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Research Article

Antioxidant Activities of Phenolic Compounds in Green and White Faba Bean (*Vicia faba* L.)

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Abstract: Polyphenols and tannins have implications for health and nutrition because of their antioxidant activities. Foods with high content of phenolics, such as fruits, vegetables, grains and legumes, show decreasing incidence of several diseases upon their consumption. However, there are limited reports on ant oxidative properties of tannins present in legumes. Faba bean seed has been known for high content of condensed tannin which is attributed as one of the ant nutritional factors in this highly proteinaceous pulse crop. Therefore, the objective of this study was to estimate and characterize the phenolic compounds in different tissues of this pulse and their ant oxidative activities. Fairly good amount of phenolics were observed in all tissues extract which was quite evident from their high FRAP (Ferric reducing antioxidant power) value. It was further, observed that the extract prepared from its seeds presented a potent radical scavenger activity as indicated by its high capacity to reduce the free radical diphenylpicrylhydrazyl, whereas the tannin-free extract indicated loss of ant oxidative activities. The seed extract also interacted with superoxide anions, hydroxyl radicals as well as the oxidant species, hydrogen peroxide. Thus, our results provide evidence that the extract prepared from different tissues of faba bean shows antioxidant and radical scavenging activities largely because of its condensed tannins (proanthocyanidins).

Keywords: Antioxidant, faba bean, phenolic

INTRODUCTION

Legumes are important sources of both macro and micro nutrients and also play important role in the traditional human diet of many regions throughout the world. In addition to their nutritive value, some of them have health benefits and therapeutic properties (Geil and Anderson, 1994). They have been shown to have low glycemic indexes (Foster-Powell and Miller, 1995), hypocholestrolaemic effects (Anderson *et al*., 1999), breast cancer prevention (Adebamowo *et al*., 2005) and health benefits with respect to cardiovascular disease (Kushi *et al*., 1999). A decreasing incidence of such diseases have been observed in epidemiological studies, upon consumption of foods with high content of phenolics, such as fruits, vegetables, grains and legumes (Kris-Etherton *et al*., 2002; Miller *et al*., 2000).

A recent epidemiological study showed that among several common fruits and vegetables, only the consumption of bean and lentil is related to lower incidence of breast cancer (Adebamowo *et al*., 2005). Recently it has been reported in several experimental studies that legumes exhibit significant antioxidant activity (Heimler *et al*., 2005; Madhujith and Shahidi, 2005; Xu and Chang, 2007). These reports suggest that legumes may serve as an excellent dietary source of natural antioxidants for disease prevention and health promotion.

It is widely known that significant antioxidant activity of food is related to high Total Phenolic Contents (TPC). Condensed and hydrolysable tannins of relatively high molecular weight have been shown to be effective antioxidants with greater activities than simple phenolics (Hagerman *et al*., 1998). However, the antioxidant properties of tannins present in legumes have not been well investigated. A very few reports on antioxidant activities of condensed tannins (proanthocyanidins) from legumes have been reported in the recent past (Beninger and Hosfield, 2003; Xu andChang, 2007).

Faba bean (*Vicia faba* L.) is one of the underutilized pulse crops in China which is known to have considerable amount of compounds especially condensed tannin (proanthocyanidin) in its seeds. Tannins are mainly located in testa and play an important role in the defense system of seeds that are exposed to oxidative damage by many environmental factors (Troszynska and Ciska, 2002). The objectives of this study were to investigate major phenolic

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compounds and the antioxidant capacity of different tissues viz. leaf, stem and seed of different color dull (green and white). Furthermore, it is attempted in this study to establish the relationship between its polyphenolic constituents and antioxidant capacity by preparing a seed extract with depleting amount of tannins and its reacting efficiency with DPPH. The potential ability of seed extract of both genotypes (named Green and White) to scavenge diverse reactive species namely superoxide anion, hydrogen peroxide and hydroxyl ions was also studied. The findings from this study may also indicate the possible *in vivo* role of tannins/phenolic compounds in defending various stresses caused due to different environmental factors.

