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Chemical Composition, Antioxidant and Anticancer Effects of the Seeds and Leaves of Indigo (*Polygonum tinctorium Ait.*) Plant

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Abstract Seeds and leaves of indigo (*Polygonum tinctorium Ait*.) plant were investigated and compared with another medicinal plant named prolipid for their properties such as chemical composition, antioxidant, and anticancer effects by Fourier transform infrared, three-dimensional fluorescence spectroscopy, and electrospray ionization-MS in negative mode. It was found that polyphenols, flavonoids, and flavanols were significantly higher in prolipid ($P<0.05$), following by indigo mature leaves, immature leaves, and seeds. Methanol extract of mature indigo leaves in comparison with the ethyl acetate extract showed higher

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inhibition of proliferation. The interaction between polyphenol extracts of indigo mature leaves and BSA showed that indigo has a strong ability, as other widely used medicinal plants, to quench the intrinsic fluorescence of BSA by forming complexes. In conclusion, indigo mature leaves were compared with prolipid. High content of bioactive compounds, antioxidant, fluorescence, and antiproliferative properties of indigo justifies the use of this plant as a medicinal plant and a new source of antioxidants.

Keywords Indigo · Extracts · Bioactive compounds · Antioxidant and anticancer activities

Introduction

Indigo (*Polygonum tinctorium Ait.*) is an 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 [1, 2]. The composition of the natural dyes was determined after the extraction procedures with different solvent systems [3]. Indigo naturalis is used by traditional Chinese medicine to treat various inflammatory diseases [4]. Some wild indigo species as herbal drugs were evaluated [5]. The data concerning the anticancer activity of indigo are very limited [6]. There are still few data on indigo plant; therefore, it is possible to compare it with other better investigated medicinal plants. Plants are a source of compounds that may be used as pharmacologically active products. *Cytisus multiflorus*, *Filipendula ulmaria* and *Sambucus nigra* have been used as important medicinal plants in the Iberian Peninsula for many years and are claimed to have various health benefits as indigo plant [7]. It was shown using chemical, biochemical, and electrochemical assays that these wild plants are source of phytochemicals and antioxidant potential [7]. Also Dall'Acqua, Cervellati, Loi, and Innocenti [8] examined the antioxidant capacities of 11 botanical species used in the tradition of Sardinia as tea beverages or as decoction for medicinal purposes. The anti-metastasis and immune-stimulating activities of EtOH extracts of fermented Korean red ginseng (FRG-E) in animal and human subjects was investigated [9]. The antioxidant properties of phenolic compounds from olive pulp of *chamlal* variety and those of individual phenolic compounds were evaluated and compared with that of vitamin C [10]. Generalic et al. [11] studied the phenolic profile and antioxidant properties of Dalmatian sage. Another plant as *Lithospermum erythrorhizon* could be a promising rich source of natural antioxidants [12]. It was of interest to know if also methanol and ethyl alcohol extracts of indigo have the same properties as some other plants. Therefore, in addition to determination of the bioactive compounds content, especially phenolics, and antioxidant activity of indigo, also its anticancer properties were examined. As far as we know, there are no published results of such investigations.

Methods and Materials

Chemicals

6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox); gallic acid; quercetin; Tris, tris(hydroxymethy1)aminomethane; 2,2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS); Folin–Ciocalteu reagent; lanthanum (III) chloride heptahydrate; $FeCl_3 \cdot 6H_2O$; $CuCl_2 \cdot 2H_2O$; 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. Deionized and distilled water was used throughout.

Testing Samples Preparation

There are three samples of indigo plant (*P. tinctorium*): seeds and two samples 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. Two samples of leaves were used because one was mature and the other—immature. The most important is to find exact data when the leaves can be harvested. 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 [13]. 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 *S. arvensis* (10 g/100 g dw). Prolipid capsules were obtained as a gift from the drug importer COWIK (Warsaw, Poland).

Extraction of Polyphenols

The extracts from seeds and leaves were prepared by the same way for all tests (bioactive compounds, antioxidant, and anticancer assays). The phenols were extracted with methanol and ethyl acetate from the indigo powder, seeds, or the prolipid (concentration 25 mg/mL) at room temperature twice for 3 h [14]. The prolipid capsules were opened and the content was dissolved in the same solvents at the same conditions.

Extraction of Phenolic Compounds for MS

The lyophilized sample of indigo plant (1 g) was 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 extracted with diethyl ether and ethyl acetate, and then the remainder of the aqueous solution was freeze-dried. The organic fractions were dried and redissolved in methanol. These extracts were used for MS, for determination of bioactive compounds and Fourier transform infrared (FTIR) analyses [15].

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 3,000 V, sheath gas pressure 35 AU, ion sweep gas pressure 0 AU, auxiliary gas pressure 30 AU, capillary temperature 200 °C, and skimmer offset 0 V.

Total Phenolic Content

The polyphenols were determined by Folin–Ciocalteu method with measurement at 750 nm with a spectrophotometer (Hewlett-Packard, model 8452A, Rockville, USA). The results were expressed as milligrams of gallic acid equivalents (GAE) per gram DW [16].

Total Flavonoid Content

Total flavonoid content was determined by an aluminum chloride colorimetric method [17]. 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 % $NaNO_2$ solution was added to the mixture. After 6 min, 150 μL of a 10 % $AlCl_3 \cdot 6H_2O$ solution was added, and the mixture was allowed to stand for another 5 min. Half of a milliliter of 1 M NaOH was added, and the total was made up to 2.5 mL with distilled water. The solution was well mixed, and the absorbance was measured immediately against the prepared blank at 510 nm in comparison with the standards prepared similarly with known (+)-catechin concentrations. The results are expressed as milligrams of catechin equivalents.

Total Tannins Content

To 50 μL of methanol extract of the plant sample, 3 mL of a 4 % methanol vanillin solution and 1.5 mL of concentrated hydrochloric acid were added. The mixture was allowed to stand for 15 min. The absorption was measured at 500 nm against methanol as a blank. The amount of total condensed tannins is expressed as (+)-catechin equivalents per gram of the sample [18].

Total Flavanols Content

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 [19]. As it was mentioned previously, (+)-catechin served as a standard for flavonoids, flavanols, and tannins, and the results were expressed as catechin equivalents (CE).

Antioxidant Activity by 2,2-Azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid) Diammonium Salt ($ABTS^{\cdot+}$) Method

$ABTS^{\cdot+}$ radical cation was generated by the interaction of ABTS (7 mM/L) and $K_2S_2O_8$ (2.45 mM/L). This solution was diluted with methanol until the absorbance in the samples reached 0.7 at 734 nm [20].

Antioxidant Activity by Ferric-Reducing/Antioxidant Power

This assay measures the ability of the antioxidants in the investigated samples to reduce ferric-tripyridyltriazine (Fe^{3+}–TPTZ) to a ferrous form (Fe^{2+}). FRAP reagent (2.5 mL of a 10 mM ferric-tripyridyltriazine solution in 40 mM HCl plus 2.5 mL of 20 mM $FeCl_3 \cdot H_2O$ and 25 mL of 0.3 M acetate buffer, pH 3.6) of 900 μL was mixed with 90 μL of distilled water and 30 μL of plant samples or methanol as the appropriate reagent blank. The absorbance was measured at 595 nm [21].

Antioxidant Activity by Cupric Reducing Antioxidant Capacity

This assay is based on utilizing the copper(II)-neocuproine reagent as the chromogenic oxidizing agent. To the mixture of 1 mL of copper (II)-neocuproine and NH_4Ac buffer

solution, acidified and non-acidified methanol extracts (or standard) solution (x, in milliliters) and H_2O [$(1.1-x)$ in milliliters] were added to make the final volume of 4.1 mL. The absorbance at 450 nm was recorded against a reagent blank [22].

