproine [Cu (II)-Nc] reagent as the chromogenic oxidizing agent. To the mixture of 1 mL of copper (II)-neocuproine and NH₄Ac buffer solution, acidified and non acidified water extracts (or standard) solution (x, in mL) and H₂O [(1.1 - x) mL] were added to make the final volume of 4.1 mL. The absorbance at 450 nm was recorded against a reagent blank (Apak, Guclu, Ozyurek, & Karademir, 2004).
- (4) Scavenging free radical potentials were tested in solution of 1, 1-Diphenyl-2-picrylhydrazyl (DPPH). The degree of decoloration of the solution indicates the scavenging efficiency of the added substance. In its radical form, DPPH has an absorption band at 515 nm which disappears upon reduction by an antiradical compounds. DPPH solution (3.9 mL, 25 mg/L) in methanol was mixed with the samples extracts (0.1 mL), then the reaction progress was monitored at 515 nm until the absorbance was stable (Brand-Williams, Cuvelier, & Berset, 1995).

Samples with different concentrations of indigo and prolipid water extracts (1, 2.5, 5, 10, 15, 20 and 30 mg/mL) were analyzed by DPPH antioxidant activity assay (Ozgen, Reese, Tulio, Scheerens, & Miller, 2006). In the kinetic studies two variables were used: the change in the concentration of the applied samples and the change in reaction time of the extracts with the scavenging radical: 1, 10, 30, 60 and 90 min.

2.5. Fluorimetry

Two dimensional (2D-FL) and three dimensional (3D-FL) fluorescence measurements were done using a model FP-6500, Jasco Spectrofluorometer, serial N261332, Jasco International Co., LTD, Tokyo, Japan. Fluorescence emission spectra for all water extracts of indigo and prolipid samples at a concentration of 0.25 mg/mL were taken at emission wavelength (nm) of 330, and recorded from wavelength of 265 to a wavelength of 310 nm, at emission wavelengths of 685 nm from 300 to 750 nm; and at excitation of 350 nm from 370 to 650 nm (Im et al., 2011). Quercetin was used as a standard. 3D-FL spectra of the investigated plant extracts were collected with subsequent scanning emission spectra from 250 to 750 nm at 1.0 nm increments by varying the excitation wavelength from 230 to 350 nm at 10 nm increments. The scanning speed was set at 1000 nm/min for all measurements. All measurements were performed with emission mode and with intensity up to 1000. All solutions for protein interaction were prepared in 0.05 mol/L Tris-HCl buffer (pH 7.4), containing 0.1 mol/L NaCl. The final

concentration of BSA was 2.0×10^{-4} mol/L. All solutions were kept in dark at 0–4 °C. The BSA was mixed with quercetin. The samples were mixed in the proportions of BSA:extract = 1:1. The samples after the interaction with BSA were lyophilized and subjected to FTIR (Kumari, Yadav, Pakade, Singh & Yadav, 2010; Yin, Li, Ding, & Wang, 2009).

2.6. Fourier Transform Infrared (FT-IR) spectra studies

The presence of polyphenols in the investigated indigo samples and the interaction between polyphenols and bovine serum albumin (BSA) was studied by Fourier Transform Infrared (FT-IR) spectroscopy. A Nicolet iS 10 FT-IR Spectrometer (Thermo Scientific Instruments LLC, Madison, WI, USA), with the smart iTR™ ATR (Attenuated Total Reflectance) accessory was used to record IR spectra (Natarajan, Krithica, Madhan, & Sehgal, 2011).

2.7. Extraction of phenolic compounds for MS

The lyophilized samples of seeds and leaves (1 g) were extracted with 100 mL of methanol/water (1:1) at room temperature and in darkness for 24 h. The extracts were filtered in a Buchner funnel. After removal of the methanol in a rotary evaporator at a temperature below 40 °C, the aqueous solution was then freeze-dried. These extracts were used for MS.

MS analysis. A mass spectrometer, a TSQ Quantum Access Max (Thermo Fisher Scientific, Basel, Switzerland) was used. Analytes were ionized by electrospray ionization (ESI) in negative mode. Vaporizer temperature was kept at 100 °C. Settings for the ion source were as follows: spray voltage 3000 V, sheath gas pressure 35 AU; ion sweep gas pressure 0 AU; auxiliary gas pressure at 30 AU; capillary temperature at 200 °C, skimmer offset 0 V (Sanz et al., 2010).

2.8. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay

Antiproliferative activity of water extracts of the studied plants on human cancer cell lines (Calu-6 for human pulmonary carcinoma and SNU-601 for human gastric carcinoma) were measured using MTT assay. The cell lines were purchased from Korean Cell Line Bank (KCLB) for MTT assay. Cells were grown in RPMI-1640 medium at 37 °C under 5% CO₂ in a humidified incubator. Serum (10 g/100 g) and antibiotics (1 g/100 g) were added to the RPMI-1640 medium. Cells were trypsinized and then centrifuged to harvest.

Cells were harvested, counted (3×10^4 cells/mL), and transferred into a 96-well plate, and incubated for 24 h prior to the addition of test compounds. Serial dilutions of test samples were prepared by dissolving compounds in dimethyl sulfoxide (DMSO) followed by dilution with RPMI-1640 medium to give final concentration at 25, 50, 100, 200, 400, and 800 μg/mL. Stock solutions of test samples were prepared for cell lines of 90 μL of medium and 10 μL of samples, and incubated for 72 h.

MTT solution at 5 mg/mL was dissolved in 1 mL of Phosphate Buffer Solution (PBS), and 10 μL of it was added to each of the 96 wells. The wells were wrapped with aluminum foil and incubated at 37 °C for 4 h. The solution in each well containing media, unbound MTT and dead cells were removed by suction and 150 μL of DMSO was added to each well. The plates were then shaken and optical density was recorded using a micro plate reader at 540 nm. Distilled water was used as positive control and DMSO as solvent control. Controls and samples were assayed in duplicate for each concentration and replicated three times for each cell line. The cytotoxicity was obtained by comparing

the absorbance between the samples and the control (Chon, Heo, Park, Cho, & Gorinstein, 2008; Heo et al., 2007).

2.9. Statistical analyses

To compare, distinguish and discriminate the antiradical activity among plant aqueous extracts multivariate analysis, employing methods of principal component, canonical discriminant analysis and classification were performed by means of Unistat v. 5.6 (Unistat, London, United Kingdom) statistical software, taking into consideration all the experimental data obtained from DPPH kinetic measurements. The methods are designed in a way that enables the enhancement of hidden properties of the original data and allows the reduction of multi-dimensional data set to only a few dimensions, which can sufficiently explain all the original data.

To verify the statistical significance, mean \pm SD of five independent measurements were calculated. Differences between 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. P -values of <0.05 were considered significant.

