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Anticancer and antioxidant effects of extracts from different parts of indigo plant

Buk-Gu Heo[a], Yun-Jum Park[b], Yong-Seo Park[c], Jong-Hyang Bae[b], Ja-Yong Cho[d,*], Kun Park[e], Zenon Jastrzebski[f], Shela Gorinstein[g,*]

[a] Naju Foundation of Natural Dyeing Culture, Naju 520-931, Republic of Korea
[b] Department of Horticulture, Wonkwang University, Iksan 570-749, Republic of Korea
[c] Department of Horticultural Science, Mokpo National University, Muan 534-729, Republic of Korea
[d] Department of Medicated Diet & Food Technology, Jeonnam Provincial College, Damyang 517-802, Republic of Korea
[e] Department of Dermatology, School of Medicine, Wonkwang University, Iksan 570-749, Republic of Korea
[f] National Medicines Institute, 00-725 Warsaw, Poland
[g] Institute for Drug Research, School of Pharmacy, The Hebrew University – Hadassah Medical School, Jerusalem, Israel

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ABSTRACT

The water, methanol and ethanol extracts of flowers, leaves, stems, and seeds from indigo (*Polygonum tinctorium Lour.*) were tested *in vitro* to verify cytotoxic effects on human renal cell line (HEK 293) and inhibitory effects on the proliferation of colon cancer cell line (HCT-116), cervical carcinoma cells (HeLa), liver carcinoma cells (Hep3B), breast carcinoma cells (MCF-7), laryngeal cancer cell line (SNU-1066), and gastric cancer cell line (SNU-601), in order to further increase the use of the indigo plant. Significant differences were found in polyphenols and flavonoids in extracts of different parts of indigo. The highest amount of total polyphenols (22.94 ± 2.18 and 22.81 ± 2.04 mg GAE/g DW) were found in methanol extracts of leaves and flowers and flavonoids (4.02 ± 0.43, 3.70 ± 0.34 and 3.28 ± 0.26 mg CE/g DW) – in methanol extracts of leaves, flowers and seeds. The highest antioxidant capacities by $ABTS^{\bullet+}$ (99.12 ± 9.18 and 96.35 ± 8.75 μM TE/g DW) and CUPRAC (78.37 ± 7.86 and 86.22 ± 8.43 μM TE/g DW) were also found in methanol extracts of leaves and flowers. The correlation coefficient (R^2) between antioxidant capacities and polyphenols of indigo parts in different extracts varied from 0.7142 to 0.9765, showing the highest correlation for leaves and flowers in methanol extracts. The survival rates at a concentration of 800 mg/L for human renal cell line (HEK 293) in indigo plant water extracts from flowers and stems were 17.58% ± 9.29 and 61.78% ± 11.00 and in methanol extract of leaves and seeds estimated as 36.47% ± 18.70 and 28.7% ± 16.68, respectively. The extracts of stem prolonged the overall survival of cancer cells. The survival rates at a concentration of 800 mg/L of colon cancer cell line (HCT-116) and cervical cancer cell line (HeLa) in the ethanol extract of indigo flowers were 5.10% ± 0.32 and 8.85% ± 0.11, respectively, and in seeds on HeLa cells was 16.93% ± 3.09. The survival rates of HeLa and HCT-116 in methanol extracts of indigo leaves at a concentration of 800 mg/L were 6.89% ± 0.53 and 13.22% ± 8.51, respectively. Proliferative and antioxidant activities were correlated. The highest R^2 between the antioxidant capacities and survival rates was in methanol extracts of leaves on HeLa cells. Therefore, investigation *in vitro* revealed that the extracts of the indigo plant were found to be effective in suppressing the proliferation of cancer cells, although the results varied depending on the different parts of *P. tinctorium Lour.*, the concentrations of solvent and extracts, the antioxidant activities and the types of cancers.

1. Introduction

Natural antioxidants, such as vitamins and polyphenols, have high antioxidant capacities and are abundant in many fruits and vegetables, whose consumption has been demonstrated to be inversely associated with the cardiovascular disease and some cancers (Duthie et al., 2000).

* Corresponding authors.
E-mail addresses: jy200@hanmail.net (J.-Y. Cho), shela.gorin@mail.huji.ac.il, gorin@cc.huji.ac.il (S. Gorinstein).

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Realizing the importance of medicinal plants, extracts of plant parts are extensively explored for different bioactivities including antioxidants (Kumari and Kakkar, 2008; Mahomoodally et al., 2012). Gan et al. (2010) compared 50 medicinal plants using similar methods shown in our report. Indio plant (*Polygonum tinctorium Lour.*) is an annual herbaceous plant of the family *Polygonaceae* originated from Central Asia and China (Kim et al., 2012a). Indigo plant has been used as blue dye and for medication in Korea, China, and Japan for over 1000 years (Heo et al., 2012). The leaves of the indigo plant have been widely used as anodyne, anti-febrile, detoxicant, and anti-inflammatory drugs in oriental medicine. The indigo seeds have also been used as anti-febrile and detoxicant medicines (Heo et al., 2011). The sprouts of indigo plants have been used as one of ingredients in salad, in addition to a coloring dye and medication, in Korea. It is commonly said that the extracts of indigo plants are highly beneficial for treating cancers (Heo et al., 2011). *P. tinctorium Lour.* has been used as a crude drug in Asia, and its remarkable physiological activity has been proved in a recent study (Heo et al., 2013). The main bioactive substances in different extracts of indigo plant, investigated in our previous report, were mostly polyphenols, flavonoids, flavanols, and tannins (Kim et al., 2012b; Jang et al., 2012; Heo et al., 2013). The useful contents in this plant are indigo, indirubin, tryptanthrin and kaempferol (Cooksey, 2012; Iwaki et al., 2011; Kukula-Koch et al., 2013). Also Marino et al. (2009) showed that the polyphenols in *Hamamelis virginiana* L. and other seven similar medicinal plants were the main bioactive compounds. The determination of polyphenol contents in aqueous extracts of these plants was based on the reduction of Cu (II) to Cu (I) by polyphenols, in the presence of bicinchoninic acid (BCA) in a buffered medium with the formation of Cu (I)/BCA complexes. Djidel et al. (2010) determined polyphenols, flavonoids contents, antioxidant and free radicals scavenging capacities in the decoctions of 40 plants from 21 botanical families grown in Algeria. These plants were used traditionally for gastro-intestinal disorders and hypertension. However, the effects of indigo extracts on different cancer cell lines have been rarely studied.