Fluorimetry

Two-dimensional- and three-dimensional (3D-FL) fluorescence measurements were done using a model FP-6500, Jasco Spectrofluorometer, serial N261332, Japan [23]. Fluorescence emission spectra for all indigo samples at a concentration of 0.001 mg/mL were taken at emission wavelength (in nanometers) of 330 and recorded from wavelength of 265 nm to a wavelength of 310 nm, at emission wavelengths of 685 nm from 260 to 750 nm, and at excitation of 350 nm from 250 to 500 nm. 3D-FL spectra of the investigated plant extracts were collected with subsequent scanning emission spectra from 260 to 750 nm at 1.0 nm increments by varying the excitation wavelength from 260 to 500 nm at 10 nm increments. The scanning speed was set at 1,000 nm/min for all measurements. All measurements were performed with emission mode and with intensity up to 1,000. 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^{-5} mol/L. All solutions were kept in dark at 0–4 °C. The BSA was mixed with methanol or with ethyl acetate extracts of 20 μl. The samples were mixed in the properties of BSA/extract=1:1. The samples after the interaction with BSA were lyophilized and subjected to FTIR.

Fourier Transform Infrared Spectra Studies

The presence of polyphenols in the investigated extracts of indigo samples was studied by FTIR spectroscopy. A Nicolet iS 10 FTIR Spectrometer (Thermo Scientific Instruments LLC, Madison, WI, USA), with the smart iTR™ attenuated total reflectance accessory, was used to record IR spectra [24–26].

MTT Assay

Anticancer activity of methanol and ethyl acetate extracts of the studied plants on human cancer cell lines (Calu-6 for human pulmonary carcinoma and SNU-601 for human gastric carcinoma) was measured using MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay. The cell lines were purchased from the Korean Cell Line Bank for MTT assay. Cells were grown in RPMI-1640 medium at 37 °C under 5 % CO_2 in a humidified incubator. 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 a final concentration at 25, 50, 100, 200, 400, and 800 μg mL^{-1}. Stock solutions of samples were prepared for cell lines at 90 μL and samples at 10 μL and incubated for 72 h. MTT solution at 5 mg mL^{-1} was dissolved in 1 mL of phosphate buffer solution, 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 anticancer activity was obtained by comparing the absorbance between the samples and the control [27].

Statistical Analyses

To verify the statistical significance, mean ± SD of five independent measurements was calculated. Differences between groups were tested by two-way 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.

Results

Bioactive Compounds

The results were summarized in the Table 1. As can be seen, the significantly highest content of polyphenols and flavonoids was in prolipid and mature leaves, flavanols—in seeds, and tannins—in prolipid, immature, and mature leaves ($P<0.05$ in all cases).

Mass Spectra

Gallic acid (Fig. 1a) and quercetin (Fig. 1b) were used as standards. The spectrum shows the main m/z peaks found in seeds (Fig. 2a) in methanol fraction: at 106, relative abundance (RA)=58 %; benzoic acid at 120 has RA=100 %; and methyl vanillate at 180 has RA=18 %, at 214 RA=40 %. The peaks in seeds were not found in immature leaves at the same location (Fig. 2b): at 104, RA=20 %; in comparison with the one at 106, RA was higher as twice as in leaves; instead of peak at 120, the peak appeared at 134 (RA=75) of p-hydroxybenzoic acid; at 192 (RA=100 %) of scopoletin; at 356 (RA=40 %) and at 365 (RA=18 %). The same fraction for mature leaves showed the following peaks (Fig. 2c): one of the main peaks was located at 134 for p-hydroxybenzoic acid with RA=95 %, slightly higher than for immature

Table 1 Bioactive compounds in methanol extracts of the studied samples per dry weight (DW)

Sample	Polyphenols mg GAE	Flavonoids mg CE	Flavanols μg CE	Tannins mg CE
Seeds	5.14±0.3 a	3.842±0.2 a	1,568.95±79.1 b	1.14±0.05 a
Immature leaves	11.55±0.5 b	5.175±0.2 a	432±4.4 c	2.56±0.1 b
Mature leaves	14.22±0.7 c	6.079±0.3b	213±2.1 d	2.95±0.2b
Prolipid	16.64±0.7 c	6.566±0.3 b	1,109.65±54.2 a	3.18±0.3 b

Values are means ± SD of five measurements. Values in columns for every bioactive compound with the same solvent bearing different letters are significantly different ($P<0.05$).

CE catechin equivalent, *GAE* gallic acid equivalent

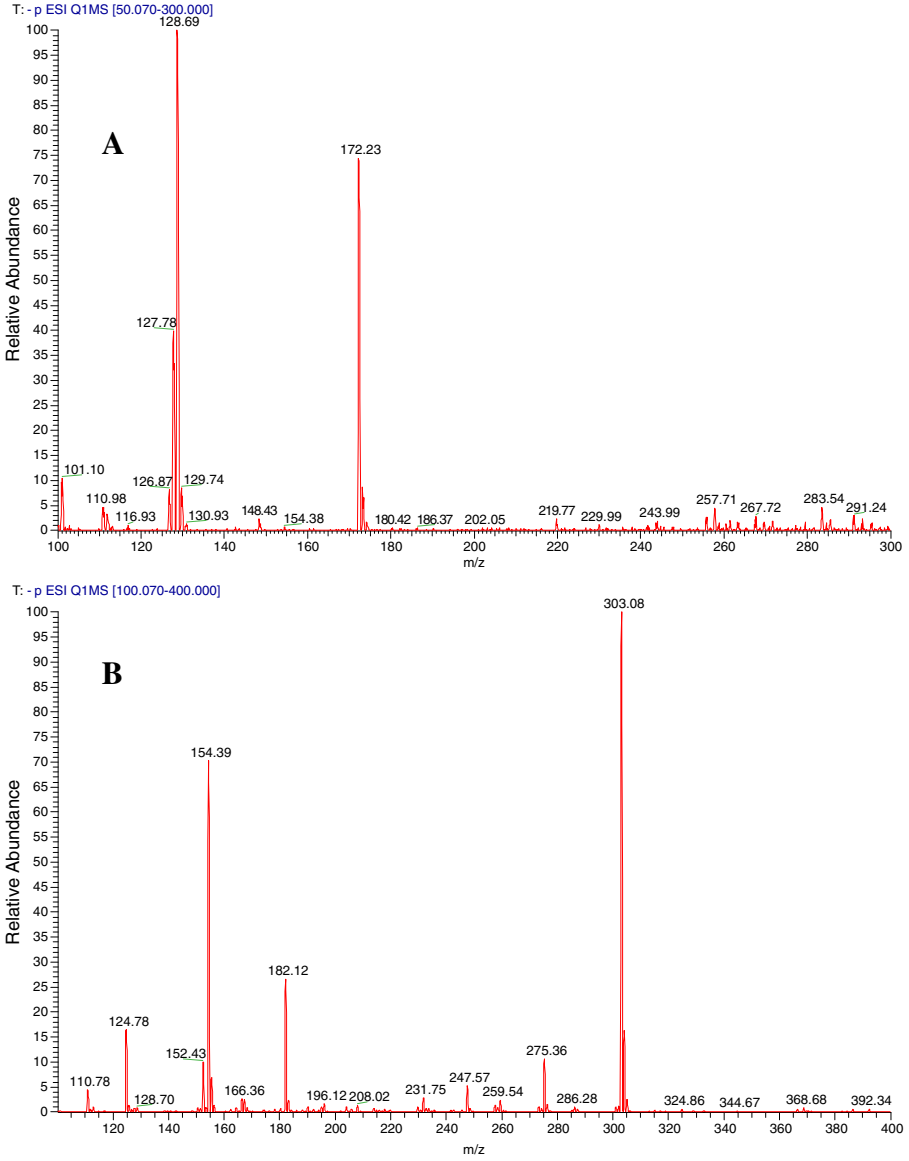
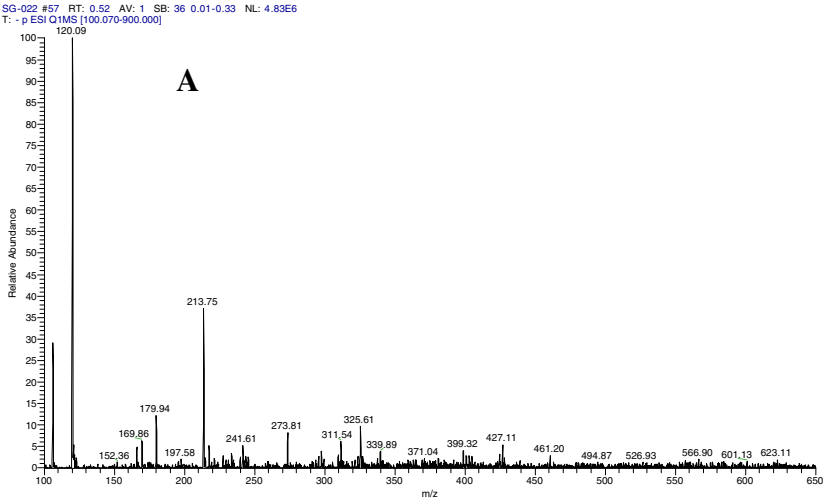
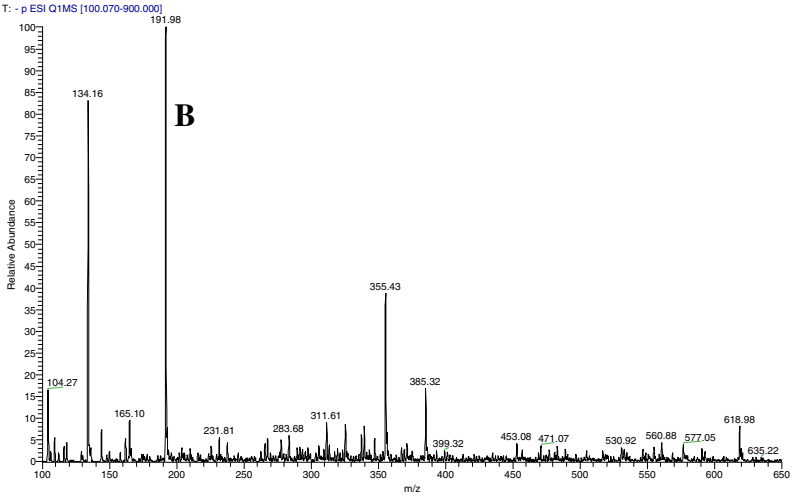


