Proximate composition and flavonoids content and in vitro antioxidant activity of 10 varieties of legume seeds grown in China

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A comparative study of several Chinese legume varieties was carried out to evaluate the proximate composition and total flavonoid contents (TFC) and in vitro antioxidant activities of the selected legume seeds. Defatted legume seed flour was extracted with ethanol. The ethanolic extracts were then subjected to evaluating their in vitro antioxidant activities using 1,1-diphenyl-2-picrylhydrazyl (DPPH) free-radical scavenging and Rancimat methods. The different legumes showed considerable variations in their flavonoid contents (0.30 to 3.38 mg rutin equivalents/g legume flour) as well as antioxidant activities (3.5 to 60.1% of DPPH radical scavenging and protection factor values of 2.0 to 4.9 from Rancimat test). Legume samples were effectively distinguished into four groups depending upon the principal component and cluster analyses. This study suggests that the Chinese kidney and rice beans varieties are rich sources of flavonoids. The selection of the right legume varieties could provide good sources of antioxidants for nutraceutical applications.

Key words: Legume seeds, flavonoids, antiradical/antioxidant activity.

INTRODUCTION

Legumes, such as soybeans, kidney beans, lentils and chickpeas, contain many important nutrients and phytochemicals; and are present in most Chinese daily diets as good sources of protein, generous amounts of dietary fiber, starch, lipids and minerals. Many researchers have shown the relationship between legume consumption and health benefits, such as, protection from cardiovascular disease, breast cancer, colon cancer, other cancers and diabetes (Kushi et al., 1999; Mathers, 2002; Messina, 1999). Including the functions above, traditional Chinese medicine dietary therapy suggests that legumes also tend to correct some disharmony-induced health problems; for example, cold-natured mung beans and soybeans have the effect of “clearing heat” and warm-natured black beans can promote diuresis and clear vision.

Phenolic compounds present in legumes with their antioxidative effects on human health, include phenolic acids, such as gallic acid, caffeic acid, syringic acid, etc., flavonoids and other polyphenolic compounds, whereas, many researchers indicated that flavonoids are mainly responsible for the antioxidant activity, and less correlation between total phenolics and the activity (Dueñas et al., 2006; Lin and Lai, 2006; Segev et al., 2010). Flavonoids are common constituents of legume, and can provide the most health-promoting functions of legume mentioned above. Dietary flavonoids present in legume seeds are usually glycosylated with six subclasses, viz, anthocyanins, flavanols, flavones, flavanones, flavonols and isoflavones (Robards and Antolovich, 1997), and have received considerable attention due to their effects on maintaining bodily health, which are partly explained by their antioxidant activity. Epidemiological research suggested that flavonoid intake is positively associated with a reduction in the risks of coronary heart disease.
and certain types of cancer (Kris-Etherton et al., 2002) induced by free radicals. Isoflavones are structurally isomeric to flavonoids and found in significant concentrations mainly in soy and soy products (Franke et al., 1994); moreover, they are believed to help prevent prostate and breast cancers (Dwyer et al., 1994). The antioxidative properties of flavonoids are considered to be due to radical scavenging by donating hydrogen. Metal-chelating is another feature of certain flavonoids, and those with the catechol structure in the B-ring or probably with both 5-hydroxyl and 4-oxo groups can suppress the iron- or copper-catalyzed Fenton reaction (Rice-Evans et al., 1996; Mira et al., 2002). In addition, flavonoids also prevent the oxidation of lipids (Kumamoto and Sonda, 1998). The importance of the antioxidant constituents of legumes and their crude extracts in the maintenance of health and nutritive value of food is also increasingly of interest among food manufacturers and consumers as the future trend toward developing functional food. Nonetheless, little information of the in vitro antioxidant activity with respect to flavonoids was known in some common Chinese legumes.