Comparison of antioxidant effect of the extracts of indigo with survival of cancer cells is limited. In order to receive the reliable results of total antioxidant capacities two generally accepted assays ABTS and CUPRAC were used (Re et al., 1999; Apak et al., 2004).

Under current circumstances, the purpose of this study was to examine the effects of various extracts from different parts of indigo plant in tumor cell lines to further increase the use and availability of *P. tinctorium Lour.* The correlations between antioxidant capacities and polyphenols in different extracts of indigo plant and antioxidant capacities and cytotoxicity on cells were determined.

2. Materials and methods

2.1. Samples and preparation

The flowers, leaves, stems, and seeds of the indigo plant were used (Fig. 1). The leaves and stems were collected in the middle of August, flowers in early October, and seeds – in early November, 2012, from cultivation package in Naju location, Korea.

2.2. Preparation of extracts

The gathered samples were dried at 50 °C for 5 days, pulverized, and then passed through a 1 mm strainer. A 200 g sample of each part of indigo plant was extracted with 2 L of 95% ethanol for 24 h. The same procedure was done with methanol and water. The extracts of methanol and ethanol were taken for decompression concentration process at 50 °C by an evaporator (IKA-Werke GmbH & Co. KG), lyophilized by a freeze dryer (Samwon Co., Korea), and then pulverized into fine powder form. The water extract was lyophilized.

2.3. Chemicals

6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox); Tris, tris(hydroxymethy1) aminomethane; 2,2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS); Folin–Ciocalteu reagent (FCR); $CuCl_2{\cdot}2H_2O$; and 2,9-dimethyl-1,10-phenanthroline (neocuproine) were purchased from Sigma Chemical Co., St. Louis, MO, USA. Deionized and distilled water was used throughout.

2.4. Determination of the contents of the main bioactive compounds

Bioactive compounds, total antioxidants and anticancer activities were determined in water, methanol and ethanol extracts from flowers, leaves, stems, and seeds of the indigo plant. The total polyphenols were determined by Folin–Ciocalteu method (Singleton et al., 1999) with measurement at 750 nm with spectrophotometer (Hewlett–Packard, model 8452A, Rockvile, USA). The results were expressed as mg GAE/g DW. Total flavonoid content was determined by an aluminum chloride colorimetric method (Zhishen et al., 1999) with some modifications (Liu et al., 2002). Briefly, 0.25 mL of the indigo 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 $AlCl_3{\cdot}6H_2O$ solution was added, and the mixture was allowed to stand for another 5 min. Half of a milliliter of 1 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 results are expressed as milligrams of catechin equivalents (CE).

The antioxidant capacities (AC) were determined by two complementary assays: (1) 2,2-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt (ABTS) method for the screening of antioxidant capacity 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 capacity. ABTS radical cation was generated by the interaction of ABTS (7 mmol/L) and $K_2S_2O_8$ (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) 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 NH_4Ac buffer solution, acidified and non acidified methanol, water or ethanol extracts of indigo (or standard) solution (x, in mL) and H_2O [$(1.1 - x)$ mL] were added to make the final volume of 4.1 mL. The absorbance at 450 nm was recorded against a reagent blank (Apak et al., 2004).

2.5. Tumor cell lines and incubation of cell lines

All tumor cell lines used in this investigation were originated from human bodies and purchased from Korean Cell Line Bank (KCLB). To identify cellular cytotoxicity of the different cancer cell samples, a human renal cell line (HEK 293) was used for normal cells. Human cancer cell lines in the experiment were colon cancer

Fig. 1. Different parts of Indigo plant: A, B, C, D, flowers, leaves, stems, seeds.

cell line (HCT-116), cervical cancer cell line (HeLa), liver cancer cell line (Hep3B), breast cancer cell line (NCF-7), gastric cancer cell line (SNU-601), and laryngeal cancer cell lines (SNU-1066). For incubation of each cell line in the experiment, different mediums were used such as DMEM (Dulbeco's Modified Eagle's Medium), RPMI-1640, MEM (Minimum Essential Medium) after adding 10% FBS and 1% penicillin/streptomycin (100 U/mL). Cell lines were adapted and subcultured in mediums at 37 °C and 5% CO_2 incubator (MCO-17 AIC, Sanyo, Tokyo, Japan).

2.6. Inhibition of cancer cell proliferation

Inhibitory effects on the hyperplasia of cancer cells were assessed using MTT assay (Mosmann, 1983; Choi et al., 1989) to examine the survival rates of cancer cells. The tumor cells were adjusted at a concentration of 3×10^4 cells/mL, divided per 90 μL/well in 96 well microplates, cultivated in an incubator (Forma, Germany) at 37 °C, 5% CO_2 for 12 h to attach the cells, and added 10 μL each to adjust extracts at concentrations of 50, 100, 200, 400, and 800 mg/L. Distilled water with an equal amount of the sample was added to the control group, and then cultivated for 72 h. A 10 μL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution at a concentration of 5 mg/mL was added to each well, and then cultured in an incubator for 4 h. Culture medium with MTT solution was eliminated, and 150 μL DMSO was added and stirred for 30 min to dissolve each cell. Absorbance was measured at 540 nm using a microplate reader (Bio-Rad, USA), and then obtained values were converted to relative cell growth rates by taking each cell of the non-sample group as 100% as shown below (Mosmann, 1983). Thus, inhibition of cancer cell proliferation (%) = {(absorbance of control group–absorbance of sample treated group)/absorbance of control group} × 100.

