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Antioxidant and Antiproliferative Properties of Extract and Fractions from Small Red Bean (*Phaseolus vulgaris* L.)

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Abstract

Crude polyphenolic extract was extracted from small red bean, and was further semi-purified by adsorption-desorption. The resultant semi-purified concentrate was fractionated to five fractions (Fr I – Fr V) with Sephadex LH-20. Phytochemical contents, antioxidant and antiproliferative properties of extracts and fractions were evaluated. Results indicated that the Fr V had the highest total phenolic content and condensed tannin content, and exhibited strong free radical scavenging activity against DPPH and ABTS radicals, with the IC_{50} of 0.128 and 0.036 mg/mL, respectively. Furthermore, the Fr V showed considerable antiproliferative effects against SK-OV-3, SW480, CAL 27, and Hep G2 cancer cells with IC_{50} of 0.035, 0.111, 0.035, and 0.008 mg/mL, respectively. In conclusion, the Fr V, whose constituents were mainly condensed tannins, possessed the highest antioxidant and antiproliferative effects, and thus could be potentially applied as natural antioxidant and antiproliferative agents in both food, cosmetic, or pharmaceutical fields for health promotion.

Keywords: Small red bean; Polyphenolics; Antioxidant; Antiproliferative; Fractionation

Introduction

Reactive oxygen species (ROS) are oxygen derived species, including superoxide anion ($O_2^{\cdot-}$), hydroxyl ($HO^{\cdot}$), peroxyl ($ROO^{\cdot}$), alkoxyl ($RO^{\cdot}$), nitric oxide ($NO^{\cdot}$), singlet oxygen, hydrogen peroxide, and hypochlorous acid [1]. ROS are very highly reactive intermediates that can damage cellular constituents such as DNA, proteins, amino acids and lipids when overproduced. The consequence of the damage include altered cell signaling, enhanced mutations rates, and accelerated cellular degeneration, thus induce degenerative disease including mutagenesis, carcinogenesis, coronary heart disease, diabetes, Alzheimer's Disease, and Parkinson's disease [2]. Intake of dietary antioxidant such as polyphenolics from medicinal plants, sea weeds, fruits and vegetables has been considered as an important approach in the prevention of those chronic diseases, which maybe attribute to the antioxidant activity of polyphenols.

Common beans (*Phaseolus vulgaris* L.), known also as dry beans, including small red bean, red kidney bean, navy bean, pinto bean, black bean, and pink bean, are the world's second most important beans after soybeans. Common beans are cultivated and consumed throughout the world, especially in South-American and African countries, for their excellent source of protein, soluble fibers, carbohydrates, vitamins, minerals, and various bioactive phytochemicals with health-promotion effects [3,4]. Consumption of common beans has been known to reduce the risk of coronary heart disease, diabetes, and obesity, as well as lowering serum cholesterol concentration [5]. A growing body of evidence suggests that the greatest consumption of beans had the lower incidence of stomach, prostate, breast, and colon cancer [6]. Epidemiological surveys and case-control studies also demonstrated the protective effects of beans against several types of cancer [7].

Small red bean, with shape being broad and oval, and seed coat being burgundy red, possessed higher concentrations of phenolic contents and antioxidant activity compared with other common beans, as shown in

our previous study [8]. Furthermore, the small red bean ranks the first in the USDA's top 20 lists of foods with the highest antioxidant capacity [9]. Although some studies have been conducted on the antioxidant and antiproliferative properties of common beans [10,11], to our knowledge, only works by Cardador-Martiänez (2002) and Aparicio-Fernández [12,10]. showed the phenolic content and antioxidant activity of fraction obtained by chromatographic separation of dry beans extracts over silica gel, and studies on the fractionation of small red bean extracts into different groups, then subsequently characterization their antioxidant and antiproliferation properties are lacking. In order to elucidate which groups of phytochemicals were most pronounced with regard to the antioxidant and antiproliferation potential of the small red bean, the phenolic substances in the crude small red bean extract were concentrated by adsorption-desorption and fractionated into different groups by an open chromatographic method, and then the antioxidant and antiproliferative properties of these extracts and fractions were determined in this study.

Materials and Methods

Materials and chemicals

Small red bean was purchased from Red River Commodity (Fargo, ND). The small red bean were ground to powder with an IKA® A11 basic mill (IKA Works Inc., Wilmington, NC) and to pass through a 60-mesh sieve. The powders were stored at -20 ˚C before use.

XAD-7 was purchased from Sigma-Aldrich (St. Louis, MO), and Sephadex LH-20 from Pharmacia LKB Biotech (Uppsala, Sweden). 2-diphenyl-1-picryhydrazyl radical (DPPH), fluorescein disodium (FL), 6-hydroxy-2,5,7,8-tetramethlchroman-2-carboxylic acid (Trolox), 2,2´-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), potassium persulfate, trifluoroacetic acid (TFA), Folin-Ciocalteu reagent, sodium carbonate, 2, 4, 6-tri(2-pyridyl)-s-triazine (TPTZ), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl (MTT), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Inc (St. Louis, MO). The 2, 2´-azobis (2-amidino-propane) dihydrochloride (AAPH) was purchased from Wako Chemicals USA (Richmond, VA). Cell culture media including McCoy's 5A Medium Modified, L-15 medium, Dulbecco's Modified Eagle's Medium (DMEM), and Eagle's Minimum Essential Medium (EMEM), fetal bovine serum (FBS), penicillin-streptomycin mixed antibiotics, phosphate buffer solution (PBS), and 0.25% trpsin-EDTA, were from Hyclone Laboratories Inc (Logan, Utah). Other chemicals were of the highest quality commercially available.

Extraction of crude extract from small red bean

Small red bean powders (200 g) were extracted with a solvent mixture (acetone/water/acetic acid 70:29.5:0.5, v/v/v) with a solid to solvent ratio of 1:10 (w/v), and subsequently placed on a magnetic stirrer (Thermolyne, Dubuque, IA) at room temperature for 12 h. The extract was filtered through Whatman No. 1 filter paper in a Buchner funnel. The residues were re-extracted twice under the same conditions, and all of the supernatants were combined and concentrated to a small volume at 40 ˚C using a rotary evaporator (Labconco Co., Kansas City, Mo) under vacuum. Then the crude extract (CE) was obtained by lyophilizing the concentrated extract and stored at -20 ˚C until use.