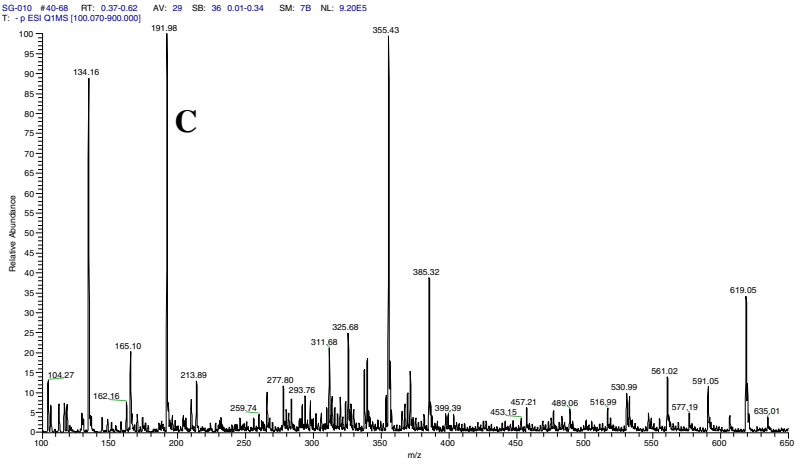
Fig. 1 ESI-MS spectra of **a** gallic acid; **b** quercetin

ones; at 192 (RA=100 %) of scopoletin, than the other ones at 355 (RA=100 %), 365 (RA=40 %), and 611 (RA=20 %).

The spectrum shows the main *m/z* peaks found in seeds (Fig. 3a) in ethyl acetate fraction: at 174 (RA=20 %) for coniferaldehyde, at 188 (RA=100 %), at 312 (RA=45 %), at 330 (RA=65 %), and at 340 (RA=40 %). Only two peaks were in immature leaves (Fig. 3b) with slightly different abundance such as at 314 with RA of 50 % and at 340 with RA of 75 %. Other peaks were for *p*-hydroxybenzoic acid at 138 (RA=20 %); vanillic acid at 166 (RA=100 %); at 266 (RA=40 %) for apigenin; at 294 (RA=100 %) for catechin, at


SG-022 #57 RT: 0.52 AV: 1 SB: 36 0.01-0.33 NL: 4.83E6
T: - p ESI Q1MS [100.070-900.000]
A
Relative Abundance
m/z
120.09
213.75
179.94
169.86
152.36
197.58
241.61
273.81
311.54
325.61
339.89
371.04
399.32
427.11
461.20
494.87
526.93
566.90
601.13
623.11


T: - p ESI Q1MS [100.070-900.000]
B
Relative Abundance
m/z
191.98
134.16
104.27
165.10
231.81
283.68
311.61
355.43
385.32
399.32
453.08
471.07
530.92
560.88
577.05
618.98
635.22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T: - p ESI Q1MS [100.070-900.000]
C
Relative Abundance
m/z
191.98
355.43
134.16
385.32
619.05
325.68
311.68
165.10
104.27
213.89
277.80
293.76
162.16
259.74
399.39
453.15
457.21
489.06
516.99
530.99
561.02
577.19
591.05
635.01

Fig. 2 ESI-MS spectra of methanol fractions of seeds (**a**), immature leaves (**b**), and mature leaves (**c**) of indigo plant in negative ion mode

326 with RA=70 % and 619 with RA of 15 %. The peaks appeared for mature leaves in the same fraction were the following (Fig. 3c): at 134 (RA=55 %) for *p*-hydroxybenzoic acid, the same peak was in immature leaves with slight shift. Another peaks appeared at 165 (RA=100 %) for vanillic acid for both leaves; at 192 (RA=50 %) for scopoletin only in immature leaves; at 215 (RA=50 %); at 286 (RA=50 %); at 294 (RA=85 %) for catechin for both leaves; and at 330 (RA=60 %) and at 618 (RA=50 %) which were shown in both leaves. The obtained results showed the same location of the peaks in both leave samples, only with higher amounts of the compounds showing different relative abundances.