3. Results

3.1. The main bioactive compounds

The results of the determination of the bioactive compounds of the water extract in studied samples are summarized in the Table 1. The following order of the value of polyphenols was obtained in water extracts such as Prolipid > Mature leaves > Immature leaves > Seeds. As can be seen, the significantly highest content of polyphenols and flavonoids was in prolipid, flavanols in indigo seeds and vitamin C in indigo mature leaves and prolipid ($P < 0.05$). The yield of polyphenols from seeds was 3.71%, for immature leaves it was 25.43% and from mature leaves it reached 26.52%. The results of the yield of extracted polyphenols showed that in seeds the polyphenols were less soluble than in both leaves.

3.2. The antioxidant activity, kinetic measurements by DPPH, multivariate and canonical discriminant analyses

The results of the determination of the antioxidant activity in the studied samples are summarized in the Table 1: the

Table 1
Bioactive compounds and the antioxidant activity in water extract of the studied plant samples per g dry weight.

Indices	Seeds	Immature leaves	Mature leaves	Prolipid
Polyphenols, mg GAE	5.59 \pm 0.1 ^a	14.76 \pm 0.3 ^b	17.74 \pm 0.5 ^c	19.87 \pm 0.9 ^d
Flavonoids, μ g CE	1.29 \pm 0.05 ^a	2.09 \pm 0.1 ^b	2.50 \pm 0.1 ^b	3.39 \pm 0.1 ^c
Flavanols, μ g CE	201.70 \pm 9.1 ^c	59.45 \pm 0.9 ^b	5.12 \pm 0.2 ^a	7.58 \pm 0.3 ^a
Ascorbic acid, mg AA	1.01 \pm 0.05 ^a	8.130 \pm 0.4 ^b	8.72 \pm 0.4 ^b	8.71 \pm 0.4 ^b
Anthocyanin, mg CGE	ND	0.023 \pm 0.002 ^a	ND	0.018 \pm 0.001 ^a
ABTS, μ mol TE	39.18 \pm 0.2 ^a	75.93 \pm 0.3 ^b	81.24 \pm 0.3 ^b	117.79 \pm 5.2 ^c
FRAP, μ mol TE	10.53 \pm 0.5 ^a	21.38 \pm 0.9 ^b	40.33 \pm 1.8 ^c	46.29 \pm 1.6 ^d
CUPRAC, μ molTE	1.06 \pm 0.04 ^a	8.13 \pm 0.4 ^b	8.80 \pm 0.7 ^b	34.72 \pm 1.5 ^c
DPPH, μ mol TE	8.15 \pm 0.5 ^a	15.17 \pm 0.9 ^b	24.46 \pm 1.8 ^c	37.79 \pm 1.6 ^d

Values are means \pm SD of 5 measurements. Values in rows with different superscript letters are significantly different ($P < 0.05$). CE, catechin equivalent; GAE, gallic acid equivalent; CGE, cyanidin-3-glucoside equivalent; TE, trolox equivalent; ABTS, 2,2-Azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt; CUPRAC, Cupric reducing antioxidant capacity; FRAP, Ferric-reducing/antioxidant power; DPPH, 1, 1-Diphenyl-2-picrylhydrazyl method.

significantly highest antioxidant activity according to all methods was in prolipid, following by mature leaves ($P < 0.05$).

As was calculated, a very good correlation was found between the antioxidant activities and the contents of total polyphenols (Table 2, R^2 from 0.99 to 0.83) in water. Other polyphenolics (flavonoids and flavanols) showed similar correlation. The correlation between the antioxidant activities and ascorbic acid was lower than with polyphenols (Table 2, R^2 from 0.99 to 0.67). Among the POL \times AA the significantly low data were in FRAPW ($P < 0.05$). Among the FLAVON \times AA not significant changes were registered ($P > 0.05$). Among the FLAV \times AA the significantly low data were in FRAPW ($P < 0.05$). Among the AC \times AA the significantly low data were in FRAPW ($P < 0.05$).

A combination of Box and Dot plots is presented in Fig. 1A, where the balls are dot plots. Dot plots provide an easy and powerful mean of sequence analysis to depict data distributions. Dot plot is one of the simplest statistical plots, and is suitable from small to moderate sized data sets and is useful for highlighting clusters and gaps, as well as outliers. Fig. 1B demonstrates the IC₅₀ (amount of samples which reduce 50% of DPPH) values of examined water extracts of Prolipid and Indigo herbal constituents. Prolipid herbal mixture revealed the highest antiradical action in comparison to the Indigo's constituents, while Indigo seeds showed the lowest activity. Oil matrix character of Indigo brown seeds is the explanation of low antioxidant yield obtained by water extraction. The IC₅₀ values were calculated from the dependency of extract concentration (mg/mL) on DPPH absorbance. The IC₅₀ as extract concentration was read at 50% of absorbance of control samples, therefore IC₅₀ is an extract concentration (mg/mL). The IC₅₀ of Indigo seeds is 857 \pm 30 mg/mL, Immature leaves 85.4 \pm 8.5, Mature leaves 55.9 \pm 5.5 and Prolipid 26.6 \pm 2.7 mg/mL.

A multivariate hierarchical cluster analysis was applied for all measured and calculated DPPH-radical scavenging parameters in order to produce a similarity matrix (dendrogram) by means of Euclidean distance measure and average between groups as the clustering method. In hierarchical cluster analysis (HCA), samples are grouped on the basis of similarities, without taking into account the information about the class membership. The results obtained following HCA are shown as a dendrogram (Fig. 2A), in which four well-defined clusters, are visible in a distance of about 50. The first cluster, which comprises samples with the lowest antiradical activity, is formed of Indigo seeds (S). The last cluster with the highest antiradical activity consists of Prolipid (P) herbal mixture samples. The second and third clusters are composed of Indigo mature (M) and immature leaves' (I) constituents with some similar values of antiradical activity.

Principal components 1 and 2 (Fig. 2B) are new uncorrelated variables, and they are linear combinations of all original variables (absorbance, inhibition and antiradical activity data). The first principal component accounts as much as possible the variability of the data (75.8%). The second component accounts the remaining variability (14.9%). Principal component analysis (PCA) is a mathematical tool which performs a reduction in data dimensionality and allows the visualisation of underlying structure in experimental data and relationships between data and samples. PCA was applied to all analyzed antiradical parameters of Indigo seeds, mature and immature leaves and Prolipid herbal constituents. PCA revealed that the first two principal components explain 90.8% of total variance (PC1 explained 75.8% while PC2 14.9%). A plot of the scores of PC1 versus PC2, is the projection of the samples along the directions of principal components, is shown in Fig. 2B. It is apparent that samples according to their antiradical ability are grouped in a similar way to cluster analysis. Indigo seeds (S) with the lowest and Prolipid (P) samples with the highest antiradical activity (Fig. 2B) were fully isolated from

Table 2
Correlation coefficients between bioactive compounds and the overall antioxidants activities in water extracts of investigated plants.