Fractionation of crude extraction

The CE was firstly concentrated by adsorption-desorption over a macroporous XAD-7 resin, and subsequently fractionated by Sephadax-LH 20 column chromatography. In brief, four g of CE was suspended in 20 mL of water by vortexing vigorously. The suspension was centrifuged to remove the insoluble part and the supernatant was filtered to get a clear solution. The residue was suspended in water twice, and all the supernatants were combined. The clear solution was poured in a column previously packed with a macroporous resin XAD-7 (column of 20 × 1.6 cm, i. d., bed volume (BV) = 33.5 mL). The solution was pumped down through the column at a speed of 1.8 bed volumes/h (BV/h). The resin was washed with 2 BV distilled water to remove the sugars, organic acids and other water-soluble compounds (water eluate). The 80% methanol was used to elute the phenolic compounds at a speed of 4 BV/h. The eluate was rotary-evaporated under vacuum to remove solvents, and then freeze-dried to yield semi-purified extract (SPE) of small red bean. A quantity of 0.3 g SPE was re-dissolved in water, and the obtained solution was further fractionated over a Sephadex LH-20 column (35 × 2.6 cm, i. d., BV = 185 mL). The column was eluted successively with H_2O (600 mL), 50% aqueous ethanol (700 mL), ethanol/methanol (1:1, v/v, 500 mL), and 50% aqueous acetone (600 mL) at a flow rate of 2 mL/min, and 240 fractions of 10 mL each fraction were collected. The absorbance of each fraction was determined at 280 nm for phenolic compounds, 360 nm for flavonoids, and 520 nm for anthocyanins, respectively, using a Multiskan Spectrum microplate reader (Thermo Electron Corporation, Asheville, NC). Moreover, each fraction was reacted with Folin–Ciocalteau reagent, and the absorbance of the obtained solution was determined at 765 nm. The fractions were combined according to their absorbance at 280, 360, 520, and 765 nm into five fractions: Fr I -V (Figure 1).

Determination of phytochemicals content

Total Phenolics Content (TPC)-Total phenolics in the all samples was determined with Folin–Ciocalteau assay with minor modifications using gallic acid as a standard phenolic compound. Sample preparation and detail of the procedure were carried out according to our previous study [13]. The TPC was expressed as mg gallic acid equivalents (mg GAE/g) using an equation obtained from the standard gallic acid calibration curve. Linearity range of the calibration curve was 62.5-2000 μg/mL (R^2 = 0.999).

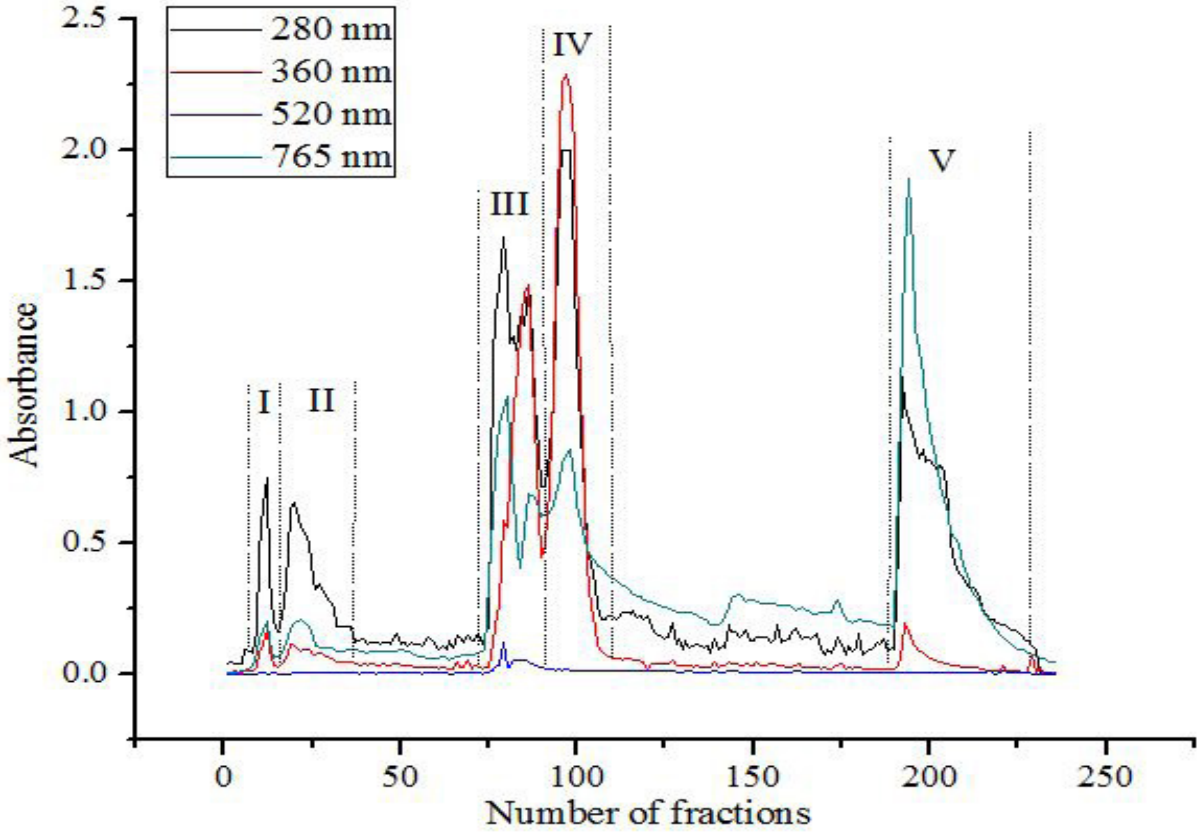


Figure 1: Elution curve of fractionation of SPE with Sephadex LH-20.
Ten mL per fraction was collected. Each fraction was monitored at 280, 360, and 520 nm, and each fraction was also detected at 765 nm after reacted with Folin-Ciocaltaeu reagent. Fractions were combined according to their absorbance. Peaks at the line of wavelength of 280 and 765 nm represented phenolics compounds such as phenolic acids, flavonoids, or condensed tannins, peaks at 360 nm represented flavonoids, and peaks at 520 nm represented anthocyanins.

Total flavonoids content (TFC)-Total flavonoids in the extract and fractions were determined using the colorimetric method described previously with slightly modification using catechin as the standard [13]. The absorbance was determined at 510 nm versus a prepared water blank using a Multiskan Spectrum microplate reader. All values were expressed as milligrams of catechin equivalents per 1 g sample (mg CE/g sample) through the calibration curve of catechin. Linearity range of the calibration curve was 7.83-1000 μg/mL (R^2 = 0.999).