2.7. Statistical analyses

To verify the statistical significance, mean ± 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

The extraction yields (mg dry extract/g of investigated plant material) for seeds, stems, leaves and flowers were the following: in water extract as 60.3, 54.8, 172.1 and 171.2; in ethanol extract 32.2, 30.2, 49.1, and 44.1; in methanol extract 40.8, 37.2, 123.3 and 110.5, respectively. The results of the bioactive compounds are presented in Fig. 2. Significant differences were found in polyphenols and flavonoids in extracts of different parts of indigo. The highest amount of total polyphenols (22.94 ± 2.18 and 22.81 ± 2.04 mg GAE/g DW) were found in methanol extracts of leaves and flowers and flavonoids (4.02 ± 0.43, 3.70 ± 0.34 and 3.28 ± 0.26 mg CE/g DW) – in methanol extracts of leaves, flowers and seeds. The highest antioxidant capacities by $ABTS^{\bullet+}$ (99.12 ± 9.18 and 96.35 ± 8.75 μM TE/g DW) and CUPRAC (78.37 ± 7.86 and 86.22 ± 8.43 μM TE/g DW) were also found in methanol extracts of leaves and flowers.

The effect of distilled water extracts of leaves, stems and flowers of *P. tinctorium Lour.* was identified by examining the survival rate of carcinoma cells. The most effective was extract of flowers in a human renal cell line (HEK-293). Survival rates were 57.3% ± 8.0 at a 50 mg/L, 35.3% ± 15.9 at a 200 mg/L and 17.6% ± 9.3 at 800 mg/L of the extract (Table 1). Insignificant influence was shown on breast cancer cell line (MCF-7) with a survival rate of 84.6% ± 2.1 at 800 mg/L. Anticancer activity of leaves was considerably higher in cervical cancer cell line (HeLa, 26.5% ± 14.3) and liver cancer cell line (Hep3B, 34.1% ± 4.3) at 800 mg/L of the extract (Table 2). Extract of stems at concentration of 800 mg/L showed survival rate of 55.9% ± 9.0 on HeLa (Table 3).

In contrast, the ethanol extract of flowers had a strong anticancer effect on colon cancer cell line (HCT-116) and HeLa and the survival rates were 5.1% ± 0.3 and 8.9% ± 0.1 at 800 mg/L of extract