Assays	POL × AA	FLAVON × AA	FLAV × AA	AC × AA
ABTSW	0.9853 ± 0.08758 ^c	0.9509 ± 0.08511 ^a	0.9763 ± 0.0871 ^a	0.9978 ± 0.0897 ^a
DPPHW	0.8717 ± 0.0783 ^b	0.9318 ± 0.08418 ^a	0.8932 ± 0.0798 ^a	0.7401 ± 0.0633 ^a
FRAPW	0.8172 ± 0.0755 ^a	0.8890 ± 0.07213 ^a	0.8423 ± 0.0712 ^a	0.6714 ± 0.0582 ^a
CUPRACW	0.9748 ± 0.0865 ^c	0.9332 ± 0.08319 ^a	0.9597 ± 0.0814 ^a	0.9999 ± 0.0899 ^a

Values are means ± SD of 5 measurements. Values in columns with different superscript letters are significantly different ($P < 0.05$). Abbreviations: POL × AA, polyphenols vs antioxidant activities; FLAVON × AA, flavonoids vs antioxidant activities; FLAV × AA, flavanols vs antioxidant activities; ND, not detected; ABTS, 2,2-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; CUPRAC, Cupric reducing antioxidant capacity; DPPH, Radical Scavenging Activity Using 1,1-diphenyl-2-picrylhydrazyl; FRAP, Ferric-reducing/antioxidant power; W, water extract; AC, ascorbic acid.

Indigo mature leaves (I) and immature leaves (L) samples with similar antiradical action. The first principal component was associated with absorbance reading values measured at time of 30 and 90 min, while second component (PC2) was strongly correlated with Trolox equivalent values read at time of 60 and 90 min. A canonical discriminant analysis was also performed, giving satisfactory results of Indigo constituents: seeds (S), mature (M) and immature leaves (I) as well as Prolipid (P) herbal mixture. All constituents were clustered in four individual groups (Fig. 3) obtained through the definition of two discriminant functions (Wilks' Lambda ~ 0; $P < 0.001$), which accounted for 99.9% of the total experimental antiradical activity variance. Canonical discrimination selected absorbance and inhibition data for scavenging time of 10 min as the variables of the highest discriminant capacity. This discrimination procedure using all antiradical variables demonstrated a good recognition and classification performance, allowing correct classification of 100% of the samples for the original Indigo and Prolipid herbal groups.

3.3. Fluorimetry

Three-dimensional fluorescence (3D-FL, Fig. 4A) spectra illustrated the elliptical shape of the contour maps of the main peaks for indigo mature leaves, seeds and immature leaves (Fig. 4B–D). The

main peaks appeared at $\lambda_{ex}/\lambda_{em}$ of 280/345 nm. One of the main peaks for BSA was found at $\lambda_{ex}/\lambda_{em}$ of 225–230/335 nm. The second main peak appeared for these samples at $\lambda_{ex}/\lambda_{em}$ of 280/345 nm. The interaction of BSA and quercetin (Fig. 4E) and BSA and indigo (Fig. 4F) showed the peak of 335 nm, and a decrease in the fluorescence intensity (FI). The fluorescence intensity initially for BSA was 881.97 (Fig. 4E, upper curve) and after quenching for 1 h at 37 °C with quercetin reached 748.61 (Fig. 4E, lower curve). The decrease was about 15.1%. In case of indigo mature leaves from the initial value to the end of the reaction, when the intensity decreased to 693.85, the decrease was 16.7% (Fig. 4F, lower curve). 3-D fluorescence was used as an additional tool for the characterization of aqueous polyphenols extracts.

The interaction between water polyphenol extracts of indigo mature leaves and BSA showed that indigo has a strong ability as other studied medicinal plants to quench the intrinsic fluorescence of BSA by forming complexes.

3.4. FTIR spectra

The FTIR spectra of BSA and quercetin (Fig. 5A, upper curve) were compared with pure quercetin and BSA (Fig. 5A, middle curve, and lower curve). The amide I and amide II peaks of BSA (Fig. 5A, lower curve) were shifted from 1544 to 1540 cm^{-1} and from 1654 to 1626 cm^{-1} upon interaction with quercetin (Fig. 5A, upper curve) and to 1544 and 1630 cm^{-1} upon interaction with indigo mature leaves extract. FTIR of quercetin (Fig. 5A, middle curve) shows broad phenolic OH band centered around 3404 cm^{-1} , characteristic –CO stretching at 1663 cm^{-1} aromatic bending and stretching around 1091 and 1663 cm^{-1} , –OH phenolic bending around 1197 and 1374 cm^{-1} . FTIR of quercetin could confirm the relative chemical stability of quercetin. FTIR spectra of water extracts of mature and immature indigo leaves (Fig. 5B, upper, and middle curves) showed a peak characteristic –CO stretching at 1634 cm^{-1} aromatic bending and the peaks at 2925 and 2852 cm^{-1} are related to the C–H bond of saturated carbons. Similar peaks are fixed in water extract of prolipid (Fig. 5B and C, lower curves). As it was shown previously that the characteristic 1663 cm^{-1} of quercetin –CO stretching (Fig. 5A, middle curve) is seen as small shoulder due to the overlapping of the dominant –CO stretching of indigo mature leaves (1744 cm^{-1} , Fig. 5C, middle curve), but the phenolic OH, corresponding to quercetin is seen around 3405 cm^{-1} , for the quercetin-BSA at 3183 cm^{-1} (Fig. 5A, upper curve). Matching between the peaks in the range from 4000 to 400 cm^{-1} between (BSA + Quercetin):(Quercetin) = 12.4%; (BSA + Quercetin):(BSA) = 49.1% (Fig. 5A and B). Matching between the peaks of the water extracts between indigo mature leaves and indigo immature leaves was 83.49%; water extract of indigo immature leaves and prolipid was 72.00%; and water extract of indigo mature leaves and prolipid was 78.38% (Fig. 5B). The matching of peaks between the dry matter of indigo mature leaves and indigo dry immature leaves was 72.44%; dry matter of indigo