Monomeric Anthocyanins Content (MAC)-The MAC was determined by a spectrophotometric pH differential method [14]. In brief, each sample was thoroughly mixed with 0.025 M potassium chloride buffer (pH = 1.0) in an appropriate dilution factor, and the absorbance of the mixture was measured at both 520 and 700 nm using an UV spectrophotometer (UV160, Shimadzu, Japan). Another aliquot of sample was mixed with sodium acetate buffer (pH = 4.5) with the same dilution factor, and the absorbance was measured at 520 and 700 nm. The absorbance of the diluted sample (*A*) was calculated as follows: $A = (A_{520} - A_{700})$ pH 1.0 - $(A_{520} - A_{700})$ pH 4.5. The MAC was expressed as mg of cyanidin 3-glucoside equivalents per gram of sample (mg CGE/g) and calculated as follows: MAC (mg CGE/g) = $(A \times MW \times DF \times 1000)/ (\varepsilon \times 1)$, where A is the absorbance of diluted sample and DF is the dilution factor, MW and ε corresponded to the molecular weight (449.2) and molar extinction coefficient (26900) of cyanidin 3-glucoside, respectively.

Condensed Tannins Content (CTC)-The CTC in extracts and its fractions was determined using the vanillin assay with slightly modification in our lab [13]. Different concentrations of (+)-catechin ranged from 31.25 to 1000 μg/mL were used as standard compound for the quantification of total condensed tannins. All values were expressed as milligrams of catechin equivalents per 1 g sample (mg CE/g).

Determination of antioxidant activity

DPPH radicals scavenging activity-The DPPH radical scavenging assay was performed as reported previously [15] with slight modification. In brief, 10 µl of the samples at different concentrations was added to 190 µl of a 105.3 µM DPPH solution in a well of 96-well plate.

The mixture was shaken gently and left to stand at room temperature in the dark for 30 min. Thereafter, the absorbance at 517nm was measured against methanol using a Multiskan Spectrum microplate reader. Controls containing methanol instead of the sample solution was also analyzed. Ascorbic acid, vitamin E, and Trolox were used as positive control. The DPPH scavenging activity of the samples was calculated according to the following formula: DPPH scavenging activity (%) = (1 - Abs. of sample/Abs. of control) × 100. The percentage of scavenging activity was plotted against the sample concentration to obtain the IC_{50}, defined as the concentration of samples necessary to cause 50% scavenging.

ABTS radicals scavenging activity-ABTS radicals scavenging activity was performed as reported previously [13]. The absorbance was recorded at 734 nm using a Multiskan Spectrum microplate reader. Ascorbic acid, vitamin E, and Trolox were used as positive control. The ABTS scavenging activity of the samples was calculated according to the following formula: ABTS scavenging activity (%) = (1 - Abs. of sample/Abs. of control) × 100. The percentage of scavenging activity was plotted against the sample concentration to obtain the IC_{50}.

Ferric reducing antioxidant power assay (FRAP)-The ferric reducing antioxidant power assay was performed as previously described with slightly modification in our lab [13]. The absorbance was read using a Multiskan Spectrum microplate reader at 593 nm. The FRAP value was calculated and expressed as millimoles of Fe^{2+} equivalents per 100 g of sample (mmol Fe^{2+} equivalents/100 g) based on a calibration curve plotted using $FeSO_4{\cdot}7H_2O$ as standard at a concentration ranging from 0.125 to 2 mM.

Oxygen radical absorbing capacity (ORAC)-ORAC assay was performed using a Fluostar Optima plate reader (BMG Labtech, Durham, NC) equipped with an incubator and two injector pumps. The procedure was the same as our previous report [13]. The ORAC values were calculated as trolox equivalents per gram sample (μmol TE/g) using a standard curve prepared with 6.25 - 50 μM trolox.

Determination of antiproliferative activity of human cancer cells

Cell lines and cell culture-Four human cancer cell lines including ovarian SK-OV-3, colon SW480, tongue CAL 27, and liver Hep G2 were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in McCoy's 5a Medium Modified, L-15 medium, Dulbecco's Modified Eagle's Medium (DMEM), and Eagle's Minimum Essential Medium (EMEM), respectively. All the media were supplemented with 10% heated-inactivated fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL). The cells were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2, and the medium was changed every other day.

Determination of cell viability by MTT assay-Cell viability was measured using the MTT assay, which is based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenases. To evaluate the antiproliferative effects of extracts and fractions, the cells were plated at density of about 1×10^5 cells/well in 96-well plates for 24 h. The extracts and fractions at different concentrations were added to the wells and incubated for 48 h, then the culture medium was aspirated, and the cells were washed two times with cold phosphate buffer saline (PBS), and 100 μl MTT solution (5 mg/mL stock solution in PBS, diluted with culture medium to the final concentration of 0.5 mg/mL) were added. After 4 h incubation at 37 °C, this solution was removed, and the produced formazan was solubilized in 150 μl dimethyl sulfoxide (DMSO). Absorbance was measured at 540 nm using a Multiskan Spectrum Microplate reader. Cell viability was expressed as a percentage of the control cells which were considered as 100% viable.

Statistical analysis

Data were expressed as mean ± standard deviation of triplicate measurements. The data were statistically analyzed using statistical software, SAS Version 9.1, (SAS institute Inc. Cary, NC). One-way analysis of variance (ANOVA) was conducted, and p values of < 0.05 were considered significant.

Results and Discussion

Yield and phenolic content of crude extract

Large amounts of solvents are employed in the extraction of antioxidant from food matrix. The yield of extraction is largely dependent on the solvent systems with different polarity and pH, extraction time, and extraction temperature. According to our previous report, the solvent mixture (acetone/water/acetic acid 70:29.5:0.5, v/v/v) ensured the complete extraction of phenolics from lentils and common beans [16]. Therefore, acidic 70% acetone was used for the extraction of phenolics from small red bean. The yield of CE based on the dry weight of small red bean was 10.9%, which was comparable to the yield of crude morton lentil extract [13], and much higher than that of red bean and adzuki bean (6.12 and 4.55%, respectively) [17] and several improved common bean cultivars from Mexico (ranged from 6.03 to 6.83%) [18], but lower than that of four bean varieties (ranged from 12.2 to 20.6%) as reported by Madhujith [19].

The contents of phenolic components including the total phenolic, flavonoids, anthocyanins, and condensed tannins in the CE were measured according to the colorimetric assay. The TPC, TFC, MAC, and CTC in the CE of small red bean was 51.0 mg GAE/g, 24.8 mg CE/g, 0.9 mg CGE/g, and 31.8 mg CE/g, respectively. The TPC was obviously higher than the extracts from beans with less colored seed coats such as white bean extract (4.9 mg CE/g), but lower than that of red bean and brown bean extract, although the results were expressed in a different way [19]. The flavonoids and anthocyanins in common beans are usually identified by HPLC or HPLC-

MS [4], few studies are conducted on the total flavonoids and anthocyanins of crude extract of common beans, so it is difficult to compare. The difference in the yield and phenolic content might be attributed to the beans cultivated in different environmental conditions, different genotypes, and different extraction methods.