MATERIALS AND METHODS

Chemicals: Catechin hydrate (+), 1, 1-Diphenyl-2-Picrylhydrazyl (DPPH), xanthine oxidase, hypoxanthine, nitroblue tetrazolium, Superoxide Dismutase (SOD), guaiacol, horseradish peroxidase, deoxyribose, hide powder were purchased from Sigma-Aldrich, Germany. Ascorbic acid was obtained from Nanjing. China. All the other chemicals used were of Biochemical grade.

Seed sample: The investigations were carried on different tissues of faba bean with green hull named Green faba bean which was collected from farmer's field of Nanjing district of Jiangsu Province, China. Faba bean with white hull named White faba bean (Faba bean variety) was obtained from Nantong city, Jiangsu Province, China. Both the genotypes were grown in College of Jinling Institute under normal agronomic conditions and the dried seeds and other tissues were used in present study.

Preparation of tannin extract from faba bean seeds: Extract was prepared according to the method described by Makkar and Goodchild (1996), using 70% aqueous acetone. Two hundred milligram finely ground different tissue sample was mixed with 10 mL of 70% aqueous acetone and kept for 2 h in shaking water bath with 130 rpm at 30ºC. The aqueous phase was concentrated under reduced pressure almost to dryness and dissolved in methanol-water (2:3, v/v) solvent and stored at -20ºC for further use. Pigments from leaf tissue were removed by extracting with diethyl ether containing 1% acetic acid before extracting tannin. Tannin-free extract was prepared from aliquots of extract using 100 mg hide powder/mL diluted methanol-water extract. The mixture was kept for 15 min at 4ºC and then vortexed and finally centrifuged. The resulting solution was filtered and used for determination of free radical scavenger activity.

Determination of Total Phenolic Contents (TPC) and proanthocyanidins: Total phenolic content of tissue extract was determined by spectroscopy at 725 nm, on the basis of a colorimetric reaction with Folin-Ciocalteu reagent, as described by Makkar and Goodchild (1996). Different volume of extracts was taken and made to 1 mL by distilled water. (0.5 mL) of Folin Ciocalteu reagent (1N) and 2.5 mL of 20% sodium carbonate solution were added in each extract sample. The absorbance of thoroughly mixed reaction mixture was taken after 40 min. The results were expressed as tannic acid (0.5 mg/mL) equivalent on a dry matter basis. Proanthocyanidin (condensed tannin) content was determined colorimetrically at 550 nm using butanol-HCl and ferric reagent (Makkar and Goodchild, 1996; Porter *et al*., 1986). (3.0 mL) of butanol-HCl reagent (butanol: HCl, 95:5 v/v) and 0.1 mL of ferric reagent (2% ferric ammonium sulphate in 2N HCl) was added in all reaction mixture and vortexed properly. The mouth of each tube was covered and then the tube was incubated in a water bath at temperature 97ºC for 60 min. Absorbance was taken after cooling the reaction mixture. Proanthocyanidins content was expressed as leucocyanidin equivalent on a per cent dry matter basis.

***In vitro* antioxidant and radical scavenging activity:** Ferric Reducing Antioxidant Power (FRAP) assay (Total antioxidant activity).

For the FRAP assay of all tissue extract, a modified method of Benzie and Strain (1996) was adopted. The stock of FRAP solution included 300 mM acetate buffer pH 3.6, 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3.6H_2O$ solution. The fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ and 2.5 mL $FeCl_3.6H_2O$. The temperature of solution was kept at 37ºC before using it. 150 µL of different tissue extract were allowed to react with 2850 µL of FRAP solution for 30 min in the dark condition. Absorbance of colored product of different samples (ferrous tripyridyltriazine complex) was taken at 593 nm. The results were expressed as mM Fe (II) /g tissue basis and compared with that of ascorbic acid and (+) catechin hydrate.