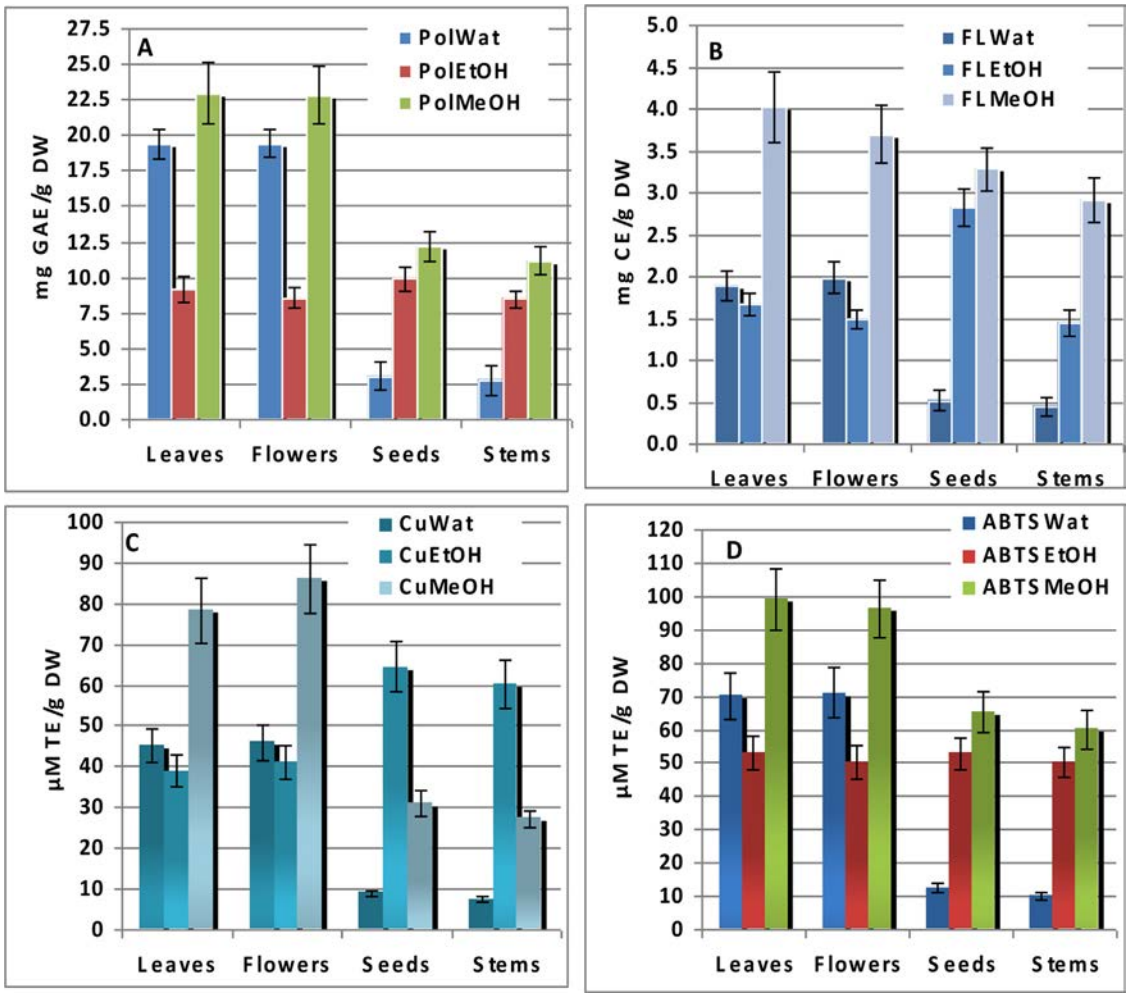

Fig. 2. Bioactive compounds and antioxidant activities in water (Wat), ethanol (EtOH) and methanol (MeOH) extracts of different parts of indigo plant: A, polyphenols (Pol); B, flavonoids (FL); C, antioxidant capacity by CUPRAC (Cu); D, antioxidant capacity of ABTS.

Table 1
Survival rate of cancer cells as affected by the concentration of indigo flower extract.

Solvent	Cancer cell	Concentration of extract (mg/L)				
		50	100	200	400	800
Water	HEK-293	57.26 ± 8.02b	64.80 ± 4.77a	35.27 ± 15.93c	39.12 ± 17.10c	17.58 ± 9.29d
	HCT-116	75.40 ± 14.47ab	77.60 ± 13.24ab	78.16 ± 18.14ab	88.31 ± 18.22a	72.34 ± 23.04ab
	HeLa	52.35 ± 1.81b	59.99 ± 0.74a	56.72 ± 3.98a	47.33 ± 3.50c	33.22 ± 13.59d
	Hep3B	92.91 ± 9.25a	87.69 ± 3.27a	70.21 ± 6.13b	50.47 ± 15.50c	55.15 ± 3.50c
	MCF-7	101.3 ± 0.69a	87.30 ± 3.54b	87.84 ± 3.43b	88.07 ± 1.10b	84.55 ± 2.11c
	SNU-1066	98.75 ± 8.18a	98.36 ± 9.81a	90.60 ± 6.24a	78.16 ± 10.80b	68.06 ± 7.74b
	SNU-601	93.45 ± 5.82a	92.69 ± 1.88a	81.63 ± 8.18ab	59.68 ± 2.26c	46.81 ± 7.39d
Ethanol	HEK-293	130.87 ± 10.12a	103.40 ± 5.48b	88.28 ± 4.68c	73.39 ± 18.99d	25.27 ± 14.95e
	HCT-116	51.84 ± 13.98a	46.26 ± 16.10a	19.86 ± 9.83b	9.84 ± 4.67c	5.10 ± 0.32d
	HeLa	57.69 ± 2.60a	51.12 ± 2.13b	34.47 ± 8.62c	13.36 ± 1.81d	8.85 ± 0.11e
	Hep3B	74.06 ± 16.14a	51.29 ± 10.86b	29.57 ± 1.78c	29.12 ± 2.00c	22.95 ± 1.94d
	MCF-7	110.07 ± 2.87a	103.44 ± 3.10b	80.85 ± 11.56c	79.82 ± 8.14c	78.17 ± 5.47c
	SNU-1066	71.08 ± 3.16a	70.86 ± 7.70a	57.84 ± 2.50b	57.72 ± 7.49b	21.14 ± 3.37c
	SNU-601	100.59 ± 7.95a	84.43 ± 3.31b	71.57 ± 6.15c	51.18 ± 7.04d	45.91 ± 2.62e
Methanol	HEK-293	76.08 ± 11.01a	76.54 ± 5.45a	53.61 ± 22.44b	24.83 ± 15.54c	24.08 ± 3.51c
	HCT-116	65.78 ± 8.84a	66.13 ± 2.78a	59.35 ± 12.01ab	59.03 ± 17.44ab	53.31 ± 12.56b
	HeLa	55.47 ± 0.94a	58.88 ± 13.45a	20.91 ± 3.49b	21.61 ± 4.94c	13.37 ± 2.48d
	Hep3B	63.90 ± 16.20a	48.70 ± 7.77b	35.20 ± 3.99c	56.29 ± 14.85ab	32.22 ± 12.63c
	MCF-7	99.86 ± 7.03a	96.78 ± 4.92a	80.15 ± 11.93ab	76.14 ± 10.57bc	73.47 ± 19.12bc
	SNU-1066	89.29 ± 17.24a	83.55 ± 18.44a	58.47 ± 12.16b	54.82 ± 12.43b	25.02 ± 4.93c
	SNU-601	94.83 ± 10.57a	78.76 ± 16.01b	78.37 ± 6.24b	54.23 ± 5.39c	35.52 ± 4.46d

Abbreviations: Cell lines: human renal, HEK 293; colon cancer, HCT-116; cervical carcinoma, HeLa; liver carcinoma, Hep3B; breast carcinoma, MCF-7; laryngeal cancer, SNU-1066; gastric cancer cell, SNU-601. All values are mean ± SD of five independent measurements. Values within different superscripts are different within the same column at $p < 0.05$ by Duncan's multiple range test.

Table 2
Survival rate of cancer cells as affected by the concentration of indigo leaves extracts.