Fractionation of crude extract

Evidence from literatures reveals that the activities of crude extract are mainly attributed to the presence of phytochemicals such as flavonoids, tannins, and phenolics. Furthermore, the bioactivities of the extract are positively correlated with the content of these phytochemicals [8]. When the matrixes are extracted with mixture of organic solvent and water, some non-phytochemical components such as proteins and carbohydrates are co-extracted, which bulked up the extract and lowered their bioactivities. The major components in CE of small red bean was sugars (75.6 g glucose/100 g) when determined by the phenol-sulfuric acid assay. In order to fractionate the CE into different fractions, the non-phytochemical components were firstly removed by macroporous resin XAD-7.

Macroporous resin, a highly cross-linked polymer with a large pore structure, is extensively used in food, pharmaceutical, and cosmetic industry for the purification or concentration of natural phytochemicals. The purification is mainly through the adsorption capacity of resins for compounds with different molecular weight, polarity, or shape of the molecules in the solution, which leads to differences in affinity for the resins [20]. In our experiment, after adsorbed by the XAD-7 resin, washed with water, and desorbed with 80% MeOH successively, the resultant yield of water eluate (WE) and 80% MeOH eluate (SPE) was 75.3% and 6.2%, respectively. The yield of SPE was much lower than that of WE, but the TPC (238.7 mg GAE/g) in SPE was almost 44 times higher than that of WE (5.4 mg GAE). The TFC, MAC, and CTC in SPE was 154.8 mg CE/g, 7.1 mg CGE/g, and 169.5 mg CE/g, respectively, which was increased by 6.2, 7.9, and 5.3 folds compared to those in the crude extract (Table 1).

The SPE obtained from the macroporous resin was further separated into five different fractions with H_2O (Fr I and Fr II), 50% ethanol (Fr III and Fr IV), ethanol/methanol (1:1, v/v), and 50% acetone (Fr V) over a Sephadex LH-20 column. The fractions were collected and combined from this column according to their absorbance at 280, 360, 520, and 765 nm (Figure 1), and the total recovery was 97.4% of the SPE applied to the column. Fr I, eluted with water, was the main fractions, which averaged almost 3 to 4 times the yield of other fractions. Whereas Fr I contained almost no phenolic compounds since the TPC and TFC were significantly lower compared with those of other fractions. The highest TPC was found in Fr V, which contained 536.4 mg/g. The TPC in fractions and extracts decreased in the following order: Fr V > Fr IV > Fr III > SPE > Fr II > CE > Fr I (Table 1). In the case of flavonoids content, Fr III and Fr IV contained higher TFC (194.1 and 167.9 mg CE/g, respectively), which suggested that flavonoids other than phenolic acids or condensed tannins was mainly eluted by 50% EtOH. Anthocyanins, one category of flavonoids, were also eluted by 50% EtOH. Only Fr III contained the highest MAC, in which the anthocyanins content were 6.2 and 48.8 times, respectively, of the SPE and CE. However, Fr I and Fr II did not contain any anthocyanins. The CTC, measured using the vanillin/HCl method, was shown in Table 1. Fr I and Fr II contained no condensed tannins. The CTC in Fr V was the highest (591.6 mg CE/g). Although Fr III and Fr IV showed positive color reaction with vanillin/HCl reagent, it might be caused by the reaction between catechin or other monomeric flavanols and vanillin/HCl reagent [21].In summary, after fractionation of SPE over Sephadex LH-20, the main constituents in Fr III, Fr IV, and Fr V were anthocyanins, flavonoids, and condensed tannins, respectively.

Table 1: The yield, TPC, TFC, MAC, and CTC of extracts and fractions from small red bean.

	Yield (%)	TPC (mg GAE/g)	TFC (mg CE/g)	MAC (mg CGE/g)	CTC (mg CE/g)
CE	10.9 [#]	51.0 ± 1.8 d	24.8 ± 1.6 d	0.9 ± 0.1 d	31.8 ± 1.9 d
SPE	6.2 [$]	238.7 ± 18.9 c	154.8 ± 23.8 b	7.1 ± 0.2 b	169.5 ± 7.8 b
Fr I	43.8 [*]	11.6 ± 0.4 e	1.9 ± 0.2 e	nd	nd
Fr II	14.9 [*]	63.8 ± 2.2 d	21.4 ± 0.9 d	nd	nd
Fr III	11.5 [*]	394.6 ± 21.9 b	194.1 ± 6.7 a	43.9 ± 0.9 a	82.5 ± 7.0 c
Fr IV	10.4 [*]	434.5 ± 20.9 b	167.9 ± 9.4 a b	4.9 ± 0.7 c	139.4 ± 16.2 b
Fr V	16.8 [*]	536.4 ± 30.6 a	101.8 ± 12.4 c	0.1 ± 0.0 e	591.6 ± 34.1 a

[#] based on small red bean powders, [$] based on CE, [*] based on SPE. GAE, gallic acid equivalents; CE, catechin equivalents; CGE, cyanidin 3-glucoside equivalents. nd: not detectable. Results were expressed as means ± standard deviation (n = 3), values with different letters within a column were significantly different ($p < 0.05$).

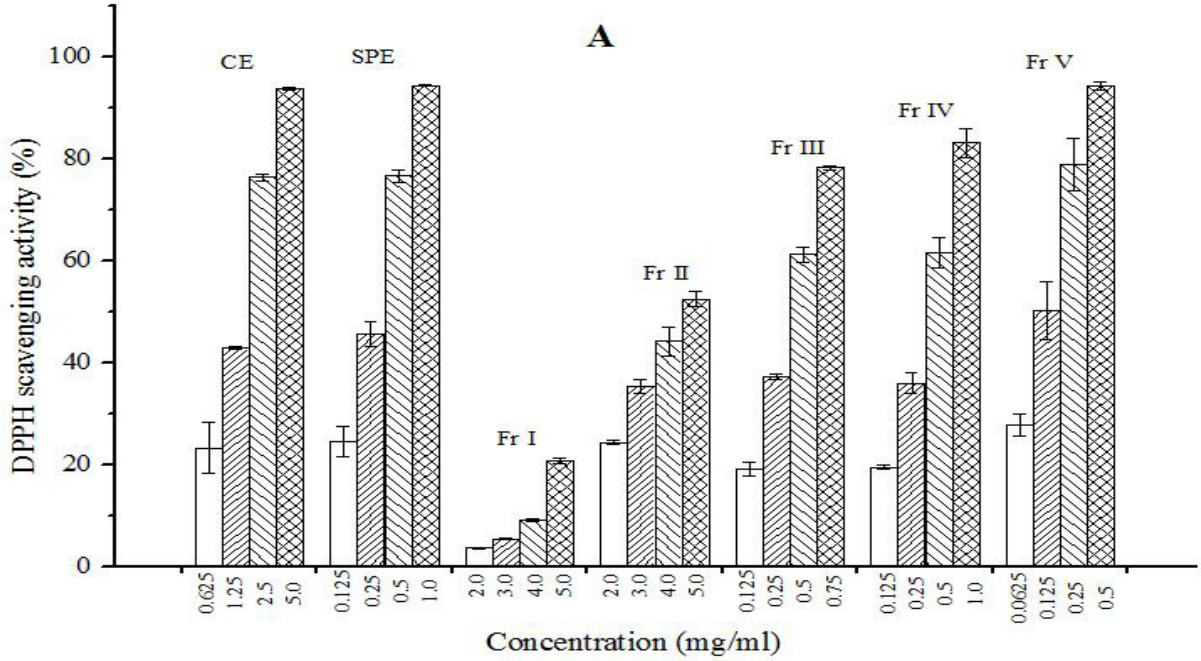
Free radical scavenging activity

The free radical scavenging activity of CE of small red bean and its fractions were determined by the DPPH and ABTS assays. Both assays are based on the transfer of hydrogen between the free radicals and antioxidants,

