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308 Lasani Town, Sargodha Road, Faisalabad - Pakistan  
Mob: +92 300 3008585, Fax: +92 41 8815544  
E-mail: [editorpjn@gmail.com](mailto:editorpjn@gmail.com)

## Nutritional and Antioxidant Profiling of Vitamin K Dietary Sources

Muhammad Yasin<sup>1</sup>, Masood Sadiq Butt<sup>1</sup>, Faqir Muhammad Anjum<sup>1</sup> and Muhammad Shahid<sup>2</sup>

<sup>1</sup>National Institute of Food Science and Technology, University of Agriculture, Faisalabad, Pakistan

<sup>2</sup>Department of Chemistry and Biochemistry, University of Agriculture, Faisalabad, Pakistan

**Abstract:** The present investigation was an effort to explore the nutritional profile and antioxidant indices of vitamin K dietary sources i.e., spinach and soybean. For the purpose, spinach and soybean were nutritionally characterized with special reference to vitamin K content. The proximate composition of spinach exhibited that moisture, crude protein, crude fat, crude fiber, ash and Nitrogen Free Extract (NFE) as 90.71±4.14, 2.03±0.95, 0.32±0.007, 0.58±0.02, 1.24±0.06 and 5.01±0.11%, respectively. However, tested soybean indicated the values respective traits as 8.96±0.45, 32.28±1.99, 18.64±1.02, 2.93±0.16, 3.38±0.19 and 33.79±1.15%. Furthermore, spinach showed good mineral profile dominated by magnesium, potassium and calcium whilst soybean is abundant in potassium, zinc and magnesium. The HPLC quantification of vitamin K revealed that spinach contained 379.09 µg/100g phyloquinone as compared to soybean 29.79 µg/100g. Amongst antioxidant extracts, methanolic extracts of spinach and soybean showed higher total phenolic, DPPH scavenging and antioxidant activities. In conclusion, spinach showed higher antioxidant status and phyloquinone as compared to soybean however, soybean contained ample amount of protein and fat contents.

**Key words:** Spinach, soybean, vitamin K, nutritional profile, antioxidant status

### INTRODUCTION

The micronutrients are essential for regulating numerous metabolic pathways hence their deficiencies may lead to various physiological threats (Willershausen *et al.*, 2011). Amongst those, inadequacy of vitamin K is generally associated with malnutrition, drugs interaction and sedentary lifestyle (Booth, 2012). Principally, dietary modifications are considered as a vanguard remedy to alleviate various lifestyle related disorders (Yasin *et al.*, 2012).

The concept of vitamin was firstly introduced in 1912 and engrossed great attention of the researchers due to their positive impact on physiological activities. The vitamins requirements are influenced by various factors like presence of antagonists, drugs and body vitamin reserves (McDowell *et al.*, 2006). Nevertheless, vitamin K has lime lighted owing to its active participation in normal blood coagulation, bone strengthening and homeostasis (Truong and Booth, 2011).

Vitamin K exists in two naturally occurring bioactive forms; phyloquinone (vitamin K<sub>1</sub>) and menaquinone (vitamin K<sub>2</sub>). The phyloquinone is the most common form of vitamin K present in green leafy vegetables like spinach, kale, broccoli (Kamao *et al.*, 2007) and certain vegetable oils (Bolton-Smith *et al.*, 2000). Likewise, menaquinone is present in fermented soybean/natto and animal products (Schurgers *et al.*, 2007). The natto is a popular breakfast food of Japanese cuisine made from soybean fermented with *Bacillus subtilis* containing high protein and menaquinones content. However,

differences in the amount and form of menaquinones may vary in different dietary sources. Natto restrains sufficient amount of menaquinone-7 (MK-7), menaquinone-8 (MK-8) and menaquinone-9 (MK-9) and plays preventive role against vitamin K deficiency (Katsuyama *et al.*, 2002; Wu and Chou, 2009). Likewise, spinach (*Spinacia oleracea*) belongs to the family *Amaranthaceae* is also recognized as vitamin K enriched vegetable. Generally, phyloquinone (vegetables) and MK-7 (pulses including fermented soybean) are considered as major sources of vitamin K for the humans (Kamao *et al.*, 2007). Besides, fermented soybean (natto) is also rich in protein, calcium and vitamin K<sub>2</sub>, involved in the activation of osteocalcin (Booth, 2012).