Solvent	Cancer cell	Concentration of extract (mg/L)				
		50	100	200	400	800
Water	HEK-293	103.58 ± 9.12a	104.26 ± 16.77a	106.02 ± 14.60a	89.81 ± 9.97ab	71.35 ± 8.02b
	HCT-116	76.59 ± 6.44a	71.85 ± 3.63a	49.84 ± 3.85b	49.21 ± 1.86b	45.44 ± 2.63bc
	HeLa	44.73 ± 6.51b	47.44 ± 3.27b	55.63 ± 11.38a	42.11 ± 8.62b	26.48 ± 14.33c
	Hep3B	91.37 ± 21.07a	73.89 ± 17.56ab	67.78 ± 18.42bc	50.74 ± 15.66 cd	34.13 ± 4.30d
	MCF-7	114.14 ± 18.92a	95.37 ± 17.02ab	82.52 ± 12.95c	77.71 ± 15.49 cd	73.70 ± 17.55 cd
	SNU-1066	102.82 ± 12.63a	100.09 ± 17.16a	87.40 ± 20.28b	79.00 ± 15.11bc	62.96 ± 5.82c
	SNU-601	86.86 ± 7.34a	85.24 ± 17.79a	69.13 ± 10.94b	53.93 ± 5.84c	35.56 ± 3.29d
Ethanol	HEK-293	129.28 ± 11.11a	105.75 ± 19.46b	133.53 ± 11.34a	82.74 ± 13.89c	83.44 ± 18.70c
	HCT-116	54.86 ± 8.89a	50.84 ± 13.50a	44.57 ± 17.66b	26.06 ± 10.99c	17.66 ± 3.51 cd
	HeLa	30.49 ± 2.60a	24.83 ± 0.40b	29.16 ± 1.44a	29.16 ± 1.46a	25.96 ± 3.87b
	Hep3B	47.52 ± 5.37a	40.31 ± 2.24ab	38.93 ± 3.28c	28.28 ± 3.02d	23.82 ± 6.26e
	MCF-7	85.11 ± 4.41a	86.19 ± 3.49a	79.47 ± 5.35b	75.71 ± 4.42bc	67.46 ± 12.60c
	SNU-1066	68.87 ± 8.79a	58.23 ± 6.88b	58.74 ± 4.50b	56.37 ± 8.76b	48.55 ± 9.18c
	SNU-601	63.11 ± 2.97a	58.93 ± 6.88b	49.14 ± 5.01c	49.68 ± 5.87c	39.29 ± 2.63d
Methanol	HEK-293	91.82 ± 18.70a	93.47 ± 17.30a	91.10 ± 10.53a	82.03 ± 17.30b	36.47 ± 18.70c
	HCT-116	55.91 ± 20.00a	57.47 ± 23.13a	31.71 ± 19.37b	16.42 ± 6.51c	13.22 ± 8.51c
	HeLa	31.97 ± 2.65b	37.19 ± 3.13a	26.93 ± 4.73c	24.87 ± 0.82c	6.89 ± 0.53d
	Hep3B	68.29 ± 13.71a	55.53 ± 5.75ab	38.50 ± 6.66c	35.18 ± 3.78c	26.47 ± 5.19d
	MCF-7	114.86 ± 5.79a	115.62 ± 3.09a	111.13 ± 3.91ab	110.14 ± 5.57b	96.68 ± 4.79c
	SNU-1066	105.86 ± 7.96a	99.64 ± 15.61ab	71.88 ± 8.01c	60.13 ± 10.65 cd	21.39 ± 9.72e
	SNU-601	119.36 ± 5.90a	99.87 ± 4.24b	98.54 ± 6.06b	97.87 ± 13.16b	35.00 ± 10.26c

Abbreviations: Cell lines: human renal, HEK 293; colon cancer, HCT-116; cervical carcinoma, HeLa; liver carcinoma, Hep3B; breast carcinoma, MCF-7; laryngeal cancer, SNU-1066; gastric cancer cell, SNU-601. All values are mean ± SD of five independent measurements. Values within different superscripts are different within the same column at $p < 0.05$ by Duncan's multiple range test.

(Table 1). Leaves showed a strong anticancer activity on HeLa and Hep3B with survival rates of 29.9% ± 3.9 and 23.8% ± 6.3 at 800 mg/L of ethanol extract (Table 2). The survival rates were 19.8% ± 2.8 in cell line HeLa and 54.6% ± 9.7 in Hep3B cell line at 800 mg/L of stem extract (Table 3). The effect of ethanol extract of seeds of *P. tinctorium Lour.* was examined and the survival rate of HeLa was the lowest: the survival rates were 22.9% ± 1.6 at a 200 mg/L and 16.9% ± 3.1 at 800 mg/L extract (Table 4). The use of ethanol extract at low concentrations requires caution since cells proliferated to 137.7 ± 0.7 at 50 mg/L (Table 4) of human renal cell line (HEK 293).

Inhibitory effect of methanol extracts of leaves was also stronger in colon cancer cell line (HCT-116) and cell line HeLa with survival rate of 13.2% ± 8.5 and 6.9% ± 0.5 at 800 mg/L, respectively (Table 2) than on Hep3B cell line with survival rate of 29.3% ± 8.4 at 800 mg/L. The methanol extract of seeds showed low survival rates in cell lines of HeLa and Hep3B as 22.9 ± 5.4 and 28.1 ± 2.6, respectively, and demonstrate potential applicability. Moreover, survival rate was 13.4% ± 2.5 in HeLa at 800 mg/L of methanol extract of flower, exhibiting significant *in vitro* anticancer activity (Table 1). The correlation coefficients (R^2) between antioxidant capacities and polyphenols of indigo plant parts in different extracts varied

Table 3
Survival rate of cancer cells as affected by the concentration of indigo stem extracts.