The Antioxidant Activity

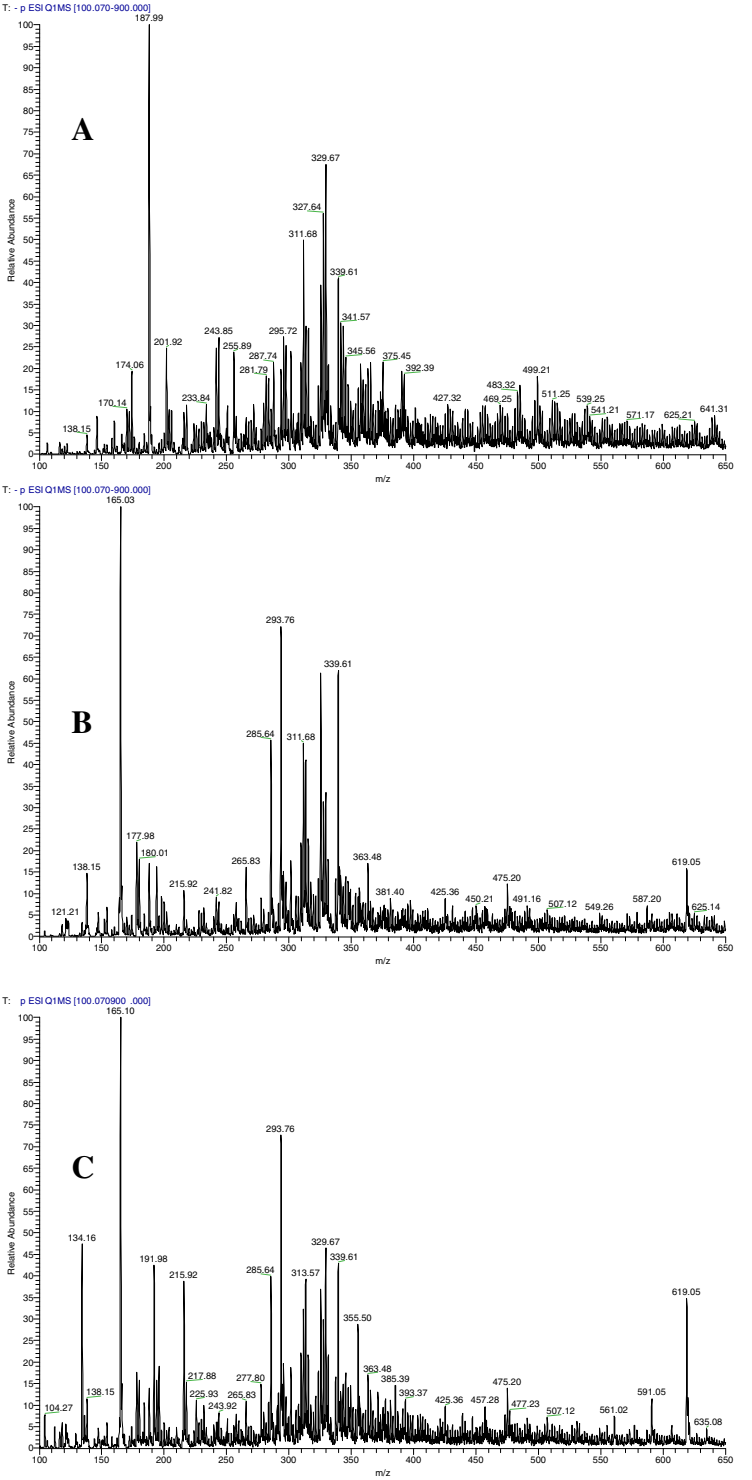
The results of the determination of the antioxidant activity in the studied samples are summarized in the Table 2. As can be seen, according to ABTS test, the significantly higher antioxidant activity was in mature leaves, according to CUPRAC—in prolipid and mature leaves, and according to FRAP—in prolipid, mature, and immature leaves ($P<0.05$ in all cases).

Fluorimetry

3D-FL (Fig. 4A, B) spectra illustrated the elliptical shape of the contour maps (Aa, Ba) and cross maps (Fig. 4Ab, Bb) of the main peaks for indigo methanol and ethyl acetate extracts of mature leaves. The main peaks for methanol extracts appeared at λex/em of 260/310 with fluorescence intensity (FI) of 889.58 and another one at λex/em of 260/360 nm with FI of 776.07 (Fig. 4Aa, C, Ab). The ethyl acetate fraction had slightly different peaks: at λex/em of 260/320 with FI of 169.59 and at λex/em of 260/360 nm with FI of 165.94 (Fig. 4Ba, D, Bb). One of the main peaks for 2×10^{-5} M/L BSA was found at λex/em of 225–230/335 nm with FI of 877.60 (Fig. 4E, upper curve). The interaction of BSA and ethyl acetate extract of indigo mature leaves (Fig. 4E, middle curve, with FI=715.61) and BSA and indigo (Fig. 4E, lower curve, with FI=650.81) showed the peak of 335 nm and decrease in the fluorescence intensity (FI). These results are in correspondence with the amount of polyphenols, antioxidant activity, and MS bioactivity data that the methanol extract is more bioactive than the ethyl acetate. The decrease in fluorescence intensity of BSA was about 18.5 % for the ethyl acetate fraction and 25.8 % for methanol fraction, showing higher quenching activity of methanol extracts of polyphenols. The interaction between methanol and ethyl acetate polyphenol extracts of indigo and BSA showed that indigo has a strong ability as other studied medicinal plants to quench the intrinsic fluorescence of BSA by forming complexes.

FTIR Spectra

The FTIR spectra of methanol extract of prolipid (Fig. 5A, upper curve) was compared with ethyl acetate extract of indigo mature leaves (Fig. 5B, middle curve) and with methanol extract of indigo mature leaves (Fig. 5C, lower curve). Noticeably, the presence of wavelengths of FTIR spectra of gallic acid at 860, 1,025, 1,100, and 1,654 cm^{-1}, tannic acid at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A
Relative Abundance
m/z
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329.67
327.64
311.68
339.61
341.57
295.72
243.85
255.89
201.92
287.74
345.56
375.45
174.06
281.79
392.39
499.21
483.32
511.25
539.25
427.32
469.25
541.21
571.17
625.21
641.31
170.14
233.84
138.15
T: - p ESI Q1MS [100.070-900.000]
B
Relative Abundance
m/z
165.03
293.76
339.61
285.64
311.68
177.98
180.01
138.15
265.83
363.48
215.92
241.82
381.40
425.36
475.20
450.21
491.16
507.12
549.26
587.20
619.05
625.14
121.21
T: p ESI Q1MS [100.070900 .000]
C
Relative Abundance
m/z
165.10
293.76
134.16
329.67
339.61
191.98
215.92
285.64
313.57
619.05
355.50
363.48
385.39
475.20
217.88
277.80
138.15
225.93
265.83
393.37
425.36
457.28
477.23
591.05
104.27
243.92
507.12
561.02
635.08

◀ **Fig. 3** ESI-MS spectra of ethyl acetate fractions of seeds (**a**), immature leaves (**b**), and mature leaves (**c**) of indigo plant in negative ion mode

1,172, 1,511, and 1,627, and *p*-coumaric acid at 1,124, 1,171, 1,508, and 1,638 cm^{-1} were observed in samples analyzed. The wavelength of FTIR spectra corresponding for vanillin was 1,498, 1,534, 1,617, 1,654, and 3,392 cm^{-1} [24, 26]. The main bands presenting in the samples are the following: the band of 1,029 cm^{-1} (−C-O alcohols) is exactly found in ethyl acetate extract of mature leaves (Fig. 5, line b) with a small shift at 1,017 cm^{-1} for the dry substance (Fig. 5, line a) and for methanol extract of 1,033 cm^{-1} (Fig. 5, line c). The band of 1,280 cm^{-1} (−OH aromatic) appeared in slightly different location of 1,201 cm^{-1}. Other peaks appeared at 1,319–1,397 cm^{-1}. The peak of 1,422 cm^{-1} (−C-O alcohols) appeared only in prolipid. The peak of 1618 cm^{-1} (C=C aromatic and C=C alkenes) appeared in all the samples with a shift at 1,650 and 1,597 cm^{-1} for carbonyl substituents. The broad band of 3,309, 2,925 and 2,917 cm^{-1} belong to glycosidic groups O-H. FTIR of quercetin as a standard showed broad phenolic OH band centered around 3,404 cm^{-1}, characteristic -CO stretching at 1,663 cm^{-1} aromatic bending and stretching around 1,091 and 1,663 cm^{-1}, and -OH phenolic bending around 1,197 and 1,374 cm^{-1} [28]. FTIR spectra of water extracts of mature indigo leaves [28] showed a peak characteristic -CO stretching at 1,634 cm^{-1} aromatic bending and the peaks at 2,925 and 2,852 cm^{-1} are related to the C–H bond of saturated carbons, which are different from our results of methanol and ethyl acetate extracts. Matching between the peaks in the range from 4,000 to 400 cm^{-1} of (prolipid methanol extract)/(indigo ethyl acetate extract)=65.08 %, (prolipid methanol extract)/(indigo methanol extract)=76.52 %, and (indigo ethyl acetate extract)/(indigo methanol extract)=69.41 % (Fig. 5). Matching between the peaks of the water extracts of indigo mature leaves and the same substances in the same range of the peaks in prolipid was slightly higher of about 78.38 % [28].