The purpose of the present study is to evaluate the amount of total flavonoids in 10 common Chinese legume varieties as well as their antioxidant activities using 1,1-diphenyl-2-picrylhydrazyl (DPPH) free-radical scavenging and Rancimat test. The experimental data will be differentiated and classified using principal component and cluster analyses in an attempt to provide information of natural antioxidants in those legumes for selection of suitable legumes as health-promoting food sources.

MATERIALS AND METHODS

Legume materials and chemicals

Legumes were randomly sampled from different regions of China: yellow soybeans (Glycine max cv. Huangdadou), peas (Pisum sativum cv. Qingwandou) and mung beans (Phaseolus radiatus cv. Zaluidou) from the Henan Province; rice beans (Vigna umbellata cv. Honghuafandou) from the Hubei Province; lentils (Polygala tatarinowii cv. Youyuxiaobiandou) and broad beans (Vicia faba cv. Pinglucandou) from the Shanxi Province; chickpeas (Cicer arietinum cv. Dailyingzuidou) from the Xinjiang Province; black soybeans (G. max cv. Danboheidadou), green soybeans (G. max cv. Luiguangmaodou) and kidney beans (Phaseolus vulgaris cv. Shenhongyundou) from the Yunnan Province. Broken and cracked legume seeds were removed along with dirt and foreign materials. The cleaned legume samples were finely ground by a grinder (HUJER FW100, Zhejiang, China) and then passed through a 60-mesh sieve to obtain legume flour, which was then kept under dark and desiccated conditions until further use.

Rutin (> 99% purity) was purchased from Huashuo Fine Chemicals Co., Ltd. (Shanghai, China). DPPH were purchased from Sigma (St. Louis, MO, USA). All other chemicals and reagents were of the analytical grade.

Proximate composition analysis

Moisture content was determined by drying the legume flour after 24 h at 105°C in an air oven until a constant weight was obtained (Tang, 1997). Fat content was determined using a Soxhlet apparatus according to the procedure described by Huang (1997b). Protein content was calculated from the nitrogen content (% N x 6.25) analyzed by the Kjeldahl method (Thiex et al., 2002). Starch content was examined using the amyloglucosidase-α-amylase method (McCleary et al., 1997). Ash was measured according to the standard procedures (Huang, 1997a).

Preparation of flavonoid extracts

Crude flavonoid extracts were obtained according to the method of Ju et al. (2001) with a slight modification. Each legume flour sample (50 g) was defatted three times using 250 ml of petroleum (3 x 250 ml) at 65°C for 1 h, followed by air drying at 50°C. An aliquot of 1 g of defatted legume flour was extracted with 5 ml of 75% ethanol with continuous stirring at 60°C for 2 h, filtrated by Whatman No. 42 filter paper, then an extra 75% ethanol was added to make up the filtrate to 50 ml, and kept in the dark at 4°C until analysis.

Determination of total flavonoid content (TFC)

The TFC in 10 legume varieties were measured by aluminum nitrate colorimetry method according to the procedure described by Wang et al. (2007). Briefly, an aliquot (5 ml) of legume flour methanol extracts was mixed with 1.4 ml of solution containing 25 g/L sodium nitrate and 50 g/L aluminum nitrate. After 6 min, 5 ml of 1 M sodium hydride solution was added, and the total volume was made up to 25 ml with 75% ethanol. The solution was well mixed and incubated at room temperature for 10 min. The absorbance was measured at 510 nm on a spectrophotometer (Shimadzu UV 1601) and the flavonoid content was calculated by a calibration curve of rutin, and expressed as mg of rutin equivalent (RE) per g of legume flour.

Evaluation of the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity (RSA)

The DPPH RSA of legume extracts was evaluated by the DPPH method of Hudc et al. (2007) with a slight modification. An aliquot of 0.4 ml of 2.5 mg/L DPPH in ethanol was added to 7.6 ml of the extracts. The mixture was then shaken well and incubated at 30°C. Using a spectrophotometer, the absorbance was recorded at 517 nm every 10 min after the initial mixing and recording until the endpoint of 40 min. A control was measured using the same procedure except that ethanol was used to instead of extracts. The degree of decoloration of the solution indicates the scavenging efficiency of the extracts. DPPH RSA was calculated using the following equation:

\[ \% \text{ discoloration} = \left(1 - \frac{A_t}{A_o} \right) \times 100\% \]

where, \( A_t \) the absorbance for legume flavonoid extract; \( A_o \), the absorbance for the control.