and these assays have been extensively used *in vitro* to evaluate antiradical activities of fruit and vegetable juices or extracts because of its simplicity and sensitivity [22].

DPPH, a stable organic free radical with absorption band at 515 - 528 nm, can be reduced to a non-radical form when accepting an electron or hydrogen atom, thus losing this absorption and result in a visually noticeable discoloration from purple to yellow. The DPPH scavenging activity of all extracts and fractions was shown in Figure 2A.

Figure 2: Free radical scavenging activity of extracts and fractions from small red bean.



Free radical scavenging activity against DPPH and ABTS was measured by the colorimetric methods as described in the Materials and methods. Results were expressed as means ± standard deviation (n = 3). (A) DPPH, and (B) ABTS.

It can be concluded that the scavenging effect on DPPH increased in a concentration-dependent manner for all extracts and fractions. However, the concentration to inhibit the DPPH was much different among different extracts and fractions. For Fr I and Fr II, the scavenging effect was only exhibited at higher concentration (up to 2 mg/mL) compared with other fractions and SPE. To get an almost equivalent scavenging effect on DPPH as Fr V and SPE, ten-folds and five-folds concentration of CE was needed as compared with that of Fr V and SPE, respectively. IC_{50}, defined as the necessary concentration at which the radicals generated by the reaction systems were scavenged by 50%, could be served as an indicator of radical-scavenging activity. The higher IC_{50} value corresponds to a lower scavenging activity on DPPH radicals. The IC_{50} of CE and different fractions is shown in Table 2. Ascorbic acid, α-tocopherol, and trolox were used as positive controls for comparison. At the range of concentrations tested in the present study, it was not possible to determine the IC_{50} of Fr I. At the highest concentration (5 mg/mL), only 20.7% of DPPH radicals were scavenged by Fr I. The IC_{50} value increased in the following order: ascorbic acid < Fr V < α-tocopherol < trolox < SPE < Fr III < Fr IV < CE < Fr II. Ascorbic acid, a strong antioxidant, exhibited the highest DPPH scavenging activity as anticipated. Among all fractions, Fr V, which contained the highest levels of total phenolics and condensed tannins, had the lowest IC_{50} value.

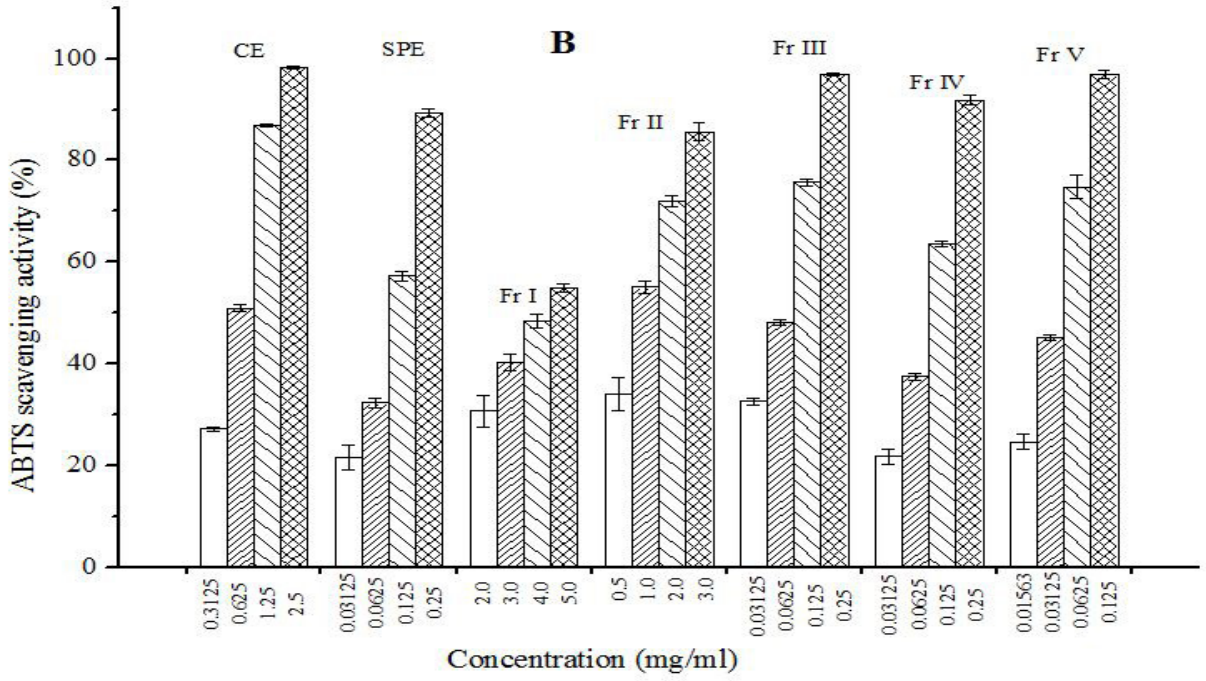
Although the ABTS assay presents a disadvantage over DPPH assay with regard to the stability of ABTS radical [23], it is still one of the popular indirect methods for determining the antioxidant activity of compounds or extracts. As shown in Figure 2B, a concentration-dependent inhibition manner was observed for CE and all fractions, and the trends were quite similar compared with that obtained in the DPPH assay. However, the corresponding extracts and fractions exhibited stronger scavenging activity against ABTS than against DPPH. For example, at 1.25 mg/mL, the CE scavenged 86.9% of ABTS compared with 42.8% for DPPH, and at 0.125 mg/mL, the Fr V scavenged 96.8% of ABTS compared with 50.2% for DPPH. Regarding the activity of different fractions, although the Fr I and Fr II exhibited slight ABTS radical scavenging activity at high concentrations, the activity of Fr I and Fr II was far lower than those of CE, SPE, and other fractions. Furthermore, the scavenging activity was undetectable below 0.25 mg/mL. The scavenging activity of CE at the concentration of 0.3125, 0.625, 1.25, and 2.5 mg/mL was 27.2%, 50.9%, 86.9%, and 98.3%, respectively, whereas the ABTS was absolutely scavenged by Fr V even at the lowest concen-

Table 2: IC_{50} (mg/mL) against DPPH and ABTS free radicals and antioxidant activity of extracts and fractions from small red bean.