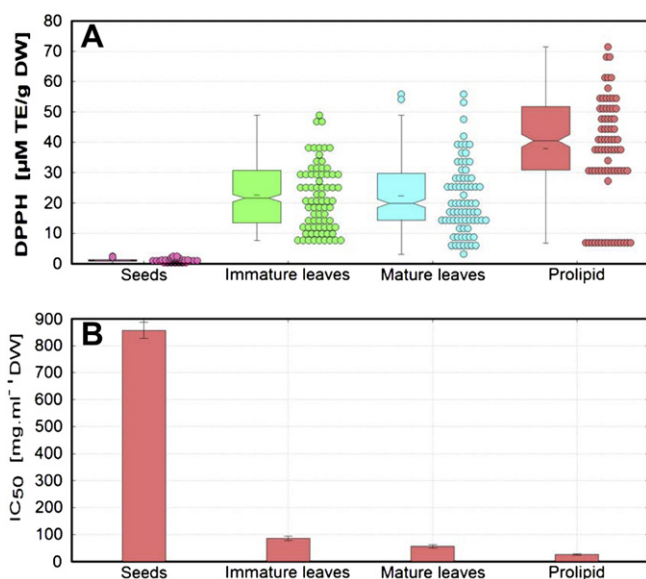


Fig. 1. A, Comparison of DPPH-radical scavenging activity ($\mu\text{mol TE/g DW}$) of Indigo seeds, immature and mature leaves and Prolipid herbal constituents in water extracts at examined conditions. The balls represent dot plots to depict distributions of samples. B, IC_{50} values of Indigo's seeds, mature and immature leaves and Prolipid's water extracts for scavenging time of 30 min.

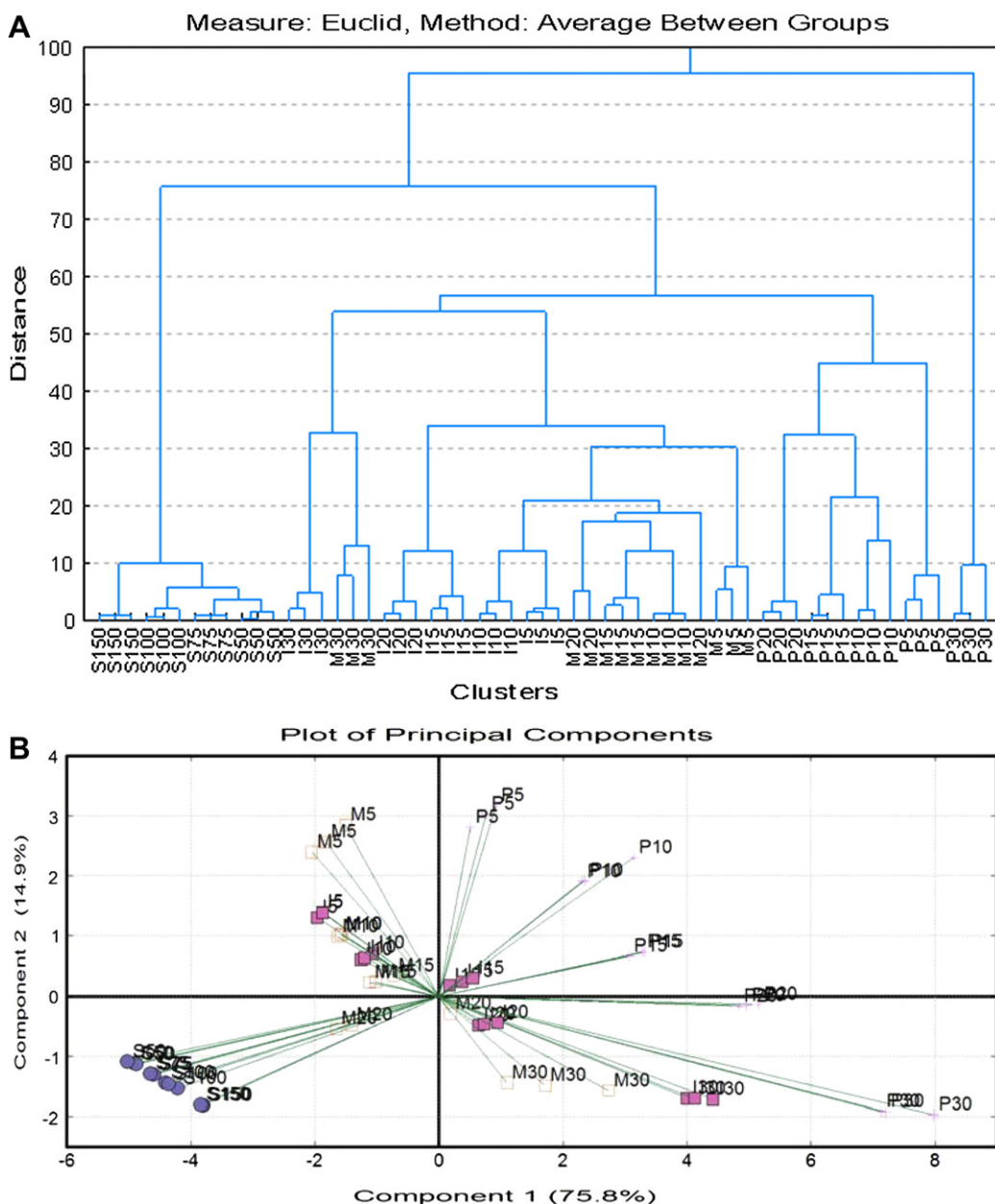


Fig. 2. A, Dendrogram representing the relationships among some Indigo and Prolipid herbal constituents (S - Seeds, I - Immature leaves, M - Mature leaves, P - Prolipid) according to their water extracts' DPPH-radical scavenging activity (extracts concentrations 5–150 mg/mL dw). B, PCA scores plot for Indigo and Prolipid herbal constituents (S - Seeds, I - Immature leaves, M - Mature leaves, P - Prolipid), comparing their DPPH-radical scavenging activity in water extracts with concentrations from 5 to 150 mg/mL (dw) at reaction times: 1, 10, 30, 60 and 90 min.

dry mature leaves and prolipid was 50.41% and indigo immature leaves and prolipid was 53.45%. The matching between the water extracts and dry matter of the samples is similar. The percentage between the dried plants (not in the water extract) of the mature leaves and the prolipid was higher than between the immature leaves and prolipid (Fig. 5C). The comparison of the peaks in the interaction between BSA and quercetin and BSA and indigo mature extract (BSA + Quercetin):(BSA + indigo) was about 87.4%. The observation that the protein conformation was not affected with the addition of flavonoid was also demonstrated by FTIR spectroscopy. If there had been a change of BSA conformation, a shift in the peak of amide I band or disappearance of the peak corresponding to the N–H residual amide II band would have been observed, neither of which occurred.