Each sample was done in triplicate, and the average was used for calculating the first-order derivative of absorbance (\( D \) which was plotted against incubation time to illustrate the kinetic scavenging rate of legume extracts.

Evaluation of the inhibitory effect on lipid oxidation by the Rancimat method

The Rancimat test of samples was performed on a 743 Rancimat...
The proximate compositions of 10 legumes are shown in Table 1. By comparing three soybeans (yellow, black and green) with other legumes, it was found that the protein and fat contents were significantly high and accounted for 37.29 to 42.2% and 14.96 to 17.86%, respectively. It was also found that total starch contents of these three soybeans were the least. Meanwhile, mung beans contain the highest total starch (52.35%), which is slightly higher than that of peas (49.97%) and lentils (49.82%), but were ranked at the bottom of fat content with broad beans among the 10 legume varieties. The ash contents varied from 2.67 to 4.99% for all legume varieties in this study.

### RESULTS AND DISCUSSION

#### Proximate composition of legumes

The proximate compositions of 10 legumes are shown in Table 1. By comparing three soybeans (yellow, black and green) with other legumes, it was found that the protein and fat contents were significantly high and accounted for 37.29 to 42.2% and 14.96 to 17.86%, respectively. It was also found that total starch contents of these three soybeans were the least. Meanwhile, mung beans contain the highest total starch (52.35%), which is slightly higher than that of peas (49.97%) and lentils (49.82%), but were ranked at the bottom of fat content with broad beans among the 10 legume varieties. The ash contents varied from 2.67 to 4.99% for all legume varieties in this study.

#### Statistical analysis

Three independent trials (n = 3) with triplicate sample analyses were performed. Data were analyzed using the general linear model procedure of the Statistix software 9.0 (Analytical Software, Tallahassee, FL, USA) and the Tukey HSD all-pairwise comparisons test was performed to identify significant differences of proximate compositions and flavonoid content between legume varieties. The principal component analysis (PCA) and cluster analysis with the hierarchical method were applied to summarize all variables to a mathematical model using SPSS 13.0 (SPSS inc., 2004).

#### Antioxidant activities of legume flavonoid extracts

Using DPPH as a stable free radical substrate and scavenged by the addition of flavonoid extracts is a simple method to estimate the antioxidant activity, which is related to the hydrogen donating ability of flavonoids and the stability of the phenoxyl radicals formed (Rice-Evans et al., 1996). The discrepancy in absorbance for each extract in intervals of time is determined, and the rate of decoloration at each sampling point was obtained and plotted versus incubation time (Figure 1). It is suggested that the larger negative slope indicates the higher rate for scavenging the radical. Kidney beans had
Figure 1. The first-order derivatives (D) of the absorbance with respect to kinetic scavenging rate of legume extracts.

The highest scavenging rate, and chickpeas were much lower than the other legumes throughout the incubation. Yellow soybeans and green soybeans demonstrated relatively high rates of free radical scavenging abilities in the initial stage as compared with chickpeas regarding to their similar significance level of flavonoid content. In addition, the declines of scavenging rates of mung beans and lentils extracts were found to be slightly lower than the legumes after 10 min, which had similar initial scavenging rates, corresponding to the secondary derivatives (data not shown). With the reaction time prolonged, the scavenging process mostly reached an approximate equivalent at 20 min. Considering the flavonoid components, mung beans are rich in vitexin and isovitexin (apigenin flavone glycoside) (Peng et al., 2008), while catechin and epicatechin (flavanols) are the major flavonoids in lentils (Bartolomé et al., 1997), but whether the discrepancy in scavenging rates can be properly explained by the variations in flavonoid components was yet to be determined.