	IC_{50} (mg/mL)		Antioxidant activity	
	DPPH	ABTS	FRAP (mmol Fe^{2+} equivalents/100g)	ORAC (μmol Trolox equivalents/g)
CE	1.444 ± 0.034 b	0.603 ± 0.006 c	49.6 ± 0.3 d	2064.1 ± 127.4 d
SPE	0.279 ± 0.008 c d	0.106 ± 0.001 d e	261.1 ± 1.5 c	11659.6 ± 1674.4 c
Fr I	> 5	4.228 ± 0.123 a	6.7 ± 0.3 e	311.3 ± 7.6 e
Fr II	4.700 ± 0.243 a	0.942 ± 0.052 b	42.3 ± 0.3 d	3103.6 ± 253.3 d
Fr III	0.373 ± 0.008 c	0.064 ± 0.001 d e	339.0 ± 7.1 b	26085.9 ± 1896.5 a
Fr IV	0.376 ± 0.019 c	0.090 ± 0.000 d e	269.1 ± 8.6 c	14255.2 ± 789.8 b c
Fr V	0.128 ± 0.009 d e	0.036 ± 0.001 e	632.0 ± 24.1 a	16890.7 ± 141.0 b
Ascorbic acid	0.086 ± 0.006 e	0.062 ± 0.001 d e		
α-Tocopherol	0.158 ± 0.003 d e	0.124 ± 0.024 d		
Trolox	0.164 ± 0.003 d e	0.092 ± 0.002 c		

Results were expressed as means ± standard deviation (n = 3), values with different letters within a column were significantly different ($p < 0.05$).

Figure2: Free radical scavenging activity of extracts and fractions from small red bean.



Free radical scavenging activity against DPPH and ABTS was measured by the colorimetric methods as described in the Materials and methods. Results were expressed as means ± standard deviation (n = 3). (A) DPPH, and (B) ABTS.

tration of 0.3125 mg/mL. The IC_{50} of CE, SPE, and different fractions against ABTS were 0.603, 0.106, 4.228, 0.942, 0.064, 0.090, and 0.036 mg/mL, respectively. The IC_{50} of Fr V was the lowest and was almost 16 times lower than that of CE , which demonstrated Fr V possessed the strongest radical scavenging activity against ABTS•+. It was noted that the IC_{50} in scavenging ABTS radical was 2 to 5 times lower than those in scavenging DPPH radical as reported in our previous study [13], suggested that the ABTS assay was more sensitive than the DPPH assay. The big difference between scavenging of ABTS and DPPH radicals was also observed when measuring the antioxidant activity of water-soluble proteins and peptides. This maybe partly due to the differences of radical's solubility and diffusivity in the reaction medium [24].

Antioxidant activity

The antioxidant activity of extracts and different fractions of small red bean was further evaluated by FRAP assay and ORAC assay. The FRAP assay is ini-

tially developed to measure the ferric reduction ability of plasma at a low pH [25]. It is based on the measurement of the ability of the substance to reduce Fe^{3+} to Fe^{2+}. When the ferric 2,4,6-tripyridyl-s-triazine complex (Fe^{3+}-TPTZ) accept an electron and thus is reduced to the ferrous form (Fe^{2+}-TPTZ), an intense blue color is developed. FRAP is the only assay that directly measures antioxidants in a sample since it uses antioxidants as reductants in a redox-linked colorimetric reaction. At the present time, ORAC is perhaps the most widely used method to evaluate the total antioxidant activity of food matrix. This assay depends on the free radical damage to a fluorescent probe, which result in a downward change of fluorescent intensity. Antioxidants can compete with free radicals, thus leading to the inhibition of decay of fluorescent probe. The ORAC assay can provide information on a sample's ability to scavenge peroxyl radical through a hydrogen atom transfer mechanisms [26].

The FRAP value ranged from 6.7 in Fr I to 632.0 mmol Fe^{2+} equivalents/100 g in Fr V (Table 2). Among all extracts and fractions tested, the Fr V exhibited the highest FRAP ($p < 0.05$), suggesting that phytochemicals in the Fr V could easily donate electrons to Fe^{3+}, thus reducing to Fe^{2+}. The result was in agreement with the highest DPPH and ABTS radical scavenging of the Fr V. The highest FRAP value of the Fr V might be attribute to the high content of condensed tannins, which can easily donate electrons, or donate more numbers of electrons to Fe^{3+} than other phenolics since condensed tannins possessed more hydroxyl groups. Maqsood and Benjakul [27] reported that tannic acid, which possessed higher number of hydroxyl groups, had the highest FRAP value compared with that of catechin, caffeic acid, ferulic acid. It was also assumed that the large contribution to the FRAP value came from tannins which were present at high levels in Cornus mas varieties [28].