Solvent	Cancer cell	Concentration of extract (mg/L)				
		50	100	200	400	800
Water	HEK-293	77.21 ± 11.21c	70.11 ± 17.02 cd	83.80 ± 4.58a	79.51 ± 1.32ab	61.78 ± 11.00e
	HCT-116	79.91 ± 6.14b	83.93 ± 3.35a	78.18 ± 9.49b	76.63 ± 7.53b	69.57 ± 7.88c
	HeLa	58.20 ± 10.78a	58.96 ± 2.05a	55.21 ± 3.02ab	54.93 ± 4.58ab	55.94 ± 8.95ab
	Hep3B	105.82 ± 8.00a	81.86 ± 11.02b	77.68 ± 12.11bc	77.91 ± 17.26bc	62.77 ± 1.19d
	MCF-7	100.18 ± 5.48a	93.95 ± 3.62b	91.51 ± 8.63b	91.37 ± 10.81b	92.69 ± 6.82b
	SNU-1066	103.74 ± 6.83ab	101.6 ± 13.74ab	98.86 ± 9.64b	110.65 ± 17.29a	95.99 ± 13.98b
	SNU-601	101.14 ± 2.00a	87.01 ± 14.05b	86.92 ± 7.00b	80.23 ± 8.49bc	77.99 ± 4.81c
Ethanol	HEK-293	99.00 ± 3.39b	104.03 ± 7.87a	104.67 ± 4.48a	109.81 ± 9.43a	100.91 ± 2.63b
	HCT-116	100.00 ± 79.57a	79.57 ± 5.36b	79.28 ± 8.03b	71.89 ± 3.04c	81.74 ± 10.40b
	HeLa	57.99 ± 3.70b	62.02 ± 12.02a	62.85 ± 7.29a	47.26 ± 6.14c	19.81 ± 2.79d
	Hep3B	83.58 ± 5.86a	78.95 ± 8.86ab	64.21 ± 3.43c	60.05 ± 3.72d	54.56 ± 9.70e
	MCF-7	112.01 ± 5.04a	107.02 ± 12.36ab	98.73 ± 7.91b	72.75 ± 13.48c	71.23 ± 14.44c
	SNU-1066	113.29 ± 5.95a	114.92 ± 8.45a	88.12 ± 17.85b	59.91 ± 20.22c	58.20 ± 14.40c
	SNU-601	107.58 ± 13.91a	99.00 ± 12.95ab	99.48 ± 12.67ab	93.79 ± 15.43b	77.63 ± 9.56c
Methanol	HEK-293	109.00 ± 13.29a	96.30 ± 8.15ab	98.69 ± 8.79ab	75.57 ± 20.21c	64.43 ± 8.98 cd
	HCT-116	101.89 ± 16.81b	119.38 ± 11.00a	108.92 ± 10.85ab	101.69 ± 13.31b	58.04 ± 9.58c
	HeLa	82.30 ± 21.04a	86.97 ± 11.39a	79.45 ± 4.60ab	75.17 ± 6.88b	43.35 ± 21.30c
	Hep3B	52.83 ± 14.50a	38.01 ± 4.10b	39.01 ± 7.06b	52.04 ± 8.35a	29.28 ± 8.40c
	MCF-7	92.38 ± 2.86a	82.90 ± 4.89b	77.86 ± 6.39c	70.29 ± 4.09d	65.48 ± 3.29e
	SNU-1066	74.96 ± 11.45a	74.24 ± 6.86a	67.28 ± 2.11b	54.48 ± 8.55c	26.94 ± 11.22d
	SNU-601	114.78 ± 18.66a	97.44 ± 16.18b	95.85 ± 3.87b	86.97 ± 15.49bc	46.53 ± 12.14d

Abbreviations: Cell lines: human renal, HEK 293; colon cancer, HCT-116; cervical carcinoma, HeLa; liver carcinoma, Hep3B; breast carcinoma, MCF-7; laryngeal cancer, SNU-1066; gastric cancer cell, SNU-601. All values are mean ± SD of five independent measurements. Values within different superscripts are different within the same column at $p < 0.05$ by Duncan's multiple range test.

Table 4
Survival rate of cancer cells as affected by the concentration of indigo seed extracts.

Solvent	Cancer cell	Concentration of extract (mg/L)				
		50	100	200	400	800
Ethanol	HEK-293	137.68 ± 10.67a	114.32 ± 21.54b	82.91 ± 12.59c	68.18 ± 18.33d	35.69 ± 14.92e
	HCT-116	56.72 ± 11.16a	52.34 ± 7.19a	50.70 ± 2.13ab	46.93 ± 11.27ab	42.38 ± 7.92b
	HeLa	34.80 ± 2.24a	27.54 ± 2.62b	22.93 ± 1.59b	28.64 ± 12.31b	16.93 ± 3.09c
	Hep3B	66.51 ± 7.19a	44.50 ± 14.78b	47.92 ± 5.16b	48.34 ± 12.87b	38.89 ± 7.57c
	MCF-7	85.09 ± 6.55a	68.81 ± 6.47b	68.86 ± 5.74b	68.96 ± 3.23b	54.57 ± 5.69c
	SNU-1066	58.50 ± 9.99a	54.74 ± 9.73a	57.41 ± 2.72a	57.41 ± 13.69a	39.42 ± 16.80b
	SNU-601	43.98 ± 7.56a	43.20 ± 5.21a	38.06 ± 2.82ab	36.26 ± 4.00ab	28.74 ± 1.04c
Methanol	HEK-293	102.88 ± 10.59a	81.38 ± 4.87b	50.49 ± 13.10d	73.00 ± 5.29c	28.70 ± 16.68e
	HCT-116	59.22 ± 2.78a	52.58 ± 0.76b	51.85 ± 3.57b	48.92 ± 1.54c	48.41 ± 1.80c
	HeLa	34.73 ± 2.15a	30.09 ± 0.52b	23.79 ± 2.79c	23.68 ± 5.05c	22.89 ± 5.37c
	Hep3B	32.03 ± 5.90a	27.65 ± 1.97c	34.07 ± 12.16a	31.29 ± 3.77ab	28.08 ± 2.59c
	MCF-7	100.92 ± 15.84a	67.12 ± 3.93c	72.31 ± 4.41b	72.67 ± 3.37b	70.29 ± 5.15b
	SNU-1066	66.68 ± 9.11a	52.31 ± 6.12b	51.66 ± 10.04b	43.92 ± 3.06c	38.54 ± 2.70d
	SNU-601	46.30 ± 7.95a	47.75 ± 3.95a	38.90 ± 2.07b	39.84 ± 9.25b	29.85 ± 2.04c