Superoxide anion scavenging activity: Superoxide anion scavenging activity of seed extract was determined following the method described by Paya *et al*. (1992) by generating superoxide anion enzymatically at room temperature by adding 0.042 U/mL of xanthine oxidase to a mixture of 2 mL containing 50 mM KH_2PO_4 pH 7.4, 1 mM EDTA, 100 µM hypoxanthine and 100 µM Nitroblue Tetrazolium (NBT). The rate of NBT reduction was recorded during 150 sec by spectrophotometer at 560 nm. Control experiments were performed to determine whether the extract directly reduced NBT or inhibited xanthine oxidase, as described by Soares *et al*. (1997). Superoxide dismutase was used as reference scavenger of superoxide anion. Reactivity of extract against $O_2^{\cdot-}$

Table 1: Polyphenol content of different tissues of faba bean (*Vicia faba* L.) genotypes

Genotypes	Tissues of faba bean used	Total extractable phenol[a]	Proanthocyanidin[b]	Proanthocyanidin[cb]
Green	Leaf	0.28±0.11	0.08±0.006	ND
	Stem	0.07±0.01	0.04±0.002	ND
	Seed	3.68±0.21	2.37±0.080	0.12±0.008
White	Leaf	0.32±0.13	0.12±0.008	ND±
	Stem	0.08±0.04	0.05±0.007	ND±
	Seed	3.89±0.23	2.48±0.090	0.13±0.009

Results are mean values of triplicate determinations, ±standard deviation; [a]: Expressed as % dry matter equivalent to tannic acid; [b]: Expressed as % dry matter equivalent to leucocyanidin; [c]: Expressed as % dry matter equivalent to leucocyanidin after hide powder treatment; ND: Not detectable

was expressed in terms of the volume of extract at which 50% inhibition of rate of NBT reduction (EC_{50}) takes place.

Hydrogen peroxide scavenging activity: Hydrogen peroxide was measured in a reaction mixture of 1 mL containing 150 mM KH_2PO_4-KOH buffer pH 7.4, 100 µL guaicol solution (prepared by adding 100 µL of pure guaicol to 50 mL deionized water) and 5 µL horseradish peroxidase (5 mg/mL in the same phosphate buffer) as described by Aruoma *et al*. (1989). The reaction was started by H_2O_2 addition. Extract was pre-incubated with H_2O_2 for 30 min at 25ºC and then, aliquots were removed and assayed for the remaining H_2O_2. Results were shown as percent H_2O_2 scavenger capacity by the equation as followed in DPPH scavenging capacity. Adequate blanks were also prepared to determine whether the control interfered with the reaction or if the seed extract was a substrate for peroxidase.

Hydroxyl radicals scavenging activity: Ascorbate/ iron/H_2O_2 system was used to generate hydroxyl radicals as referred elsewhere (Burits and Bucar, 2000). The reaction mixture in a final volume of 1 mL, contained 20 mM KH_2PO_4-KOH buffer pH 7.4, 2.8 mM 2-deoxyribose, $FeCl_3$-EDTA (100 and 104 µM, respectively), 1 mM H_2O_2 and 100 µM ascorbate. The extent of deoxyribose degradation was measured after 1 h of incubation at 37ºC by the thiobarbituric acid method by adding 1.0 mL of TBA (1% in 50 mM NaOH) and 1.0 mL of TCA (2.8 % w/v) in the reaction mixture (Buege and Aust, 1978) and tubes were heated at 1000C for 20 min. After cooling, the absorbance was read at 532 nm against blank containing only buffer and deoxyribose. The absorbance value obtained was used for calculation of percent inhibition of deoxyribose degradation by different volume of seed extracts of both genotypes. Extract, blank or Dmanitol (reference compound) were added before ascorbate addition. Solution of iron salt, H_2O_2 and ascorbate were always prepared freshly.