3.5. Mass spectra

The spectrum shows the m/z peaks found in seeds (Fig. 6A) benzyl caffeate at 134 with Relative Abundance (RA) = 30%; myricetin at 180 with RA = 8% and ferulic acid at 193 with RA = 70%. In immature leaves (Fig. 6B) were found: 134 (RA = 37%, benzyl caffeate); 166 (RA = 25%, vanillic acid); 180 (RA = 43%, myricetin); 193 (RA = 76%, ferulic acid); 290 (RA = 18%, catechin); 355 (RA = 20%, pinobanksin-3-O-pentanoate). In mature leaves (Fig. 6C) the following peaks were identified: 120 (RA = 10%, benzoic acid); 134 (RA = 47%, benzyl caffeate); 166 (RA = 20%, vanillic acid); 180 (RA = 37%, myricetin); 193 (RA = 92%, ferulic acid); 263 (RA = 5%, indigotin); 290 (RA = 20%, catechin); 279 (RA = 55%, *p*-coumaric cinnamyl ester); 355 (RA = 20%, pinobanksin-3-O-pentanoate). The obtained results were similar in both leaves

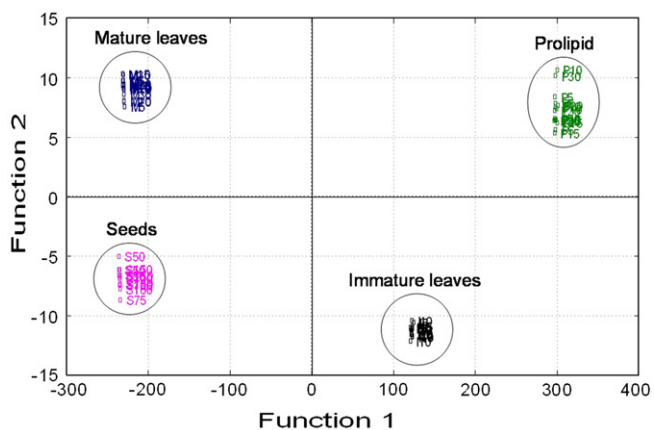


Fig. 3. Scatter plot of canonical discriminant analysis of some Indigo and Prolipid herbal constituents (S - Seeds, I - Immature leaves, M - Mature leaves, P - Prolipid herb mixture) obtained from the DPPH-radical scavenging activity measurements in water extracts with concentrations from 5 to 150 mg/mL dw and reaction times: 1, 10, 30, 60 and 90 min. Function 1, 2: canonical discriminant functions.

samples, but only with higher amount of *p*-coumaric cinnamyl ester in mature leaves.

3.6. Inhibition of proliferation

It was observed that the percentage of proliferativity of the water extracts of immature dry leaves, seeds, mature leaves and prolipid samples on two cell lines (Fig. 7A, Calu-6 for human pulmonary carcinoma and Fig. 7B, SNU-601 for human gastric carcinoma) were different. The proliferativity (%) for concentrations of 800 μ g/mL for prolipid on Calu-6 was 84.12%, and on SNU-601 was 87.21%, showing the highest antiproliferative activity in comparison with mature leaf sample for Calu-6 (86.87%) and SNU-601 (91.12%). Our investigation shows that antioxidant activity of the studied samples was not always correlated with their proliferative activity.

4. Discussion

Indigo plant was effectively used in traditional folk medicine of Far East for many centuries (Ho & Chang, 2002). The above cited authors claim that indigo plant is known for its effective treatment of various inflammatory diseases and the root of indigo plant, popularly known as Ban-Lan-Gen, is used by traditional Chinese medicine for seasonal febrile diseases, pestilence, mumps, eruptive

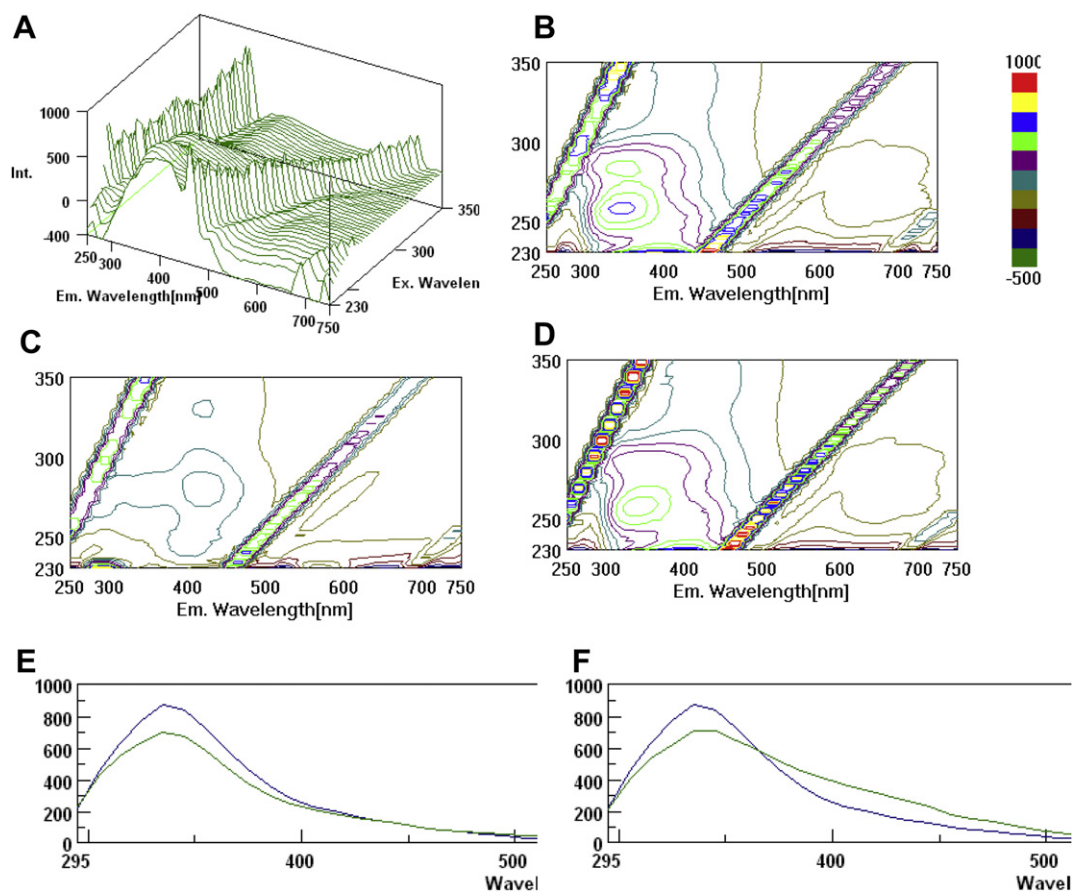


Fig. 4. A, Three-dimensional fluorescence (3D-FL) spectrum of water extract of indigo mature leaves (0.01 mg/mL); B, Elliptical shapes of the contours of water extracts of indigo mature leaves (0.01 mg/mL); C, indigo seeds (0.1 mg/mL); D, indigo immature leaves (0.1 mg/mL); E, change in the fluorescence intensity (ID) as a result of binding affinity of 2.0×10^{-4} mol/L BSA and 2.0×10^{-4} mol/L quercetin by 2D-FL (E, upper line - BSA, lower line - BSA + quercetin); F, change in the ID, as a result of binding affinity of 2.0×10^{-4} mol/L BSA and 40 μ g/mL indigo mature leaves water extract (F, upper line - BSA, lower line - BSA + indigo mature leaves water extract). The 3D-FL were run emission mode and fluorescence intensity up to 1000, emission wavelengths from 250 to 750 nm and excitation wavelengths from 230 to 350 nm; scanning speed was 1000 nm/min, B–F, emission wavelength on x-axis and fluorescence intensity on y-axis (E, F); B, C, D, emission wavelength on x-axis and excitation wavelength on y-axis.