Abbreviations: Cell lines: human renal, HEK 293; colon cancer, HCT-116; cervical carcinoma, HeLa; liver carcinoma, Hep3B; breast carcinoma, MCF-7; laryngeal cancer, SNU-1066; gastric cancer cell, SNU-601. All values are mean ± SD of five independent measurements. Values within different superscripts are different within the same column at $p < 0.05$ by Duncan's multiple range test.

Table 5
Correlation coefficients between polyphenols and the overall antioxidants capacities (AC) in water (Wat), ethanol (EtOH) and methanol (Me) extract of investigated parts of indigo plant.

PolxAC assays	Leaves	Flowers	Seeds	Stem
PolWat × ABTSWat	0.9467	0.9345	0.7254	0.7142
PolEtOH × ABTSEtOH	0.8765	0.8665	0.7444	0.7238
PolMeOH × ABTSMeOH	0.9765	0.9676	0.7565	0.7476
PolWat × CuWat	0.9543	0.9454	0.7304	0.7234
PolEtOH × CuEtOH	0.8862	0.8751	0.7523	0.7443
PolMeOH × CuMeOH	0.9832	0.9743	0.7643	0.7564

Abbreviations: PolxAC, polyphenols *vs* antioxidant capacities; ABTS, 2, 2-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt; Cu, Cupric reducing antioxidant capacity.

from 0.7142 to 0.9765, showing the highest correlation for leaves and flowers in methanol extracts (Table 5).

The highest R^2 between the antioxidant capacities and survival rates was in methanol extracts of leaves on HeLa cells.

4. Discussion

Our recent investigation has shown (Heo et al., 2013) that the highest anticancer activity was in prolipid (a mixture of medicinal herbs, which was used for the comparison of indigo bioactivity) in concentrations of 800 μg/mL against Calu-6, following by indigo brown leaves. Organic extracts of indigo brown leaves were analyzed for their antioxidant and anticancer activities and compared with prolipid, using polyphenols composition, antioxidant activities and fluorescence properties. The indigo ability to quench the intrinsic fluorescence of bovine serum albumin (BSA), relatively high content of phenolic compounds and anticancer properties indicate that indigo can be used as medicinal plant (Heo et al., 2013). Kim et al. (2012b) and Jang et al. (2012) showed the proliferative and antioxidant activities of indigo plant in water in methanol extracts. However, the obtained results were from one year collection. Therefore it was interesting to compare our recent results from 2012 of indigo plant with the previously obtained of 2010 from the same area of collection and to use water, methanol and ethanol extracts and to apply these extracts to different cells lines. Our obtained results were slightly different from the ones obtained previously (Kim et al., 2012b; Jang et al., 2012; Heo et al., 2013). Total polyphenols (mg GAE/g DW) and flavonoids (mg CE/g DW) of water extracts in leaves (19.39 ± 1.45 and 1.89 ± 0.18, Fig. 2) can be compared with the data of Djidel et al. (2010), where polyphenol (mg GAE/g DW) and flavonoid (mg QE/g DW) contents of *Origanum majorana* leaves were 19.14 ± 0.0015 and 2.96 ± 0.01, respectively. In *Inula viscosa Boraginaceae* leaves concentrations of polyphenols and flavonoids were in line with our results such as 19.88 ± 0.0026 and 1.65 ± 0.0037. Polyphenols and flavonoids in *Foeniculum* ssp. *Araliaceae* seeds (1.92 ± 0.004 and 4.71 ± 0.0055) were similar to our obtained results (3.07 ± 0.28 and 0.52 ± 0.12, Fig. 2). *Polygonum bistort Rhamnaceae* aerial parts (2.05 ± 0.0045 and 0.36 ± 0.034) were similar to our results of stem extracts (2.75 ± 0.14 and 0.52 ± 0.12, Fig. 2). Plants with the highest free radical scavenging activities had as well the highest polyphenol content (*Pistachia lentiscus* L. and *Murtus communis* L.) as shown in Fig. 2. We can conclude that some medicinal plants used traditionally for gastrointestinal disorders and hypertension exhibited radical scavenging capacities due, in part, to phenolic compounds present in these plants and showed as well proliferative properties as on gastric cancer cell line SNU-601. Our results can also be compared with results from Marino et al. (2009), where the polyphenol content was determined in aqueous extracts in medicinal plants and varied from 1.3 to 15.4%. Ndjonka et al. (2012) compared ethanolic and aqueous extracts of the 11 selected plants and pure compounds from *Phyllanthus muellerianus* and *Anogeissus leiocarpus* which were tested *in vitro* against Plasmodium falciparum 3D7. Proliferation inhibitory effects were high with phenolic acids which correspond with our results that Indigo extracts with high antioxidant activity showed as well high proliferativity. Growth inhibition was concentration-dependent. This conclusion is in accordance with our obtained results shown in Tables 1–4, where the cytotoxicity is dependent on the concentration of the added indigo extracts. Our results can be compared with the information given by Li et al. (2007), where the efficiencies of two traditional extraction methods used in Chinese medicine (the decoction method and the maceration method) were evaluated for the extraction of antioxidants from medicinal plants. The results of this investigation are in line with