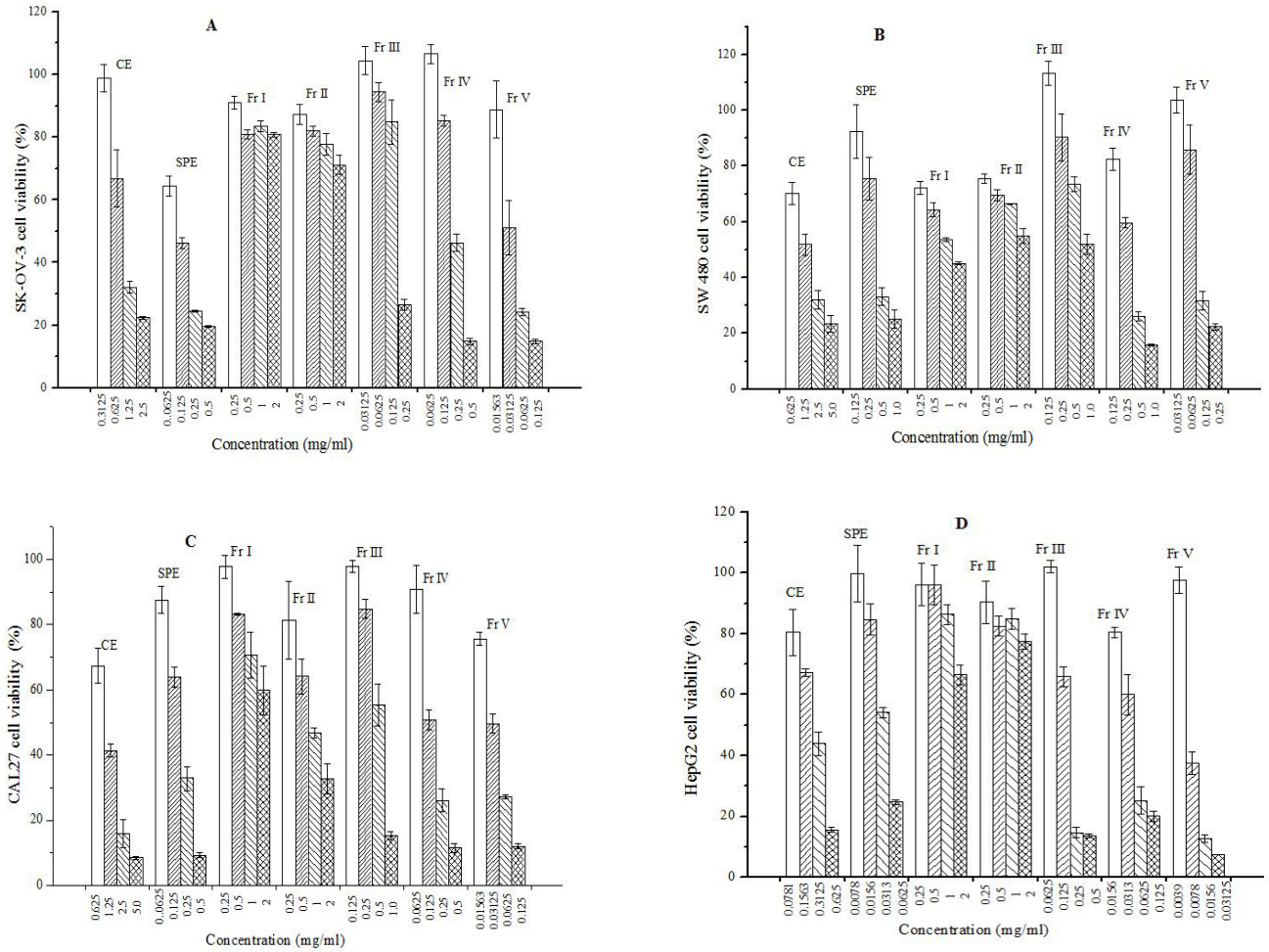
With regard to ORAC value (Table 2), the trend was a little different from those of free radicals scavenging activity and ferric reducing power. Fr V, which possessed the highest free radicals scavenging activity and reducing power, had a little lower ORAC than that of Fr III. This observation was in accordance with the conclusion that the correlation coefficient between CTC and ORAC was relatively lower than that between TFC, TPC and ORAC [8]. Among extracts and fractions, Fr III had the significantly highest ORAC (26085.9 ± 1896.5 μmol Trolox equivalents/g, $p < 0.05$), followed by Fr V (16890.7 ± 141.0), Fr IV (14255.2 ± 789.8), SPE (11659.6 ± 1674.4), Fr II (3103.6 ± 253.3), CE (2064.1 ± 127.4), and Fr I (311.3 ± 7.6). There was no significant difference ($p > 0.05$) in ORAC between Fr IV and Fr V, Fr IV and SPE, Fr II and CE.

In general, Fr V exhibited higher antioxidant activity in all assays except ORAC assay. Considering the relatively high yield of Fr V, and taking our previous results [13] into account, it might be concluded that the antioxidant activity of legumes such as lentils and dry beans may be attributed largely to the condensed tannins. However, other phytochemicals such as catechin and flavonols also contributed to the total antioxidative capacity of legumes. High-molecular-weight or condensed tannin-rich fractions from adzuki bean also exhibited the highest radical scavenging activity against DPPH and antioxidant activity in β-carotene-linoleate and reducing systems [29]. Phenolic hydroxyl groups attached to the flavanol skeleton [30], and the presence of an interflavonoid link [31] might play an important role in the higher radical scavenging activity and antioxidant activity of condensed tannins.

Antiproliferative activity

The antiproliferative activity of extracts and different fractions of small red bean against human ovarian SK-OV-3, colon SW480, tongue CAL 27, and liver Hep G2 cancer cells was measured by the MTT assay. The concentration effectiveness of extracts and fractions on the viability of SK-OV-3, SW480, CAL 27, Hep G2 cells is presented in Figure 3. Since different groups of phytochemicals were present in the extracts and fractions, four different concentrations, from 0.3125 to 5 mg/mL in CE, 0.125 to 1 mg/mL in SPE, 0.25 to 2 mg/mL in Fr I and Fr II, 0.0156 to 1 mg/mL in Fr III and Fr IV, and 0.0039 to 0.25 mg/mL in Fr V, were applied. All extracts and fractions concentration-dependently inhibited the proliferation of four cancer cells, although the concentration-dependent inhibition effect of Fr I to SK-OV-3 cells, and Fr II to Hep G2 cells was not significant. Treatment of SK-OV-3 cells with Fr V produced the highest inhibitory effect, from 14.9% to 88.8% of cell viability at concentrations ranging from 0.125 to 0.0156 mg/mL, followed by SPE from 19.5% to 64.4% of cell viability at concentrations ranging from 0.5 to 0.0625 mg/mL, Fr III from 26.5% to 94.4% of cell viability at concentrations ranging from 0.25 to 0.0625 mg/mL, Fr IV from 14.9% to 85.2% of cell viability at concentrations ranging from 0.5 to 0.125 mg/mL, and CE from 22.4% to 98.9% of cell viability at concentrations ranging from 2.5 to 0.3125 mg/mL (Figure 3A). Nearly a similar tendency was found regarding the proliferation-inhibitory effect of the crude extracts and fractions to SW480, CAL 27, and Hep G2 cells (Figure 3B-3D). However, the concentrations to inhibit the proliferation of Hep G2 cells were in average 4- or 8-folds lower than those of SK-OV-3, SW480, and CAL 27 cells except for Fr I and Fr II. In general, CE showed moderate inhibitory effect against four cancer cells. After CE was semi-purified by removing non-phenolic components with macroporous resin XAD-7, the inhibitory effect of SPE increased. Fr I and Fr II showed less inhibitory effect than the crude extracts, SPE, and other fractions. The most potential inhibitory effect was observed in Fr V, which contained the highest condensed tannins and showed the highest antioxidant activity. The IC_{50} values of the crude and semi-purified extracts and various fractions against SK-OV-3, SW480, CAL 27, and Hep G2 cancer cells are presented in Table 3. The IC_{50} values varied a lot among different cells and different fractions. IC_{50} values of Fr I against SK-OV-3,

Figure 3: Antiproliferative activity of extracts and fractions from small red bean.