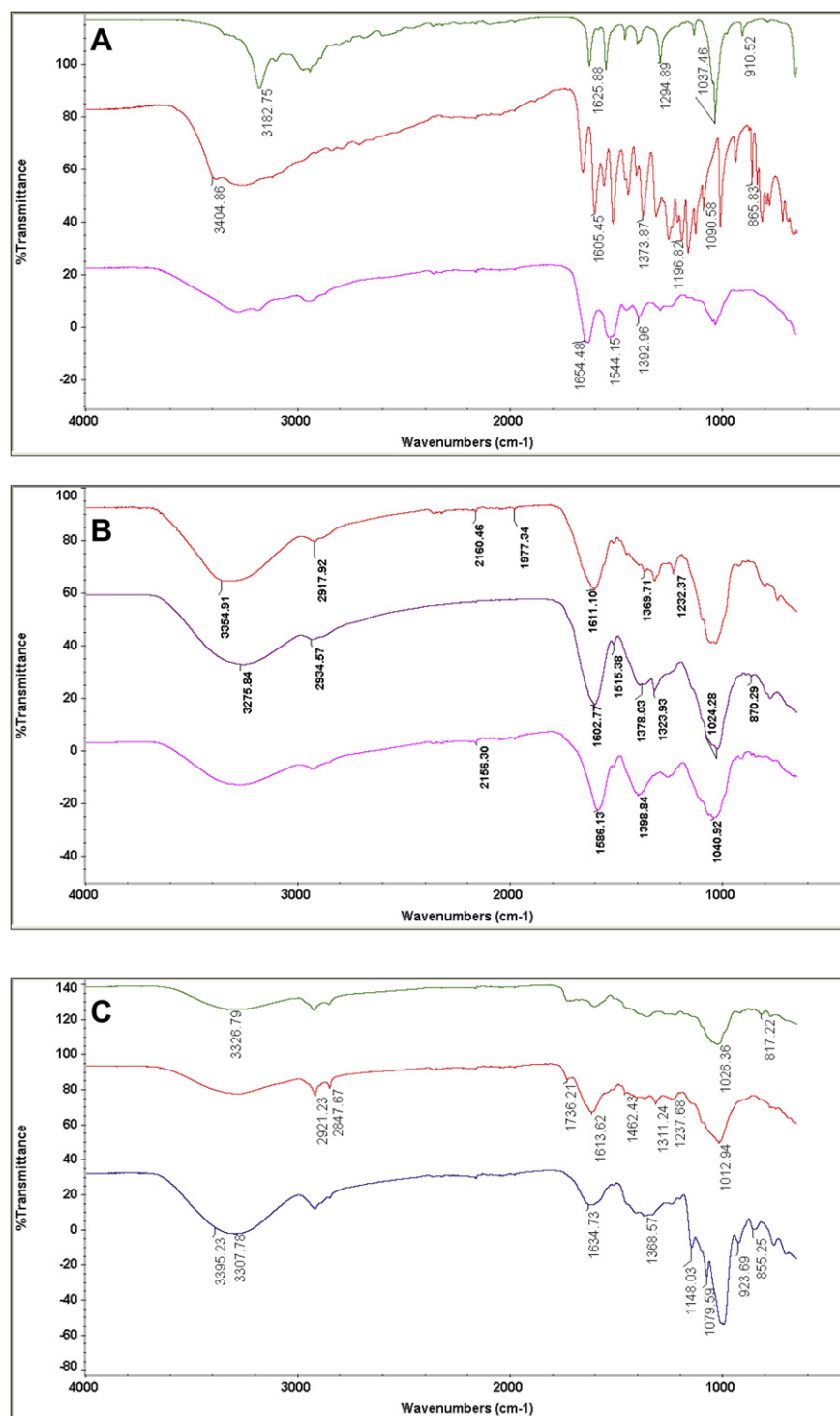


Fig. 5. Infrared study of FTIR spectra of A, BSA and quercetin (upper curve); quercetin (middle curve); BSA (lower curve); B, water extracts of: Indigo mature leaves (upper curve); indigo immature leaves (middle curve); prolipid (lower curve). C, dry matter of: Indigo mature leaves (upper curve); indigo immature leaves (middle curve); prolipid (lower curve).

diseases, inflammatory diseases with redness of skin, sore throat. However, as was stated that the content of bioactive compounds and the antioxidant and anticancer activities of indigo was less studied. Therefore, in this investigation the above mentioned indices of indigo parts were investigated using new analytical tests.

As for bioactive compounds in various parts of the indigo plant, flavanols being important anticancer compounds were the richest (202 $\mu\text{g CE}$) in seeds (Table 1). Among the four assays used for antioxidant capacity of the plant parts (Table 1), CUPRAC, FRAP, ABTS and

DPPH results ($\mu\text{mol TE/g dw}$) were distinctly higher in prolipid, following by indigo mature leaves. Our results are in accordance with Xia et al. (2011), who investigated the phenolic compounds and antimicrobial activity of ethanolic extract from seeds of *P. mume*. The results exhibited that the antimicrobial activity of *P. mume* seeds may be partly due to the phenolic compounds. The total phenolic content estimated as $188.7 \pm 14.4 \text{ mg GAE/g dw}$ of *P. mume* seeds extract, which is higher than in seeds in this research due to the extraction procedure (Table 1). Our results are in accordance with others