RESULTS AND DISCUSSION

Total extractable phenol and proanthocyanidin: Results obtained in the present study revealed that the level of total extractable phenol and proanthocyanidin (condensed tannin) in the aqueous acetone extract of different tissues of faba bean were present in a considerable amount. Among all three tissues viz. leaf, stem and seed, maximum amount of both extractable phenol as well as proanthocyanidin were observed in seeds of both the genotypes i.e., Green (3.68±0.21 and 2.37±0.08 respectively) and White (3.89±0.23 and 2.48±0.09, respectively) (Table 1). While leaf and stem tissues of Green and White genotypes contain lesser amount of total extractable phenol and proanthocyanidin. As the amount of proanthocyanidin was very less in leaf and stem, its concentration could not be detected after hide powder treatment. Whereas seed extract of both the genotypes have shown some amount of proanthocyanidin after hide powder treatment.

Table 2: Total Antioxidant Power (FRAP) value

Genotypes	Extract	Total Antioxidant activity (FRAP) mM FeII/g tissue basis
Green	Leaf	256.30±2.34
	Stem	248.40±3.67
	Seed	362.50±6.12
White	Leaf	245.40±4.16
	Stem	236.20±3.65
	Seed	472.40±9.64
Standard	Ascorbic acid (+)	78.27±2.72
	Catechin hydrate	38.94±1.96

Results are mean values of triplicate determinations, ±standard deviation; [a]: mM FeII/mg ascorbic acid basis; [b]: mM FeII/mg catechin

Ferric Reducing Antioxidant Power (FRAP) assay: FRAP assay depends on the reduction of Ferric tripyridyltriazine (Fe (III)-TPTZ) complex to the Ferrous Tripyridyltriazine (Fe (II)-TPTZ) by a reductant (antioxidants or other reducing agents) at low pH. Fe (II)-TPTZ has an intensive blue color and can be monitored at 593 nm (Benzie and Strain, 1996). FRAP values of leaf, stem and seed extract were estimated and compared with ascorbic acid and catechin hydrate. We observed lesser FRAP value for all tissues extract than that of ascorbic acid and catechin hydrate, nevertheless tissues have considerable amount of reducing ability as compared to many plants extract of medicinal importance. Although leaf and stem do not have much amount of extractable phenol and PA as compared to seed extract, they show fairly good amount of FRAP value (Table 2). However, seed extract of White genotype show higher FRAP value (472.4±9.64 mM FeII/g tissue basis) than Green seed extract (362.5±6.12 mM FeII/g tissue basis).

Table 3: DPPH reduction (%) of seed extract of green and white and equivalent amount of ascorbic acid solution

	Volume of extract (mL)					
Extract	10	20	40	60	80	100
Green extract	10.2	18.3	32.6	52.1	60.4	72.5
White extract	10.3	22.5	55.1	73.1	84.9	91.2
Ascoribic acid	18.5	22.2	61.4	72.5	83.4	92.7

Table 4: Relationship between DPPH radical quenching of seed extracts and tannin free seed extract

	Volume of extract					
Extract	10	20	40	60	80	100
Green seed extract	9.8	18.5	35.7	53.1	64.4	75.7
Green tannin free seed extract	ND	2.3	8.4	10.2	13.5	13.8
White seed extract	10.2	22.8	52.6	78.8	87.5	88.9
White tannin free seed extract	ND	2.6	7.8	12.3	14.3	15.6

Free radical scavenger activity: The general free radical scavenger activity of the extract was evaluated by its interaction with DPPH in solution. DPPH scavenging activity has been successfully used as a quick and reliable parameter to assess the in vitro general antioxidant activity of plant extracts (Goncalves *et al*., 2005; Khurana *et al*., 2010; Policegoudra *et al*., 2010) DPPH is a stable free radical that can accept an electron or hydrogen radical to become a stable diamagnetic molecule. The methanolic solution of DPPH shows a strong absorption band at 517 nm (Blois, 1958) because of its odd electron, which decreases in the presence of free radical scavengers. We compared the DPPH scavenging activity of seed extract of White with Green. White seed extract reacted with DPPH leading up to a loss of 90% in the absorbance intensity which is almost equal to ascorbic acid (Table 3) whereas the same amount of seed extract of Green showed only 72% loss in absorbance. In contrast, the tannin free extract only presented 15.6 and 13.8% of DPPH reduction capacity in White and Green genotypes respectively (Table 4).