our conclusions that any extraction procedure is suitable for the extraction of bioactive compounds. At home, people can use these methods to extract aqueous antioxidants from plants for consumption, as the bioactivity of water extracts is as efficacy in comparison with ethanol extracts, as shown in this report. In the food industry these methods could be utilized to prepare crude extracts from plants containing antioxidants for use as food additives. Kaneria et al. (2012) showed how hydroalcoholic and decoction methods influenced the extraction of bioactive compounds in different parts of plants. The results showed that the extracting solvent significantly altered the antioxidant property estimations of screened plants which are exactly correspondent with our results. Our results can be compared with a study by Mahomoodally et al. (2012), where selected traditional medicinal plants were evaluated for their antioxidant and cytotoxic potential *in vitro* using the crude methanolic and water extracts and fractions, obtained from crude methanolic extracts fractionated by solvent–solvent extraction procedure into dichloromethane, ethyl acetate, *n*-butanol and aqueous fractions. Crude extracts and fractions have potent antioxidant and antiglycation properties with no apparent cytotoxicity and might have prophylactic and therapeutic potentials in the management of diabetes and related complications. Our results are in agreement with Kaewpiboon et al. (2012), where 52 traditionally used species of Thai medicinal plants for their *in vitro* cytotoxic, antioxidant, lipase inhibitory and antimicrobial activities. In the cited research hexane, dichloromethane, ethanol and water extracts were applied to human lung (A549), breast (MDA-MB-231), cervical (KB3-1) and colon (SW480) cancers cells, using the MTT cytotoxicity assay. The Thai medicinal plant *Bauhinia strychnifolia* vines exerted strong *in vitro* cytotoxic activities against human cancer cell lines. The ethanol extract of indigo in polyphenols and ABTS antioxidant activity was similar to *Kochia scparia* (L.) Schrad with antioxidant activity of 68.83 ± 1.69 μM TE/g DW and polyphenols of 8.63 ± 0.12 mg GAE/g DW, shown in a study by Gan et al. (2010). Our data well correspond with the determined amount of polyphenols and their antioxidant activities in Kumari and Kakkar (2008), where the ethanolic extracts from five therapeutically important medicinal plants native to India showed the highest polyphenols of 195 mg GAE/g. As was calculated, a very good correlation was found between the antioxidant capacity and the contents of total polyphenols (Table 5). A positive linear correlation between antioxidant capacities and total phenolic contents implied that phenolic compounds in these plants could be the main components contributing to the observed activities. The correlation of antioxidant effect with survival of cancer cells was relatively high. So, the correlation coefficients of antioxidant capacities of water leave extract and their survival rates on SNU-1066 HCT-116 and HeLa were 0.953, 0.611 and 0.707. The $R^{2\ of}$ the antioxidant and cytotoxicity of ethanol flower extract on HCT-116 and HeLa were 0.718 and 0.810; and extracts of leaves on SNU-1066 and HCT-116 were 0.763 and 0.913. Methanol extracts of leaves on SNU-1066, HeLa and HCT-116 were 0.948, 0.921, and 0.737. Our conclusions can be compared with the results of Kumar et al. (2011), where the methanolic leaf extract of *Indigofera cassioides* (MEIC) against transplantable tumors and human cancer cell lines was evaluated. MEIC was investigated for its *in vitro* cytotoxicity on HeLa, HEp-2, HEpG-2, MCF-7, HT-29, Vero and NIH 3T3 cells by MTT assay. MEIC exhibit potent *in vitro* cytotoxicity against all the tested cancer cell lines, but it was found to be safe on normal cells. Our results can be compared as well with Utami et al. (2013), where methanolic extracts of *Elaeocarpus floribundus* were subjected to screening of antioxidant activity and cytotoxic activities by MTT assay toward human T4 lymphoblastoid (CEM-SS) and human cervical (HeLa) cancer cells. In the total phenolic content determination, methanolic extract of leaves gave higher value than stem bark. It was found direct relationship between the polyphenols and antioxidant activity which is in line with our conclusions. We can compare our results with Shivananjappa and Joshi (2012), where aqueous extracts of *Emblica officinalis Gaertn*. improved endogenous antioxidant defenses in HepG2 cells.

5. Conclusions

The water, methanol and ethanol extracts of flowers, leaves, stems, and seeds from indigo (*P. tinctorium Lour.*) were tested *in vitro* to verify cytotoxic effects on human renal cell line (HEK 293) and inhibitory effects on the proliferation of a number of cancer cells. Significant differences were found in polyphenols and flavonoids in extracts of different parts of indigo. The highest amount of total polyphenols and antioxidant capacities found in methanol extracts of leaves and flowers and flavonoids in methanol extracts of leaves, flowers and seeds. The highest correlation coefficient (R^2) between antioxidant capacities and polyphenols of indigo parts were in leaves and flowers in methanol extracts. The survival rates at a concentration of 800 mg/L of colon cancer cell line (HCT-116) and cervical cancer cell line (HeLa) in the ethanol extract of indigo flowers were $5.10\% \pm 0.32$ and $8.85\% \pm 0.11$, respectively. Proliferative and antioxidant activities were correlated. The highest R^2 between the antioxidant capacities and survival rates was in methanol extracts of leaves on HeLa cells. Therefore, investigation *in vitro* revealed that the extracts of the indigo plant were found to be effective in suppressing the proliferation of cancer cells, although the results varied depending on the different parts of *P. tinctorium Lour.*, the concentrations of solvent and extracts, the antioxidant activity and the types of cancers.

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