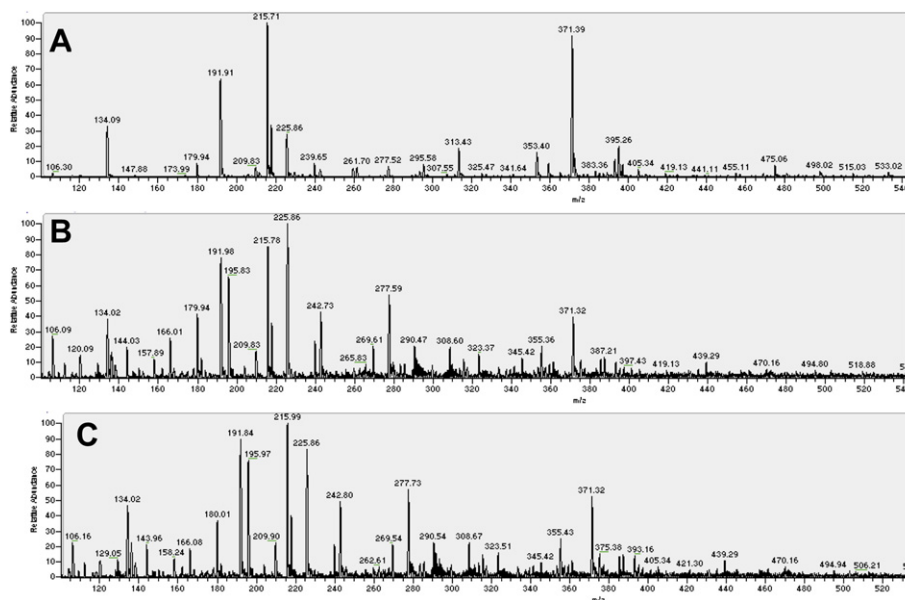


Fig. 6. ESI-MS spectra of water fractions of Seeds (A), Immature leaves (B) and Mature leaves (C) of indigo plant in negative ion mode.

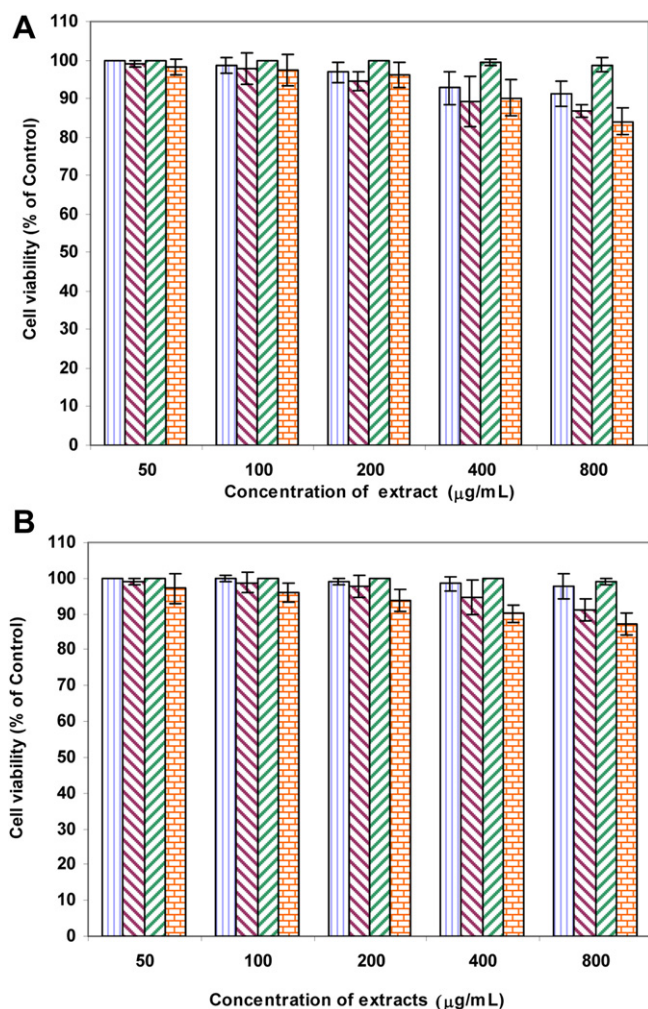


Fig. 7. Cell viability (% of control) of human cancer cells of the A, Calu-6 and B, Snu-601 lines, in the presence of indigo plant extracts: ■ Immature leaves; ▨ Mature leaves; ▩ Seeds; ▤ Prolipid. Each point represents the mean \pm SD ($n = 6$) different from the Control (■ and ▤) at a level of $p < 0.05$.

(Nantitanon et al., 2010), where hot water was the best solvent to extract the active principles. Total phenolic content and antioxidant activity with the TEAC was from 80 to 136 mg/g and 9 to 15 mmol/mg, respectively. These values are 5 times higher than the obtained in our results. Our results are in accordance with Fialova, Valigura, Tekelova, and Grancai (2009), where in leaves of *Isatis tinctoria* L. the following indices were determined: total polyphenols (3.03 g/100 g), tannins (1.05 g/100 g), total hydroxycinnamic derivatives (expressed as cyanidin-3-glucoside chloride 0.08 g/100 g), total flavonoids (expressed as isoquercitrin 0.3 g/100 g). The results obtained by ESI-MS/MS method were in correspondence with others (Falcão et al., 2010; Pawlak et al., 2006; Pellati, Orlandini, Pinetti, & Benvenuti, 2011), where typical phenolic acids and flavonoids, four of the novel phenolic compounds were methylated and/or esterified or hydroxylated derivatives of common popular flavonoids. Six peculiar derivatives of pinocembrin/pinobanksin, containing a phenylpropanoic acid derivative moiety in their structure, were also identified.

Antioxidant activity of *Cymbopogon schoenanthus* L. Spreng (lemon grass) shoots from three different locations in South Tunisia measured by DPPH assay showed that the proanthocyanidin extract exhibited higher antioxidant activity than the aqueous extract (Khadri et al., 2010). Extract concentration providing 50% inhibition (IC_{50}) ranged from 16.4 ± 6.8 µg/mL to 26.4 ± 6.8 µg/mL. The greatest acetylcholinesterase inhibitory activity ($IC_{50} = 0.23 \pm 0.04$ mg/mL) was exhibited by the ethyl acetate and methanol extracts of the plants collected from the mountainous region, as well as the polyphenols, µg pyrogallol per g dw, (methanol extract - 103.9; proanthocyanidin extract (ethyl acetate) - 129.8; aqueous extract - 61.8). The amounts of total flavonoids, µg rutin per g dw, were in methanol extract - 629; proanthocyanidin extract (ethyl acetate) - 1555; aqueous extract - 188. It seems that extracts obtained with more polar solvents gave better results. The DPPH test IC_{50} (µg/mL) for the same plant was for hexane, dichloromethane, ethyl acetate, methanol, proanthocyanidin extract and aqueous extracts were the following: 34.2, 29.8, 20.7, 17.9, 18.1, and 62.8. It was found that among 45 selected medicinal plants *Sargentodoxa cuneata* Rehd. *Et Wils*, *Fraxinus rhynchophylla* Hance, *Paeonia lactiflora* Pall, *Paeonia suffruticosa* Andr and *Scutellaria baicalensis* Georgi possessed the highest antioxidant capacities and thus could be potentially rich sources of natural antioxidants (Li et al., 2008). Our data were similar to the following medicinal plants: *Cremastra variabilis* (BL.) Nakai