Anticancer Activity

It was observed that the percentage of proliferativity of the methanol and ethyl acetate extracts of mature leaves and prolipid samples on two cell lines (Fig. 6a, Calu-6 for human pulmonary carcinoma and Fig. 6b, SNU-601 for human gastric carcinoma) was different. The proliferativity (in percent) for concentrations of 800 μg/mL for methanol and ethyl acetate extracts of prolipid on Calu-6 were 75.49 and 79.24 %, respectively, and on SNU-601 were 77.42 and 80.45 %, showing the highest antiproliferative activity in comparison

Table 2 Antioxidant activities (in micromole Trolox equivalents) in methanol extracts of the studied samples per dry weight (DW)

Sample	ABTS	CUPRAC	FRAP
Seeds	68.326±3.4 b	29.27±1.3 a	12.21±0.6 a
Immature leaves	134.438±6.6 c	30.62±1.4 a	19.79±0.9 b
Mature leaves	185.464±9.1 d	59.46±2.8 b	20.91±1.1 b
Prolipid	206.24±20.2a	64.65±3.1 b	22.68±1.2 b

Values are means ± SD of five measurements. Values in columns for every bioactive compound with the same solvent bearing different letters are significantly different ($P<0.05$).

ABTS 2,2-Azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt, *CUPRAC* cupric reducing antioxidant capacity, *FRAP* ferric-reducing/antioxidant power

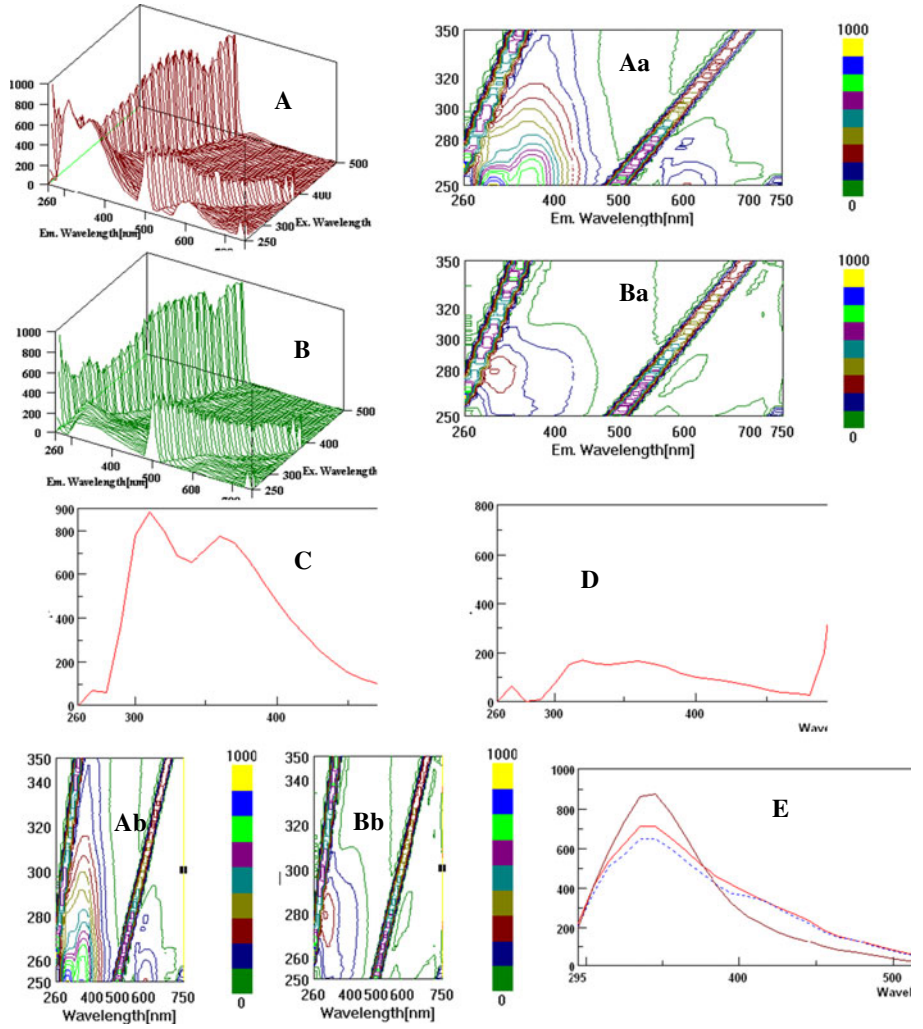

Fig. 4 *A* three-dimensional fluorescence (3D-FL) spectrum of methanol extract (0.001 mg/mL) of indigo mature leaves, *Aa* elliptical shapes of the contours of methanol extracts of indigo mature leaves, *B* 3D-FL spectrum of ethyl acetate extract (0.001 mg/mL) of indigo mature leaves, *Ba* elliptical shapes of the contour map of ethyl acetate extracts of indigo mature leaves, *C* 2D-FL of methanol extract of indigo mature leaves, *D* 2D-FL of ethyl acetate extract of indigo mature leaves, *Ab* cross maps of methanol extracts of indigo mature leaves, *Bb* cross maps of ethyl acetate extracts of indigo mature leaves, *E* change in the fluorescence intensity (ID) as a result of binding affinity of: 2.0×10^{-4} mol/L of BSA (*upper line*); BSA and 40 μg/mL of indigo leave methanol extract (*middle line*); BSA and 40 μg/mL of indigo leave ethyl acetate extract (*lower line*); the 3D-FL were run emission mode and fluorescence intensity up to 1,000, emission wavelengths from 260 to 750 nm and excitation wavelengths from 250 to 500 nm; scanning speed was 1,000 nm/min, For *Aa*, *Ba*, *C*, *D*, *Ab*, *Bb*, and *E*, emission wavelength on x-axis and fluorescence intensity on y-axis for *C*, *D* and *E*; for *Aa*, *Ba*, *Ab* and *Bb*, excitation wavelength on y-axis

with mature leave sample for Calu-6 (76.12 and 80.22 %) and SNU-601 (79.43 and 82.26 %). Our investigation shows that antioxidant activity of the studied samples was highly correlated with their antiproliferative activity.