The contribution of phyloquinone, MK-7 and menaquinone-4 (MK-4) is approximately more than 60, 24 and 7%, respectively of the total vitamin K dietary intake (Iwamoto *et al.*, 2009). Humans and animals have ability to convert phyloquinone into MK-4 by removing integral side chain thereby contributes to bone strengthening (Okano *et al.*, 2008). Physiological requirement of vitamin K for both menaquinones and phyloquinone is about 1.16 µg/kg body weight, normally stored around 90 and 10% in the liver, respectively. However, only 5 to 25% of ingested vitamin K is catabolized to MK-4 followed by conversion of menaquinones in the liver via prenylation (Shearer and Newman, 2008). The vegetables contribute about 60% of total phyloquinone where cooked green vegetables

provide around 28% (McKeown *et al.*, 2002). The phylloquinone and green leafy vegetables intake has positive correlation with plasma phylloquinone and fasting triglyceride concentrations. Considering the effectiveness of vitamin K for normal blood coagulation and bone related biomarkers, the present project was an attempt forward in this regard. In the context, spinach and soybean were characterized nutritionally with special reference to vitamin K contents.

## MATERIALS AND METHODS

The present study was carried out in the Postgraduate Research Laboratory, National Institute of Food Science and Technology (NIFSAT), University of Agriculture, Faisalabad. Indigenously cultivated spinach and soybean were used as vitamin K enriched dietary sources for *in vitro* studies. Materials used and protocols followed are discussed herein.

**Materials:** Spinach variety Desi Palak while soybean cultivar namely Faisal Soybean were procured from Ayub Agriculture Research Institute (AARI) Faisalabad. Analytical reagents and HPLC grade standards were obtained from Merck (Darmstadt, Germany) and Sigma-Aldrich.

**Characterization of spinach and soybean:** Spinach and soybean samples were analyzed for moisture, crude protein, crude fat, crude fiber, ash and nitrogen free extract by using triplicate samples.

**Moisture content:** Moisture content in spinach and soybean were evaluated by drying sample in Air Forced Draft Oven (Model: DO-1-30/02, PCSIR, Pakistan) at  $105\pm 5^\circ\text{C}$  till constant weight by following the procedure AACC (2000) Method No. 44-15A.

**Crude protein:** Estimation of crude protein contents of spinach and soybean were carried out through nitrogen determination in the sample multiplying with factor (6.25) using Kjeltex Apparatus (Model: D-40599, Behr Labor Technik, GmbH-Germany) by adopting the guidelines of AACC (2000) Method No. 46-30.

**Crude fat:** Soxtec System (Model: H-2 1045 Extraction Unit, Hoganas, Sweden) was used to determine the crude fat content in respective samples using hexane as solvent according to the procedure described in AACC (2000) Method No. 30-25.

**Crude fiber:** Crude fiber content of fat free sample was estimated by simmering initially with 1.25%  $\text{H}_2\text{SO}_4$  solution for 30 min followed by 1.25% NaOH solution in Labconco Fibertech apparatus (Labconco Corporation Kansas, USA) as method in AACC (2000) Method No. 32-10.

**Ash:** Ash contents of oven dry samples were calculated through charring followed by direct incineration at  $550^\circ\text{C}$  in Muffle Furnace (MF-1/02, PCSIR, Pakistan) till grayish white residue (AACC, 2000; Method No. 08-01).