Inhibition of cancer cell growth was measured by the MTT assay. Cells were incubated with different concentrations of extract and fractions for 48 h. Results were expressed as means ± standard deviation (n = 3). (A) human ovarian cancer cells (SK-OV-3), (B) human colon cancer cells (SW480), (C) human tongue cancer cells (CAL 27), and (D) human hepatocarcinoma cells (Hep G2).

Table 3: IC_{50} (mg/mL) of extracts and fractions from small red bean against human cancer cells.

	cell lines			
	SK-OV-3	SW480	CAL 27	Hep G2
CE	0.897 ± 0.062 a	1.404 ± 0.110 a	1.070 ± 0.122 a	0.268 ± 0.021 a
SPE	0.113 ± 0.005 c	0.378 ± 0.030 b	0.176 ± 0.014 d	0.035 ± 0.002 c
Fr I	nd	1.212 ± 0.058 a	nd	nd
Fr II	nd	nd	0.863 ± 0.053 b	nd
Fr III	0.206 ± 0.010 b	1.035 ± 0.056 a	0.562 ± 0.063 c	0.155 ± 0.004 b
Fr IV	0.238 ± 0.012 b	0.307 ± 0.003 b	0.150 ± 0.011 d	0.038 ± 0.001 c
Fr V	0.03 5± 0.005 d	0.111 ± 0.005 c	0.035 ± 0.002 e	0.008 ± 0.000 d

Results were expressed as means ± standard deviation (n = 3), values with different letters within a column were significantly different ($p < 0.05$).

CAL 27, and Hep G2, and IC_{50} of Fr II against SK-OV-3, SW480, and Hep G2 could not be determined at the concentrations tested in the present study (greater than 2 mg/mL). The IC_{50} values of Fr V were much lower than those of SPE, Fr III, Fr IV, and CE in all four cancer cells. Generally, the IC_{50} values of the extracts and fractions against four cancer cells were in the following order: Fr V, SPE, Fr IV, Fr III, and CE. The sensitivity of four cell lines to different extracts and fractions were considerably different, as illustrated from the IC_{50} values. The highest IC_{50} values of Fr V, SPE, Fr IV, Fr III, and CE were found in SW480 cancer cells, and the lowest were found in Hep

G2 cancer cells, which suggested that Hep G2 cells were the most sensitive to small red bean extracts and fractions, whereas SW480 were the least sensitive.

Some varieties of *Phaseolus vulgaris* L have been investigated to posses anticancer activities *in vitro* and in vivo. Cardador-Martinez and others [31] and Rocha-Guzmán and others [18] demonstrated antimutagenic activity of common bean extract on *Salmonella typhimurium* tester strains induced by aflatoxin B1 and1- nitropiren, respectively. Methanol extract of black bean and its toyopearl and silica gel fractions inhibited the proliferation of human adenocarcinoma HeLa cancer cells via induction of apoptosis, with 100% methanol extract and fractions contained proanthocyanidins being the highest potential [10]. Condensed tannins isolated from black bean suppressed the growth of Caco-2 colon, MCF-7 and Hs578T breast, and DU 145 prostatic cancer cells [33]. In these studies, methanol, ethanol, acetone and their aqueous solutions were used to extract polyphenolics from beans, and therefore polyphenolics were responsible for their anticancer activity, although extracts using different solvents exhibited different antiproliferative activities against cancer cells. However, in contrast to the *in vitro* studies, no association between anticancer activity and phenolic or flavonoid contents was found *in vivo* mammary carcinogenic models [34]. In another study, dietary intake of small red bean reduced the mammary cancer incidence, cancer multiplicity, and tumor burden in female Sprague Dawley rats via induction of apoptosis [35]. The authors also addressed that ethanol or acetone extracts of small red bean could not suppress the growth of human breast MCF-7 and MDA-MB-469 cancer cells in vitro, and they speculated small red bean mediated anticancer effect via perturbing systemic circulating factors. Common beans contain proteins, fats, crude fibers, carbohydrates or polysaccharides, trypsin inhibitors, and various phytochemicals. But until now, which groups in common beans attribute to their anticancer activity *in vivo* is still largely unknown.

In our studies, CE of small red bean was a rich source of phenolics, in which the TPC was 51.0 mg GAE/g. Sugar content in the CE was as high as 75.6 g glucose equivalent per 100 g. However, after removal of these sugar fractions by adsorption of CE with XAD-7 and then desorption by water and 80% MeOH successively, the resultant water eluate (mainly including sugars) had almost no effect on the growth of four cancer cells (data not shown), whereas the IC_{50} of the 80% MeOH eluate (mainly including polyphenolics) decreased from 0.897 to 0.113 mg/mL in SK-OV-3 cells, from 1.404 to 0.378 mg/mL in SW480 cells, from 1.070 to 0.176 mg/mL in CAL 27 cells, and from 0.268 to 0.035 mg/mL in Hep G2 cells, respectively. Our results partly suggested that polysaccharides were not responsible for the antiproliferative effect, and polyphenolics were effective in inhibiting the growth of these cancer cells. After fractionation with Sephadex-LH into five fractions, different antiproliferative activity of fractions was observed. Fr III, rich in anthocyanins, showed moderate activity, but the activity was lower than that of Fr IV and Fr V. The result was contrary to the study from Aparicio-Fernández and others [10] that black bean anthocyanin fraction did not have any activity against HeLa cancer cells, but was in agreement with other reports that anthocyanins from vegetables or fruits did have antiproliferative activity [36]. Fr IV, rich in flavonoids, only showed antiproliferative activity comparable to that of SPE. Meanwhile, Fr V showed the highest activity against SK-OV-3, SW480, CAL 27, and Hep G2 cells with the IC_{50} of 0.030, 0.111, 0.035, and 0.008 mg/mL, respectively. The composition analysis and antioxidant activity determination showed that Fr V presented the highest concentration of condensed tannins and the highest antioxidant activity. These results indicated that the antiproliferative activity of small red bean could be largely attributed to condensed tannins, as manifested by black bean proanthocyanidins against HeLa cells [10] and wild blueberry proanthocyanidin against human prostate and mouse liver cancer cell [37].

Conclusions

In summary, the small red bean extract was semi-purified by adsorption-desorption on macroporous resin, and further fractionated on Sephadex LH-20 into different fractions. The phytochemical contents and antioxidant activity varied widely among extracts and fractions. The antiproliferative activity depended upon the cancer cell lines and was largely influenced by the different fractions which contained different bioactive components. The main components in Fr V were condensed tannins, and Fr V exhibited the highest antioxidant and antiproliferative activity. Taken these results together, it can be concluded that small red bean could be an important source of polyphenolic compounds with potential antioxidant and antiproliferative activity, which could be potentially applied as natural antioxidant and antiproliferative agents for health promotion. However, further research is required to determine the antioxidant and antiproliferative activity *in vivo* and their involved mechanisms. The identification of polyphenolic compounds in small red bean extracts and its fractions is also needed.

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