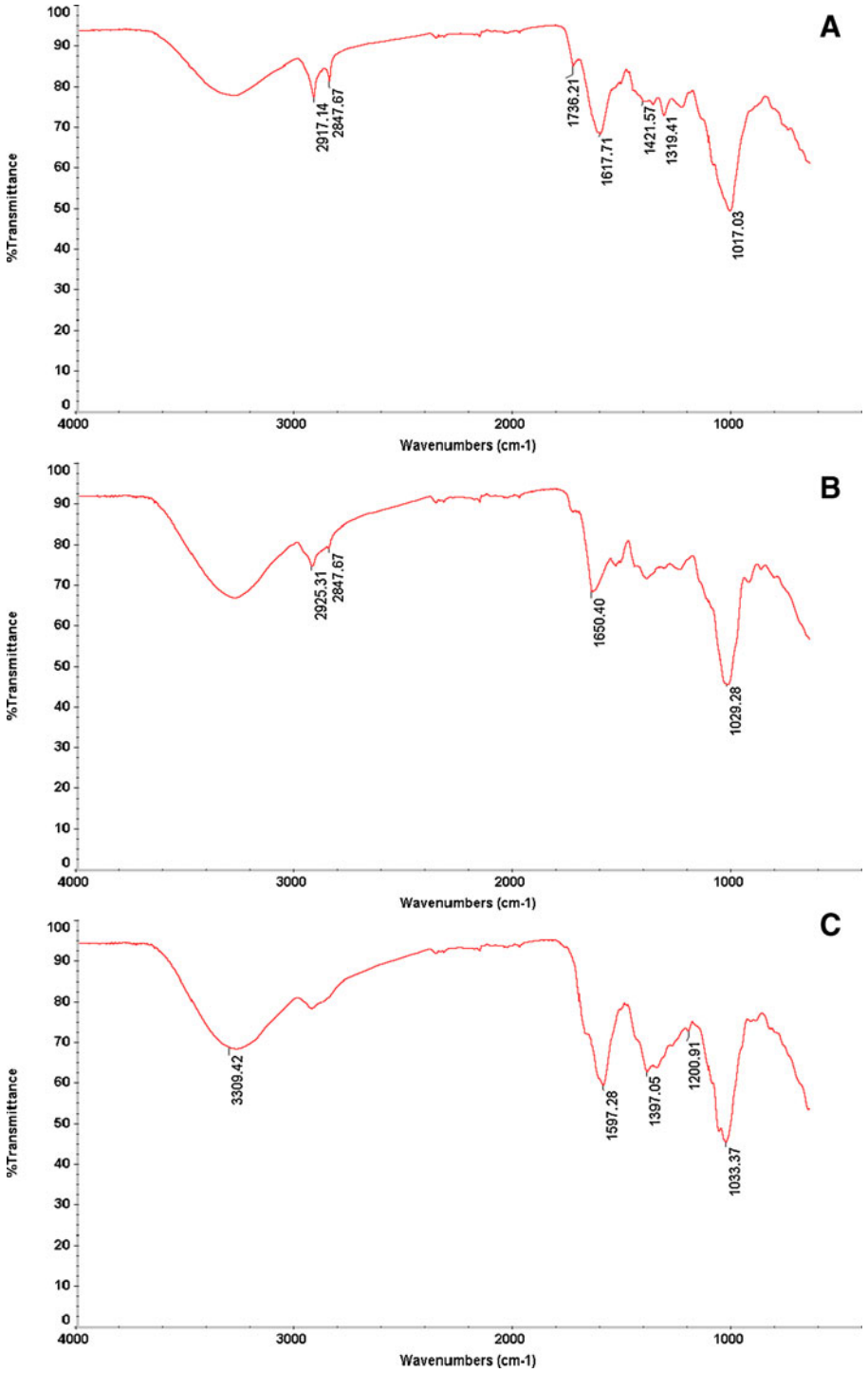

Fig. 5 Infrared study of FTIR spectra of **a** methanol extract of prolipid; **b** ethyl acetate extract of indigo mature leaves; and **c** methanol extract of indigo mature leaves

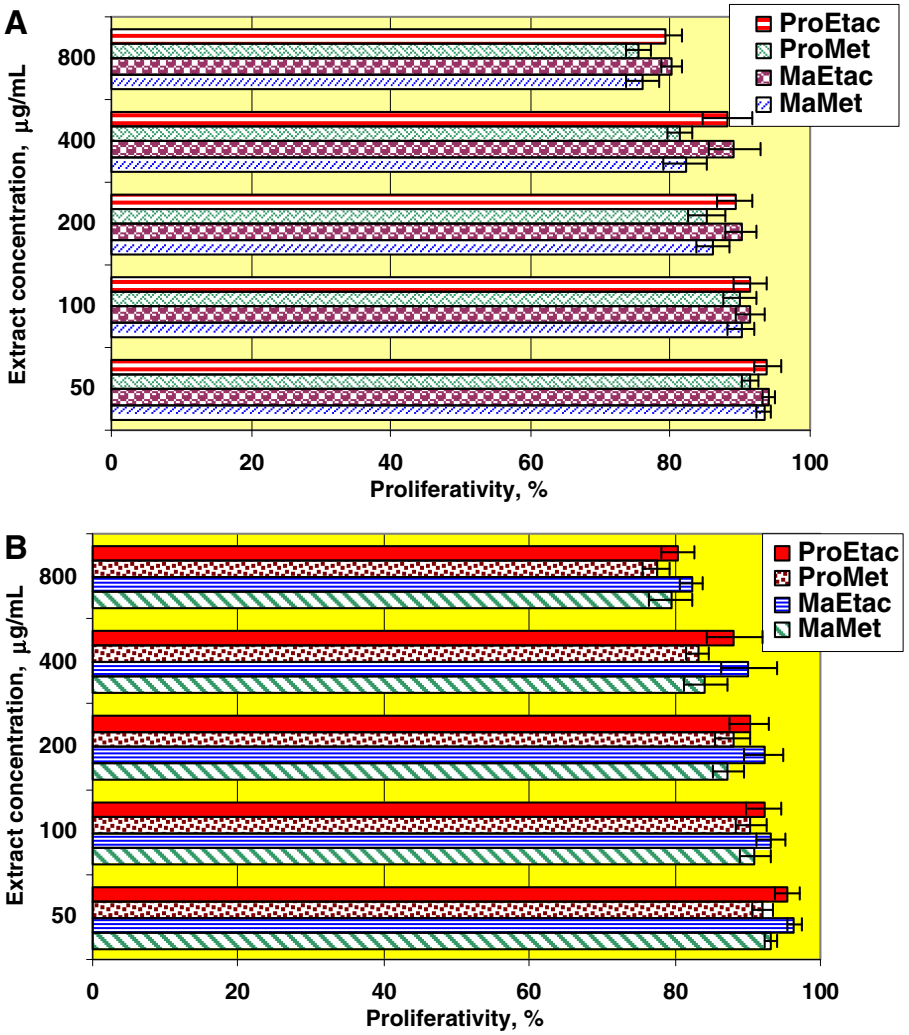

Fig. 6 The proliferativity (in percent) of human cancer cells of the **a** CALU-6 and **b** SNU-601 lines, in the presence of methanol and ethyl acetate indigo mature leaves and prolipid. Each point represents the mean ± SD (n=6). Abbreviations: *Proetac* prolipid ethyl acetate extract, *ProMe* prolipid methanol extract, *MaEtac* mature leaves ethyl acetate extract, *MaMet* mature leaves methanol extract

Discussion

The obtained results of our research can be connected with the recently performed experiments in order to find the scientific basis for the health properties of this plant [29], where the antinociceptive, anti-inflammatory, and antipyretic effects of indigo plant root methanolic extract were evaluated. The indigo plant root extracts significantly and dose-dependently inhibited the writhing responses of mice and decreased the licking time in both the early and late phases of the formalin test. However, as was stated, the research on the content of bioactive compounds and the antioxidant and anticancer activities of indigo was limited. Therefore, some other plants were

reviewed in order to compare the obtained results. So, Dall'Acqua et al. [8] evaluated in vitro antioxidant properties of some traditional medicinal plants: investigation of the high antioxidant capacity of *Rubus ulmifolius* used in Sardinia as tea beverages or as decoction for medicinal purposes. Among the various species, *R. ulmifolius* resulted as the more bioactive with all the used methods. Phytochemical investigation revealed several phenolic compounds as caffeic acid, ferulic acid, quercetin, kaempferol-3-O-glucuronide, kaempferol-3-O-(6"-p-coumaroyl)-β-D-glucopyranoside, kaempferol-3-O-(6"-caffeoyl)-β-D-glucopyranoside, and many others which are in accordance with our MS data, which are responsible for the antioxidant properties. Our results can be compared with Generalic et al. [11], who studied the phenolic profile and antioxidant properties of Dalmatian sage. The results strongly indicate that Dalmatian sage leaves are rich source of valuable phenolics, mainly phenolic acids, with extremely good antioxidant properties. The presence of resveratrol or its derivates was confirmed in all extracts. The authors found that the best results for total phenols and flavonoids, as well as the best antioxidant properties were obtained for May sage. Our results can be compared with the phytochemical composition and antioxidant activity of wild medicinal plants, based on chemical, biochemical, and electrochemical methods. So, *F. ulmaria* was found to be the richest in antioxidant phytochemicals, such as phenolics (228 mg GAE/g DW) and flavonoids (62 mg CE/g DW). The antioxidant activity was found to vary in the order: *F. ulmaria*>*S. nigra*>*C. multiflorus*, irrespective of the analysis method [7]. Seven compounds related to flavonoids and a mixture of two caffeic acid esters were isolated from *L. erythrorhizon* Sieb et. Zucc. and identified by spectroscopic methods with good radical scavenging activities toward ABTS but showed moderate inhibition of DPPH [12]. The presented MS data (Figs. 1, 2, and 3) were in accordance with others [2], where for direct identification of the organic dye compounds quercetin, indigotin, and alizarin in reference materials, in solution, by use of direct analysis in real time ionization and high-resolution time-of-flight mass spectrometry was done. These data are in accordance with Mantzouris et al. [1] that the treatment by the standard HCl dyestuff extraction method revealed different flavonoids and phenolic acids, where some of them are listed: apigenin, ellagic acid, fisetin, indigotin, indirubin, kaempferol, naringenin, quercetin, and others. Our results exactly in accordance with others [3], where the composition of the natural dyes was determined after different extraction procedures. The efficiency of eight different procedures used for the extraction of natural dyes was evaluated using contemporary wool samples dyed with cochineal, madder, woad, weld, brazilwood, and logwood. Comparison was made based on the LC-DAD peak areas of the natural dye's main components which had been extracted from the wool samples. Among the tested methods, an extraction procedure with Na_2EDTA in water/DMF (1:1, *v*/*v*) proved to be the most suitable for the extraction of the studied dyes, which presented a wide range of chemical structures [3].