**Nitrogen free extract (NFE):** Nitrogen free extract (NFE) was estimated by following expression:

$$\text{NFE (\%)} = 100 - (\text{M} + \text{CP} + \text{CF} + \text{crude fiber} + \text{ash}) \%$$

M = moisture, CP = crude protein, CF = crude fat

**Minerals profile:** The samples were subjected to mineral assay through wet digestion considering the protocols of AOAC (2006). For the estimation of sodium and potassium, Flame Photometer-410 (Sherwood Scientific Ltd., Cambridge, UK) was used whilst calcium, iron, magnesium, zinc and copper were measured through Atomic Absorption Spectrophotometer (Varian AA240, Victoria Australia).

## Extraction of vitamin K

**Preparation of sample:** For the extraction of vitamin K, spinach and soybean were ground separately in a blender to uniform consistency. The 5 g of anhydrous sodium sulfate was added in weighed amount of each raw material and further pulverized. The resultant powder and 20  $\mu\text{L}$  of internal standard (200 ng of dihydrophyloquinone) were transferred to 50 mL centrifuge tube. Afterwards, 15 mL of 2-propanol/hexane (3:2 v/v) and 32 mL of  $\text{H}_2\text{O}$  were added in the mixture and centrifuged (4000 rpm) for 5 min. The supernatant layer containing phylloquinone and dihydrophyloquinone was transferred to clean amber color tube and evaporated under nitrogen stream. The residue was redissolved in 10 mL hexane with further purification through solid-phase silica gel.

**Quantification of vitamin K:** Extracted samples were quantified by HPLC (Perkin Elmer, Series 200, USA) using  $\text{C}_{18}$  column (250 mm x 4.6 mm, 5.0  $\mu\text{m}$  particle size). For the intention, 10  $\mu\text{L}$  aliquot of sample was injected via autosampler (WISP Model 710). The column temperature was maintained at  $40^\circ\text{C}$ . The mobile phase comprised of dichloromethane (100 mL), methanol (900 mL), zinc chloride (1.37 g), sodium acetate (0.41 g) and acetic acid (0.30 g). The flow rate was adjusted at 1 mL/min. Quantification of vitamin K was carried out using UV/vis detector (model 481) at 249 nm (Majchrzak and Elmadafa, 2001).

**Antioxidant potential:** For *in vitro* studies, methanol and ethanol extracts of spinach and soybean were tested for their antioxidative properties.

**Total phenolics:** Total Phenolic Content (TPC) of each extract was determined by using Folin-Ciocalteu reagent

(Singleton *et al.*, 1999). A 50 µL sample was mixed with 250 µL of 2N Folin-Ciocalteu's reagent. Later, 750 µL of 20% Na<sub>2</sub>CO<sub>3</sub> solution and distilled water were added to make the volume 5 mL. After 2 hr, absorbance was measured at 765 nm using UV/vis Spectrophotometer (CECIL CE7200). Total phenolic content was calculated and expressed as gallic acid equivalent (mg gallic acid/100g).

**Antioxidant activity:** Antioxidant activity of both spinach and soybean extracts was assessed by coupled oxidation of β-carotene and linoleic acid as described by Taga *et al.* (1984). Briefly, β-carotene (1.0 mg) was dissolved in 10 mL of chloroform. The 1 mL prepared solution was taken in flask containing linoleic acid (20 mg) and tween 40 (200 mg). The chloroform was removed using Rotary Evaporator at 40°C. Gradually, 50 mL of distilled water was added to the flask with vigorous shaking to form an emulsion. Subsequently, 5 mL emulsion was mixed with 0.2 mL sample in test tube. After shaking, absorbance was recorded at 470 nm. Test tube was placed in a water bath equipped with agitation at 50°C and reading was measured after every 10 min interval up to 40 min.