(FRAP, $\mu\text{mol Fe (II)/g}$, 6.97 ± 0.20 ; TEAC, $\mu\text{mol Trolox Equivalent (TE)/g}$, 6.55 ± 0.41 ; phenolic content, mg GAE/g , 1.17 ± 0.05), *Dictamnus dasycarpus* Turcz. (FRAP, $\mu\text{M Fe (II)/g}$, 10.09 ± 0.40 ; TEAC, $\mu\text{mol TE/g}$, 5.49 ± 0.13 ; phenolic content, mg GAE/g , 2.92 ± 0.04), *Lycium chinense* Mill (FRAP, $\mu\text{mol Fe (II)/g}$, 37.40 ± 1.07 ; TEAC, $\mu\text{mol TE/g}$, 25.18 ± 1.00 ; phenolic content, mg GAE/g , 6.22 ± 0.20); *Prunella vulgaris* L. (FRAP, $\mu\text{mol Fe (II)/g}$, 56.08 ± 0.56 ; TEAC, $\mu\text{mol TE/g}$, 27.23 ± 0.89 ; phenolic content, mg GAE/g , 5.84 ± 0.15); *Pulsatilla chinensis* (Bge.) (FRAP, $\mu\text{mol Fe (II)/g}$, 67.81 ± 2.36 ; TEAC, $\mu\text{mol TE/g}$, 33.34 ± 1.24 ; phenolic content, mg GAE/g , 9.72 ± 0.21). A strong correlation between TEAC values and those obtained from FRAP assay implied that antioxidants in these plants were capable of scavenging free radicals and reducing oxidants. A high correlation between antioxidant capacities and their total phenolic contents indicated that phenolic compounds were a major contributor of antioxidant activity of these plants. Such conclusions were obtained as well from our results. Considerable variations were observed between Indigo and Prolipid herbal components in terms of anti-radical power examined by the DPPH-radical scavenging parameters in water extracts. Multivariate statistical and recognition techniques enabled visualisation of this complex dataset and underlying relationships responsible for clustering, differentiation and observed classification. Absorbance readings at 10, 30 and 90 min were identified as important parameters in clustering ability, according to the sample type. Prolipid herbal mixture water extracts exhibited the highest antiradical power as indicated by Trolox equivalents and IC_{50} values, whereas Indigo seeds extracts were characterised by relatively low levels of antioxidant activity. Canonical discrimination demonstrated a very good recognition and classification performance, allowing classification of 100% of the samples, according to their original groups. Multivariate techniques enabled deeper insights into the variations in the antiradical profiles between the examined samples.

High correlations between antiproliferative and antioxidant activities of medicinal plant extracts are usually encountered. In this regard, it was observed that at high concentrations of plant extract (800 $\mu\text{g/mL}$), the lowest antiproliferation against two different cancer cell lines was observed for seed extract (Fig. 7), with low antioxidant activity. The cell viability (%) of mature leaves was 86.87 ± 1.53 and 91.12 ± 3.12 in comparison with the Control sample ($p < 0.05$), showing its antiproliferation properties.

Our results on cytotoxicity are in accordance with others (Costa-Lotufu et al., 2005; Itharat et al., 2004; Lau et al., 2004). Kim, Jang, Shin, Shin, and Kim (2003) investigated ginseng radix, the root of *Panax ginseng*, the best known oriental medicinal herb, by the MTT assay, flow cytometry, in order to assess whether Ginseng radix possesses a protective effect against 1-methyl-4-phenylpyridine (MPP1)-induced cytotoxicity in PC12 neuronal cells. Cells treated with MPP1 exhibited various apoptotic features, while cell pre-treated with Ginseng radix prior to MPP1 exposure showed a decrease in the occurrence of apoptotic features. Costa-Lotufu et al. (2005) evaluated the anticancer potential of 11 plants used in Bangladeshi folk medicine. The plant extracts were tested for cytotoxicity by the brine shrimp lethality assay, sea urchin eggs assay, hemolysis assay and MTT assay, using tumor cell lines. The extract of *Oroxylum indicum* showed the highest toxicity on all tumor cell lines tested, with an IC_{50} of 19.6 $\mu\text{g/mL}$ for CEM, 14.2 $\mu\text{g/mL}$ for HL-60, 17.2 $\mu\text{g/mL}$ for B-16 and 32.5 $\mu\text{g/mL}$ for HCT-8. On the sea urchin eggs, it inhibited the progression of cell cycle since the first cleavage ($\text{IC}_{50} = 13.5 \mu\text{g/mL}$). The extract of *Aegle marmelos* exhibited toxicity on all used assays, but in a lower potency than *O. indicum*. As was recently shown by Chon et al. (2008) and Heo et al. (2007) the Korean medicinal plants, which were used for a long time as traditional seasoned salads, possess anticancer activity. Most of the cited reports indicated that tryptanthrin and indirubin are responsible for

many of the biological activities of *P. tinctorium*, which are extracted by methanol or other organic solvents (Ishihara et al., 2000; Iwaki & Kurimoto, 2002; Iwaki et al., 2006; Kimoto et al., 2001; Zhong et al., 2005). The content of polyphenols and the antioxidant activities in extracts of *P. tinctorium* are much lower than that of Chungtaejeon tea (CTJ) or green tea reported in our previous research (Park et al., 2010). The contents of polyphenols, flavonoids and flavanols in CTJ methanol were $229.30 \pm 11.3 \text{ mg GAE/g DW}$, $15.24 \pm 0.8 \text{ mg CE/g DW}$, $109.10 \pm 5.1 \text{ mg CE/g DW}$, respectively, and significantly higher than in acetone extracts ($p < 0.05$). In water extracts of the same investigated teas the amount of polyphenols was higher than in methanol and acetone. Therefore we have used the water extracts of indigo leaves based on our previous research. Our studies on cytotoxicity are in correspondence with Iwaki and Kurimoto (2002) and Iwaki et al. (2006). We consider as these authors, that it is likely that *P. tinctorium* shows cancer preventive activity as a consequence of the integral effects of polyphenol substances, such as gallic and caffeic acids, quercetin. The obtained MS data confirmed that the mature leaves have higher amount of bioactive substances than the immature ones. The ability of indigo leaves water extract to quench the intrinsic fluorescence of BSA determined by 3-FL and FTIR was relatively lower than Prolipid which is widely used as a medical plant for atherosclerosis.

5. Conclusions

The antioxidant activity of the studied water extracted samples is significantly different ($P < 0.05$): according to the used tests was significantly minimal ($P < 0.05$) in indigo seeds, the highest was in prolipid, following by indigo mature leaves. The correlations between the polyphenols compounds and the antioxidant activities were relatively high (R^2 from 0.82 to 0.99). DPPH kinetic measurements compared, distinguished and discriminated the antiradical activity among indigo and prolipid water extracts by multivariate analysis. The ability of water extract of indigo mature leaves to quench the intrinsic fluorescence of BSA, relatively high content of bioactive compounds, positive antioxidant and anti-proliferative properties of indigo justify the use of this plant as a new source of valuable antioxidants.

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