The present results can be compared with our recent ones [28], where the water extract of indigo plant was analyzed. In water extract, the polyphenols and flavonoids were significantly higher in prolipid, flavanols—in indigo seeds. Our results are in accordance with Fialova et al. [30], where in leaves of *Isatis tinctoria* L. the following indices were determined: total polyphenols (3.03 %), tannins (1.05 %), and total flavonoids (expressed as isoquercitrin 0.3 %). The phenolic compounds showed higher radical scavenging activity that vitamin C [10]. The antioxidant activity was the highest in prolipid, followed by indigo mature leaves. Exactly the same relationship was obtained in methanol and ethyl acetate extracts, but the highest

value was in methanol fraction [28]. The composition of the indigo plant depends on the extraction procedure. Results of the study of five plants, of which four are endemic to Turkish flora [31] showed that the plants were screened for their possible in vitro antioxidant activities by two complementary test systems (DPPH and β-carotene/linoleic acid). In the first case, *Pelargonium endlicherianum* extract exerted greater antioxidant activity with an IC_{50} value of 7.43±0.47 μg/mL, followed by *Hieracium cappadocicum* of 30.0±0.14 μg/mL. When compared to the synthetic antioxidant BHT (18.0±0.40 μg/mL), the methanolic extract of *P. endlicherianum* exhibited more than twofold greater antioxidant activity. In the β-carotene/linoleic acid test system, the most active plant was *P. endlicherianum* with 72.6 %±2.96 inhibition rate, followed by *H. cappadocicum* (55.1 %±2.33) and *Verbascum wiedemannianum* (52.5 %±3.11). The results of antioxidant activities of indigo plant (Table 2) are in agreement with the above-cited data. A strong correlation between TEAC values and those obtained from CUPRAC 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. Our results on cytotoxicity are in accordance with others [32–34]. The antioxidant activity of maca (*Lepidium meyenii*) was assessed by the inhibition of peroxynitrite. Maca (1 mg/mL) protected RAW 264.7 cells against peroxynitrite-induced apoptosis and increased ATP production in cells treated with H_2O_2 (1 mM). The concentration of catechins in maca was lower than in green tea (2.5 vs. 145 mg/g). Maca has the capacity to scavenge free radicals and protect cells against oxidative stress. Our results can be compared with the recent work of Lin et al. [4]. The extract of indigo naturalis (QD) and its main components indirubin, indigo, and tryptanthrin in human neutrophils were investigated for their anti-inflammatory effects. QD showed the significant inhibition of superoxide anion, attenuated the formyl-l-methionyl-l-leucyl-l-phenylalanine (FMLP)-induced phosphorylation of extracellular regulated kinase; QD inhibited calcium mobilization caused by FMLP. On the other hand, neither indirubin, indigo, nor tryptanthrin produced similar changes in human neutrophils.

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 [32]. The extract of *Oroxylum indicum* showed the highest toxicity on all tumor cell lines tested, with an IC50 of 19.6 μg/mL for CEM, 14.2 μg/mL for HL-60, 17.2 μg/mL for B-16 and 32.5 μg/mL for HCT-8. On the sea urchin eggs, it inhibited the progression of cell cycle since the first cleavage (IC50=13.5 μg/mL). As was recently shown by Heo et al. [27] that the Korean medicinal plants, which were used for a long time as traditional seasoned salads, possess anticancer activity. Our studies on cytotoxicity are in correspondence with Iwaki and Kurimoto [6], where it was shown that tryptanthrin and indirubin, both compounds originating from indican in the leaves of *P. tinctorium*, are responsible for many of the biological activities of this plant. Tryptanthrin has a potent anti-inflammatory activity and shows growth inhibitory activity against cancer cell lines in vitro. The effect of this substance on azoxymethane-induced intestinal tumorigenesis in rats with carcinogenesis in the intestines is closely associated with inflammation. Tryptanthrin inhibited the incidence of intestinal tumors. Indirubin has been reported to possess an anti-leukemic activity and *P. tinctorium* also contains various anti-oxidative substances, such as gallic acid and caffeic acid, with potential anti-tumor activity. We consider it likely

that *P. tinctorium* shows cancer preventive activity as a consequence of the integral effects of these substances. Our results are in accordance with others [35], where diploid leaf extracts of *Gynostemma pentaphyllum* Makino, which is used in tea and food, had strongest inhibition on inflammation and HT-29 proliferation, but these extracts had different order of antiproliferative properties in the LNCaP cells. The interaction between water 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 [28]. Better ability is shown by methanol extract. The application of IR spectroscopy and fluorescence in herbal analysis is still limited when compared to other areas. The representative IR spectra from the mid-IR region (4,000–800 cm^{-1}) for ethyl acetate and methanolic extracts were observed. The three extracts in the region of polyphenols showed slight variation in bands than the standards.

Conclusions

It was found that polyphenols, flavonoids, and flavanols were significantly higher in prolipid, following by indigo mature leaves, immature leaves, and seeds. The ability of indigo to quench the intrinsic fluorescence of BSA, relatively high content of polyphenols compounds and anticancer properties can be used as a new source of antioxidants.

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References

1. Mantzouris, D., Karapanagiotis, I., Valianou, L., & Panayiotou, C. (2011). HPLC–DAD–MS analysis of dyes identified in textiles from Mount Athos. *Analytical and Bioanalytical Chemistry, 399*, 3065–3079.
2. Selvius De Roo, C., & Armitage, R. A. (2011). Direct identification of dyes in textiles by direct analysis in real time-time of flight mass spectrometry. *Analytical Chemistry, 83*, 6924–6928.
3. Manhita, A., Ferreira, T., Candeias, A., & Barrocas Dias, C. (2011). Extracting natural dyes from wool—an evaluation of extraction methods. *Analytical and Bioanalytical Chemistry, 400*, 1501–1514.
4. Lin, Y.-K., Leu, Y.-L., Huang, T.-H., Wu, Y.-H., Chung, P.-J., Su Pang, J.-H., & Hwang, T.-L. (2009). Anti-inflammatory effects of the extract of indigo naturalis in human neutrophils. *Journal of Ethnopharmacology, 125*, 51–58.
5. Barros, G. M. C. C., & Teixeira, Sd. P. (2008). Pharmacobotanical study of two wild indigo species (*Indigofera suffruticosa* and *Indigofera truxillensis*, Leguminosae) with pharmacological properties. *Revista Brasileira de Farmacognosia, 18*, 287–294.
6. Iwaki, K., & Kurimoto, M. (2002). Cancer preventive effects of the indigo plant, *Polygonum tinctorium*. *Recent Research Developments in Cancer, 4*, 429–437.
7. Barros, L., Cabrita, L., Boas, M. V., Carvalho, A. M., & Ferreira, I. C. F. R. (2011). Chemical, biochemical and electrochemical assays to evaluate phytochemicals and antioxidant activity of wild plants. *Food Chemistry, 127*, 1600–1608.
8. Dall'Acqua, S., Cervellati, R., Loi, M. C., & Innocenti, G. (2008). Evaluation of in vitro antioxidant properties of some traditional Sardinian medicinal plants: investigation of the high antioxidant capacity of *Rubus ulmifolius*. *Food Chemistry, 106*, 745–749.
9. Kim, B.-G., Shin, K.-S., Yoon, T. J., Yu, K.-W., Ra, K. S., Kim, J. M., et al. (2012). Fermentation of Korean red ginseng by *Lactobacillus plantarum* M-2 and its immunological activities. *Applied Biochemistry and Biotechnology, 165*, 1107–1119.