$\ln(a/b) \times 1/t$  = degradation rate of sample

- ln = Natural log
- a = Initial absorbance (470 nm) at time zero
- b = Absorbance (470 nm) after 40 min
- t = Time (min)

Antioxidant activity (AA) was expressed as % inhibition relative to control:

$$AA = \frac{\text{Degradation rate of control} - \text{degradation rate of sample}}{\text{Degradation rate of control}} \times 100$$

**Free radical scavenging ability:** Free radical scavenging activity of spinach and soybean extract was estimated using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) as described by Muller *et al.* (2011). Shortly, 125 µL sample was mixed with 4 mL DPPH (1.2 mM) in methanol solution. Absorbance was measured after 30 min at room temperature using Spectrophotometer (CECIL CE7200) at 520 nm. The inhibition of free radicals by DPPH was calculated through following expression:

$$\text{Inhibition (\%)} = (100 \times (A_{\text{blank}} - A_{\text{sample}}) / A_{\text{blank}})$$

**Ferric Reducing Antioxidant Power (FRAP):** The ability of reduced ferric ions was measured by following the method of Muller *et al.* (2011). An aliquot of spinach and soybean (50 µL each) was taken with 3 mL of FRAP reagent following incubation at 37°C for 30 min. The increase in absorbance was noted at 593 nm using

Spectrophotometer. The results were compared with the calibration curve, prepared by using various concentrations of trolox as standard.

## RESULTS AND DISCUSSION

**Proximate composition:** The proximate composition of spinach indicated moisture, crude protein, crude fat, crude fiber, ash and Nitrogen Free Extract (NFE) as 90.71±4.14, 2.03±0.95, 0.32±0.007, 0.58±0.02, 1.24±0.06 and 5.01±0.11%, respectively. Whereas, tested soybean showed the values 8.96±0.45, 32.28±1.99, 18.64±1.02, 2.93±0.16, 3.38±0.19 and 33.79±1.15% for respective traits (Table 1).

The results of instant research are synchronized with the pervious findings of USDA (2010), reported 91.40, 2.86, 0.39, 2.2 and 3.63% of moisture, protein, total lipids, fiber and carbohydrates, respectively in raw spinach. Similarly, Hussain *et al.* (2010) investigated the proximate composition of spinach and documented the values for protein, fat, fiber and carbohydrates as 20.82, 3.325, 4.92 and 48.82%. Likewise, Bangash *et al.* (2011) also carried out a nutritional analysis of Pakistani spinach (*Spinacea oleracea*) and noticed 92.70±0.13, 2.5±0.04, 0.29±0.01, 3.91±0.02, 0.70±0.06 and 1.90±0.002% of moisture, protein, fat, carbohydrates, fiber and ash contents, respectively. The compositional variations in present study are might be due to varietal differences, climatic changes, different topographic locations and variations in agronomic practices.

The findings of Ren *et al.* (2012) are in accordance with the present observations for soybean proximate composition, they expounded that soy contains 8.43±0.44, 37.29±1.99, 17.86±1.17, 13.31±0.80 and 4.99±0.23% of moisture, crude protein, crude fat, carbohydrates and ash, respectively. Similarly, Esteves *et al.* (2010) illuminated that soy has 37.83 to 39.37% protein, 20.83 to 22.57% crude fat, 4.31 to 6.62% ash, 2.10 to 2.11% total dietary fiber and 22.98 to 25.06% carbohydrates. Earlier, Wei and Chang (2004) delineated that proximate composition of soybean is varied from 5.20-7.41, 36.49-40.29, 18.78-25.41, 5.35-5.83 and 30.35-36.04% for moisture, crude protein, crude fat, ash and carbohydrate contents, respectively.