Antioxidant activity characterization:

Interaction with superoxide anion: The reactivity of seed extract with superoxide anion was evaluated by the decrease in the rate of Nitroblue Tetrazolium (NBT) reduction which is induced by $O_2^{\cdot-}$ generated by the enzymatic system xanthine oxidase/hypoxanthine. Any compound that decreases the rate of NBT reduction should react with $O_2^{\cdot-}$ unless itself reacts directly with NBT. The production of $O_2^{\cdot-}$ in the present experiment was ascertained by use of Superoxide Dismutase (SOD), the physiological scavenger of superoxide anion, as it inhibited the reaction of NBT reduction in a concentration dependent manner with an inhibition concentration at 50% = 1.7 U/mL. It was observed that 26.7 and 22.3 µL of seed extract of Green and White respectively cause 50% inhibition of rate of NBT reduction (Table 5). The extract neither reduced NBT by itself nor inhibited xanthine oxidase was further evaluated by uric acid formation.

Table 5: Scavenging activity of superoxide anion by faba bean seed extract

Genotypes	EC_{50} value µL
Green	26.7
White	22.3

Table 6: Scavenging activity of hydrogen peroxide by faba bean seed extract

	H_2O_2 scavenger capacity	
Volume of % seed extracts (µL)	Green	White
10	20.31	32.68
20	28.72	45.76
40	30.54	58.11
60	31.96	67.26
80	33.12	65.41
100	33.57	60.23

Interaction with hydrogen peroxide: The reactivity of extract with hydrogen peroxide can be sensitively measured by the conventional peroxidase based assay which is based on the decrease in absorbance at 436 nm where guaicol is used as an electron donor. Any compound that reacts with H_2O_2 should decrease the rate of guaicol oxidation thereby decreasing the absorbance intensity at 436 nm. The results clearly show considerable level of decrease in absorbance at 436 nm indicating the interaction of extract with H_2O_2. Different volume of seed extract ranging from 10-100 µL of both Green and White genotypes were taken for this experiment. Green seed extract showed 20.31-33.57% decrease in absorbance whereas White seed extract showed per cent decrease in absorbance from 32.68 (10 µL seed extract) to 67.26 (60 µL) and followed by decreasing to the level up to 60.23% at the volume of 100 µL (Table 6). The extract neither oxidized directly guaicol nor was as substrate for peroxidase was also confirmed.

Interaction with hydroxyl radical: The interaction of the extract with hydroxyl radicals is tested in a simple assay where the radicals are formed from H_2O_2 in a reaction catalyzed by metal ions (Fe^{2+} or Cu^{+}) known as the Fenton reaction (Nordberg and Arner, 2001) in a solution from the ascorbate/iron/H_2O_2 system and are detected by their ability to degrade deoxyribose when

Table 7: Scavenging of OH^- (hydroxyl) radical by seed extract and manitol showing % reduction of absorbance intensity at 532 nm relative to control assay

	Volume of extract					
Extract	10	20	40	60	80	100
Green seed extract	22.5	38.2	58.5	45.3	45.6	47.9
White seed extract	82.2	84.9	85.2	86.2	87.1	88.3
Mannitol	81.3	85.3	84.8	86.3	88.2	88.8

on heating with TBA, forms a pink chromogen (Paya *et al*., 1992). The seed extracts used in the present study competed with deoxyribose for hydroxyl radicals demonstrating in vitro significant protective effect on the deoxyribose damage assay. However, compared to Manitol and White seed extract, seed extract of Green showed lesser percentage inhibition of deoxyribose degradation ability i.e., 22.5% inhibition in 10 µL seed extract and reaches to maximum of 58.5% inhibition in 40 µL and then started falling to 47.9% in 100 µL seed extract volume (Table 7). Manitol and White seed extract showed almost similar and higher values of percentage inhibition of deoxyribose degradation (Table 7).