10. Nadour, M., Michaud, P., & Moulti-Mati, F. (2012). Antioxidant activities of polyphenols extracted from olive (*Olea europaea*) of chamlal variety. *Applied Biochemistry and Biotechnology*. doi:10.1007/s12010-012-9633-8.
11. Generalic, I., Skroza, D., Ljubenkov, I., Katalinic, A., Burcul, F., & Katalinic, V. (2011). Influence of the phenophase on the phenolic profile and antioxidant properties of Dalmatian sage. *Food Chemistry, 127*, 427–433.
12. Han, J., Weng, X., & Bi, K. (2008). Antioxidants from a Chinese medicinal herb—*Lithospermum erythrorhizon*. *Food Chemistry, 106*, 2–10.
13. Jastrzębski, Z., Tashma, Z., Katrich, E., & Gorinstein, S. (2007). Biochemical characteristics of the herb mixture prolipid as a plant food supplement and medicinal remedy. *Plant Foods for Human Nutrition, 62*, 145–150.
14. Haruenkit, R., Poovarodom, S., Vearasilp, S., Namiesnik, J., Sliwka-Kaszynska, M., Park, Y.-S., et al. (2010). Comparison of bioactive compounds, antioxidant and antiproliferative activities of Mon Thong durian during ripening. *Food Chemistry, 118*, 540–547.
15. Sanz, M., Cadahia, E., Esteruelas, E., Munoz, A. M., Simon, B. F., Hernandez, T., & Estrella, I. (2010). Phenolic compounds in cherry (*Prunus avium*) heartwood with a view to their use in cooperage. *Journal of Agriculture and Food Chemistry, 58*, 4907–4914.
16. Singleton, V. L., Orthofer, R., & Lamuela-Raventos, R. M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin–Ciocalteu reagent. *Methods in Enzymology, 299*, 152–178.
17. Liu, M., Li, X. Q., Weber, C., Lee, C. Y., Brown, J., & Liu, R. H. (2002). Antioxidant and antiproliferative activities of raspberries. *Journal of Agricultural and Food Chemistry, 50*, 2926–2930.
18. Broadhurst, R. B., & Jones, W. T. (1978). Analysis of condensed tannins using acidified vanillin. *Journal of the Science of Food and Agriculture, 29*, 788–794.
19. Feucht, W., & Polster, J. (2001). Nuclei of plants as a sink for flavanols. *Journal of Bioscience, 56*, 479–481.
20. Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology & Medicine, 26*, 1231–1237.
21. Ozgen, M., Reese, R. N., Tulio, A. Z., Jr., Scheerens, J. C., & Miller, A. R. (2006). Modified 2, 2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) method to measure antioxidant capacity of selected small fruits and comparison to ferric reducing antioxidant power (FRAP) and 2,2'-diphenyl-1-picrylhydrazyl (DPPH) methods. *Journal of Agriculture and Food Chemistry, 54*, 1151–1157.
22. Apak, R., Guclu, K., Ozyurek, M., & Karademir, S. E. (2004). Novel total antioxidant capacity index for dietary polyphenols and vitamins C and E, using their cupric ion reducing capability in the presence of neocuproine: CUPRAC method. *Journal of Agricultural and Food Chemistry, 52*, 7970–7981.
23. Paul, B. K., & Guchhait, N. (2011). A spectral deciphering of the binding interaction of an intramolecular charge transfer fluorescence probe with a cationic protein: thermodynamic analysis of the binding phenomenon combined with blind docking study. *Photochemical & Photobiological Sciences, 10*, 980–991.
24. Kannan, R., Ragupathi, R., Arumugam, R., & Anantharaman, P. (2011). Fourier transform infrared spectroscopy analysis of seagrass polyphenols. *Current Bioactive Compounds, 7*, 118–125.
25. Lu, X., Wang, J., Al-Qadiri, H. M., Ross, C. F., Powers, J. R., Tang, J., & Rasco, B. A. (2011). Determination of total phenolic content and antioxidant capacity of onion (*Allium cepa*) and shallot (*Allium oschaninii*) using infrared spectroscopy. *Food Chemistry, 129*, 637–644.
26. Nirmaladevi, R., Padma, P. R., & Kavitha, D. (2010). Analyses of the methanolic extract of the leaves of *Rhinacanthus nasutus*. *Journal of Medicinal Plants Research, 4*, 1554–1560.
27. Heo, B. G., Park, Y. S., Chon, S. U., Lee, S. Y., Cho, J. Y., & Gorinstein, S. (2007). Antioxidant activity and cytotoxicity of methanol extracts from aerial parts of Korean salad plants. *BioFactors, 30*, 79–89.
28. Kim, K.-S., Hwang, W.-G., Jang, H.-G., Heo, B.-G., Suhaj, M., Leontowicz, H., et al. (2012). Assessment of indigo (*Polygonum tinctorium Ait*.) water extracts' bioactive compounds, and their antioxidant and antiproliferative activities. *LWT- Food Science and Technology, 46*, 500–510.
29. Ho, Y. L., & Chang, Y. S. (2002). Studies on the antinociceptive, anti-inflammatory 17 and antipyretic effects of *Isatis indigotica* root. *Phytomedicine, 9*, 419–424.
30. Fialova, S., Valigura, R., Tekelova, D., & Grancai, D. (2009). *Isatis tinctoria L*. (dyer's woad) from the standpoint of phenolic compound content and scavenging activity. *Farmaceuticky Obzor, 78*, 54–58.
31. Tepe, B., Sokmen, M., Akpulat, H. A., Yumrutas, O., & Sokmen, A. (2006). Screening of antioxidative properties of the methanolic extracts of *Pelargonium endlicherianum* Fenzl., *Verbascum wiedemannianum* Fisch. & Mey., *Sideritis libanotica* Labill. subsp. *linearis* (Bentham) Borm., *Centaurea mucronifera* DC. and *Hieracium cappadocicum* Freyn from Turkish flora. *Food Chemistry, 98*, 9–13.

32. Costa-Lotufo, L. V., Khan, M. T. H., Ather, A., Wilke, D. V., Jimenez, P. C., Pessoa, C., et al. (2005). Studies of the anticancer potential of plants used in Bangladeshi folk medicine. *Journal of Ethnopharmacology, 99*, 21–30.
33. Itharat, A., Houghton, P. J., Eno-Amooquaye, E., Burke, P. J., Sampson, J. H., & Raman, A. (2004). In vitro cytotoxic activity of Thai medicinal plants used traditionally to treat cancer. *Journal of Ethnopharmacology, 90*, 33–38.
34. Sandoval, M., Okuhama, N. N., Angeles, F. M., Melchor, V. V., Condezo, L. A., Lao, J., & Miller, M. J. S. (2002). Antioxidant activity of the cruciferous vegetable Maca (*Lepidium meyenii*). *Food Chemistry, 79*, 207–213.
35. Xie, Z., Huang, H., Zhao, Y., Shi, H., Wang, S., Wang, T. T. Y., Chen, P., & Yu, L. (2012). Chemical composition and anti-proliferative and anti-inflammatory effects of the leaf and whole-plant samples of diploid and tetraploid *Gynostemma pentaphyllum* (Thunb.) Makino. *Food Chemistry, 132*, 125–133.