**Mineral profile:** Means for mineral profile (Table 2) indicated potassium (K), zinc (Zn), magnesium (Mg), calcium (Ca), iron (Fe), sodium (Na) and copper (Cu)

Table 1: Proximate composition (%) of raw materials

Proximate composition	Spinach	Soybean
Moisture	90.71±4.14	8.96±0.45
Crude Protein	2.03±0.95	32.28±1.99
Crude Fat	0.32±0.007	18.64±1.02
Crude Fiber	0.58±0.02	2.93±0.16
Ash	1.24±0.06	3.38±0.19
NFE	5.01±0.11	33.79±1.15

Table 2: Mineral profile (mg/100g) of raw materials

Minerals	Spinach	Soybean
K	515.41±25.77	673.17±31.31
Zn	80.18±4.01	288.91±14.45
Mg	712.26±35.61	228.95±11.45
Ca	87.58±4.38	181.20±9.06
Fe	2.49±0.12	7.35±0.37
Na	76.51±3.83	2.80±0.14
Cu	6.92±0.35	1.93±0.03

Table 3: Mean squares for antioxidant potential of raw materials

SOV	df	TPC	DPPH (FRSA)	AA	FRAP
Materials (M)	1	39298.7**	2121.96**	2004.44**	3.141**
Solvents (S)	1	226.9*	64.30*	32.16*	0.058*
M×S	1	205.0*	0.54 <sup>NS</sup>	1.05 <sup>NS</sup>	0.001 <sup>NS</sup>
Error	8	21.2	6.98	4.53	0.007
Total	11				

\*\*Highly significant; \*Significant; NS: Non-significant; RPC: Total phenolic content; DPPH (FRSA): DPPH free radical scavenging activity; AA: Antioxidant Activity

contents as 515.41±25.77, 80.18±4.01, 712.26±35.61, 87.58±4.38, 2.49±0.12, 76.51±3.83 and 6.92±0.35 mg/100g, respectively in the spinach (Table 2). Besides, respective minerals in soybean were 673.17±31.31, 288.91±14.45, 228.95±11.45, 181.20±9.06, 7.35±0.37, 2.80±0.14 and 1.93±0.03 mg/100g.

The results for calcium, sodium, potassium, magnesium, iron and zinc are in accordance with the work of Bangash *et al.* (2011), they noticed that spinach contains 100±0.01, 50±0.9, 310±0.11, 137±0.16, 29±0.02, 0.17±0.01 and 1.21±0.02 mg/100g of K, Zn Mg, Ca, Fe, Na and Cu, respectively. Earlier, Singh *et al.* (2001) determined iron, copper, magnesium and zinc in Indian spinach and reported the values 35.8±0.11, 1.9±0.03, 10.2±0.05 and 6.0±0.04 mg/100g, respectively. The slight differences in mineral profile of soy and spinach are owing to variations in climate, soil and agronomic practices. Likewise, Almeida *et al.* (1996) delineated that the nutritional composition of green leafy vegetables is influenced by agrotechnics, raining condition, growing method and soil type. The instant results regarding calcium, potassium, sodium and magnesium are in harmony with the previous investigation of Esteves *et al.* (2010), they noticed variations for aforementioned minerals in soy ranging from 1.70 to 2.08, 17.40 to 18.90, 0.24 to 0.25 and 2.61 to 3.17 g/100g. One of their peers, Paucar-Menacho *et al.* (2010) evaluated different soybean cultivars for their mineral potential and recorded calcium, iron, copper and zinc as 290.41±1.47 to 335.37±1.27, 22.30±0.07 to 26.46±0.25, 2.88±0.04 to 3.14±0.06 and 7.42±0.16 to 8.27±0.10 mg/100g, respectively. Earlier, calcium and magnesium contents of four soy cultivars were estimated that varied from 175.80 to 227.90 and 251.25 to 283.05 mg/100g, respectively (Wei and Chang, 2004).