Naturally occurring phenolic compounds exert their beneficial health effects mainly through their antioxidant activity (Fang *et al*., 2002). These compounds are capable of scavenging free radicals; chelating metal catalysts, activating antioxidant enzymes, and inhibiting oxidases (Heim *et al*., 2002). Tannins are biological active compounds and may have beneficial or adverse nutritional effects. Condensed tannins, the predominant phenolic compounds in legume seeds, are widely found in lentils, peas, colored soybeans, and common beans. The results strongly suggest phenolic compounds are present in different tissues of this plant, however, more abundantly in seeds which could be attributed possibly in in vivo scavenging reactive oxygen species capability. A recent report suggests high positive relationship between total phenols and antioxidant activity in many plant species (Adedapo *et al*., 2008) which is also corroborated in our FRAP assay results. There are several tannin-complexing agents are known such as Polyvinylpyrrolidone (PVP), bovine serum albumin, lysozyme, gelatin, hide powder etc (Fickel *et al*., 1999; Gonclaves *et al*., 2005). We tried PVP and hide powder and obtained good precipitation with hide powder. The results clearly indicate that the general free radical scavenger activity of seed extract is largely because of proanthocyanidins (condensed tannin) as the loss of proanthocyanidins leads to a significant loss of antioxidant capacity. Amarowicz *et al*. (2000) also studied scavenging effect of condensed tannin of beach pea, canola hulls, evening primrose and faba bean on DPPH radical and reported polyphenolic compounds as major free radical scavenger.

The result of this study also showed that seed extract are capable of interacting with different ROS viz. O_2^{-}, H_2O_2 and OH^-, exhibiting in vitro capability to scavenge them. Besides being one of the strongest reactive oxygen species among the free radicals, superoxide anion radicals has the ability to give rise to powerful and toxic hydroxyl radicals as well as singlet oxygen, both of which contribute to oxidative stress. Therefore, superoxide radical scavenging by natural antioxidant present in seed has physiological implications. The reaction of seed extract with O_2^{-} further indicates its ability to prevent the formation of peroxynitrite (ONOO-), highly cytotoxic compound, synthesized by reaction O_2^{-} with Nitric Oxide $(NO)^-$ Although hydrogen peroxide is not free radical it has a great physiological relevance because of its ability to penetrate biological membranes and to act like an intermediate in the production of more reactive oxygen species namely hydroxyl radical and hypochlorous acid (Nordberg and Arner, 2001). Further the results also showed that seed extract of faba bean competed with deoxyribose for hydroxyl radicals demonstrating significant in vitro protecting effect on the deoxyribose damage assay.

Almost all organs (except few) of higher plants which perform aerobic metabolism generate Reactive Oxygen Species (ROS) and thereby experience 'oxygen stress'. Oxidative stress is induced by wide range of environmental factors including UV stress, pathogen invasion which are intimately connected with ROS. Stress situations are counteracted by an increase in radical scavenging processes or by pre-existing or de novo synthesized compounds functioning in detoxification of ROS. Of the ROS, hydrogen peroxide and superoxide radicals etc., are produced in a number of cellular reactions including the iron-catalysed Fenton reaction, and by various enzymes such as lipoxygenases, peroxidases, NADPH oxidases and xanthine oxidase. In plant tissues, many phenolic compounds are potential antioxidants: flavonoids, tannins etc., which may work as ROS-scavenging compounds. Condensed tannins have been proposed to play a role in the interactions between plants and microorganisms, either pathogenic or mutualistics, as well as in plant responses to abiotic stresses (Escary *et al*., 2007; Paolocci *et al*., 2005; Reinoso *et al*., 2004). Our results have shown that faba bean seeds contain adequate amounts of phenolic compounds which have fairly good amount of free radical scavenging and reducing ability. The seed extract have also been shown interaction with H_2O_2, O_2^{-} and OH^-, which indicate its possible role in combating oxidative stress that a seed often encounters.

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