**Vitamin K content:** The raw spinach and soybean were subjected to vitamin K quantification through HPLC and

results showed phyloquinone concentration in respective materials as 378.09±13.89 and 29.79±1.23 µg/100g (Fig. 1). The present finding are in accordance with the previous work of Schurgers and Vermeer (2000), they illuminated the phyloquinone contents of the fresh spinach as 299-429 µg/100g. Later, Kamao *et al.* (2007) explored phyloquinone concentration of 58 commonly consumed foods of Eastern Japan through HPLC and narrated higher values in spinach (498 µg/100 g) as compared to broccoli (307 µg/100g). Furthermore, Booth (2012) quantified phyloquinone content of soybean and its oil through HPLC and recorded the values 34.74 and 193 µg/100g for respective material. Earlier, Peterson *et al.* (2002) explored the phyloquinone contents of different oils. They elucidated that its concentration varied from 33.5 to 71.8, 50.1 to 70.3 and 4.8 to 11.1 µg/100g in vegetable, olive and corn oils, respectively. It has been documented that phyloquinone is principally present in the photosynthetic tissue of the plants and its concentration varied among different vegetables (Damon *et al.*, 2005). Furthermore, it is synthesized in the chloroplast membrane of plant cell through dogma reaction in the presence of light. In this context, chorismate is a direct precursor of phyloquinone formed through 1, 4-dihydroxy-2-naphthoate that is synthesized from α-ketoglutarate and isochorismate in the presence of Mn<sup>+2</sup> and thiamine diphosphate (Shimada *et al.*, 2005). Earlier, Koivu *et al.* (1997) recorded that phyloquinone contents of the vegetables are higher in summer season than that of winter.

**Antioxidant potential:** Mean squares in Table 3 indicated that antioxidant indices were affected significantly by raw materials and solvents however, their interactive effect was found non-momentous except for total phenolic content. Means for Total Phenolic Content (TPC) of raw materials (Fig. 2) showed the highest value 894.16±36.69 mg GAE/100g for methanolic extract of spinach followed by 701.81±23.02 mg GAE/100g in ethanolic extract of spinach whereas the lowest TPC

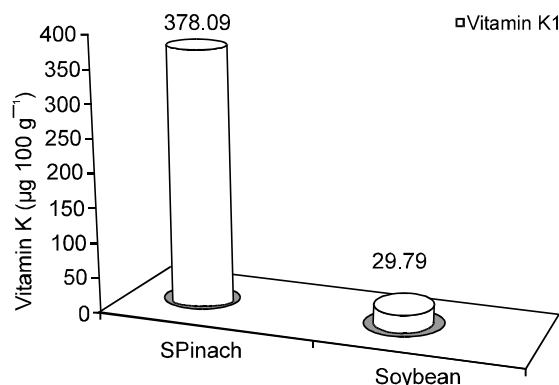


Fig. 1: Phyloquinone (µg/100g) in raw materials

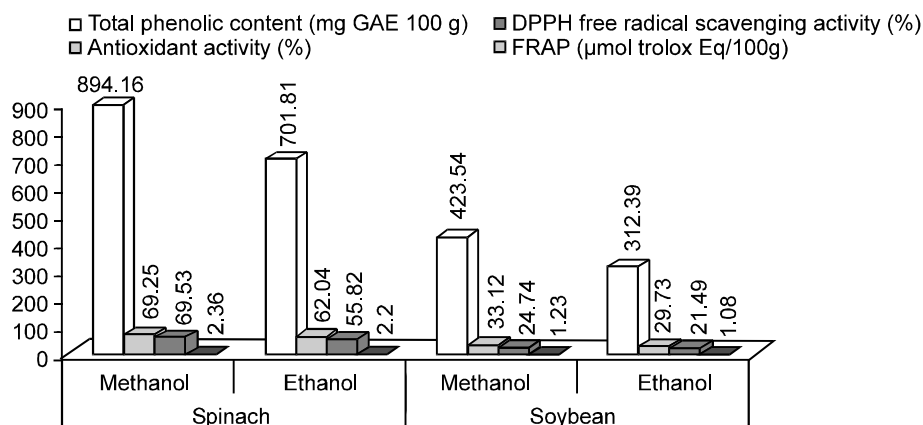


Fig. 2: Total phenolic content, DPPH free radical scavenging activity, antioxidant activity and FRAP in different extracts

value  $312.39 \pm 13.15$  mg GAE/100g for ethanolic extract of soybean. Likewise, the maximum DPPH (1, 1-diphenyl-2-picrylhydrazyl) inhibition was noticed in methanolic extract of spinach ( $69.25 \pm 3.38\%$ ) trailed by ethanolic extract of spinach ( $62.04 \pm 3.15\%$ ) whereas the minimum for ethanolic extract of soy ( $29.73 \pm 1.66\%$ ). Similarly, methanolic extract of spinach exhibited the highest values for  $\beta$ -carotene and FRAP as  $62.53 \pm 2.79\%$  and  $2.36 \pm 0.11$   $\mu\text{mol trolox Eq}/100\text{g}$  followed by ethanolic extract of spinach  $55.82 \pm 2.64\%$  and  $2.2 \pm 0.10$   $\mu\text{mol trolox Eq}/100\text{g}$  whilst, the lowest outputs were recorded for ethanolic extract of soybean  $21.49 \pm 1.18\%$  and  $1.08 \pm 0.05$   $\mu\text{mol trolox Eq}/100\text{g}$ , respectively.

The polyphenolic compounds i.e., phenolic acids, phenols, hydroxycinnamic acid derivatives and flavonoids are the promising phytonutrients that hold strong antioxidant potential. Accordingly, plant derived dietary polyphenols are gaining importance due to their high antioxidant activity. In this context, p-coumaric acid and flavonoids derivatives are the main spinach based antioxidant compounds (Bergman *et al.*, 2001). Furthermore, isoflavones and lignans are the primary polyphenols of soybean that modulate various biochemical processes (Takahashi *et al.*, 2005). Moreover, soybean also contains highly polymerized procyanidins with significant DPPH activity (Takahata *et al.*, 2001).

The results pertaining to antioxidant potential of the spinach are in line with the findings of Turkmen *et al.* (2005), they observed  $1274.8 \pm 94.09$  mg GAE/100g and  $67.4 \pm 7.82\%$  of total phenolic and DPPH activity, respectively. Later, Fan *et al.* (2011) explored total phenolic contents of the spinach as 125 mg GAE/100g on fresh weight basis. The results of the present study are in harmony with the findings of Slavin *et al.* (2009), reported 12.1 mg GAE/g of total phenolics in soybean.

Earlier, Malenčić *et al.* (2007) documented the variations in TPC and DPPH values for different soybean cultivars from  $2.70 \pm 0.15$  to  $4.72 \pm 0.07$  g catechin/kg and

$22.87 \pm 1.32$  to  $48.17 \pm 2.78\%$ , respectively. Previously, Takahata *et al.* (2001) estimated the polyphenols of black, brown and reddish brown coated soybean cultivars that ranged from  $6.55 \pm 0.40$  to  $81.3 \pm 5.5$  mg catechin Eq/g. Moreover, brown, green and yellow soybean cultivars showed variations in TPC by 0.8 to 2.2 mg GAE/g. Afterwards, Popovic *et al.* (2010) demonstrated that total phenolic contents and FRAP values are  $31.3 \pm 2.3$  to  $59.7 \pm 7.1\%$  and 307 to 480  $\mu\text{M Fe}^{2+}/\text{g}$  of different soybean varieties. Moreover, they also observed antioxidant activity of soybean 54.55 to 65.65% through  $\beta$ -carotene linoleic acid model system.

From the above discussion, it is deduced that raw spinach contains higher amount of phyloquinone as compared to soybean. Moreover, antioxidative characteristics of spinach and soybean extracts are affected by the type of solvents. In this context, methanolic extract performed better than ethanolic extract. In general, spinach showed higher antioxidant activity as compared to soybean.

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