The percentage of DPPH radical-scavenging after the 40 min reaction is shown in Figure 2. It appears that lentils, kidney and rice beans were also ranked at the top in respect to the scavenging free radicals activity (60.1, 58.4 and 53.6%, respectively) (P < 0.05), and chickpea showed the lowest activity (3.5%) among all experimental legumes. Vinson et al. (1998) reported that kidney beans had the highest antioxidant quality among their 22 selected vegetables (Vinson et al., 1998). These results are also broadly comparable to these previous studies (Dueñas et al., 2006; Lin and Lai, 2006; Xu et al., 2007).

The antioxidant activity of flavonoids is mainly due to their structural features. For example, the major flavonoids found in kidney beans and lentils are flavone, quercetin, flavanol, and catechin/epicatechin, respectively, which possess different basic structures but the same hydroxylation pattern (3,5,7,3′,4′-OH) (Bartolomé et al., 1997; Beninger and Hosfield, 2003). However, kaempferol, differing from quercetin in the absence of the 3′-OH group from the B-ring, and the isoflavone and formononetin were proved to be the major flavonoids present in chickpea (Segev et al., 2010). Strong correlation between TFCs and DPPH RSA was found in our study with the Pearson correlation coefficient 0.822 at a 95% confidence level (P < 0.05). This high correlation can be explained by the principle of the aluminum nitrate method, in which the most aluminum-flavonoid complex is formed between aluminum ion and o-dihydroxyl group (3′,4′-OH) in the B-ring (Jia et al., 1999; Kim et al., 2005), and the α-dihydroxy system can synergistically enhance the overall antiradical activity of the flavonoid compounds (Burda and Oleszek, 2001; Silva et al., 2002). A moderate or high association between certain legume seeds or other plant flavonoids and DPPH RSA has also been reported by many researchers (Lin and Lai, 2006; Oomah and Mazza, 1996; Rohman and Utari, 2006; Xu et al., 2007).

The Rancimat method was utilized to monitor the change of electrical conductivity of water. In this method, lipid oxidation gives rise to a formation of volatile secondary oxidation products under elevated temperature and accelerated aeration (Ho et al., 1992).
The inhibitory effect of flavonoid extracts on oxidation-induced lipid fragmentation by antioxidants was evaluated by the protection factor and shown in Figure 2 with DPPH RSA. It is illustrated that, except for 2 legumes with extremely high PF value (kidney bean 4.9 and rice bean 4.8) and 1 legume with the lowest PF value (broad bean 2.0), the PF values roughly ranged from 2.5 to 3.1. By the Pearson correlation coefficient (0.662), PF values were moderately correlated with TFCs at a 95% confidence level ($P < 0.05$), but a weak association (0.353, $P > 0.05$) was observed between PF values and the DPPH RSAs. This ambiguous relationship, which cannot be well explained by the flavonoid content, should be attributed to the different composition in flavonoid extracts. For instance, isoflavones are the predominant flavonoids in soybean as determined in our study using high performance liquid chromatography (HPLC) with external standards. The amount of daidzin and genistin in yellow, black and green soybean flour samples were 1.51 and 1.69, 1.18 and 1.03, and 1.18 and 1.03 mg/g, respectively, which may have been underestimated by aluminum nitrate method. Also, the contents of daidzin and genistin were found in trace amount in the other experimental legumes, confirming the findings of previously reported research (Bingham et al., 1998; Mazur et al., 1998). Additionally, the type of free radical or oxidant in the assay such as DPPH and peroxyl radicals provided different ranked antioxidant activity of legume extracts. Unlike DPPH assay, which give rise to phenoxy radicals (Antoshina et al., 2005), flavonoids in the Rancimat test are able to inhibit lipid oxidation due to their metal chelating activity (Hudson and Lewis, 1983; Ruiz-Larrea et al., 1995; Yu et al., 2007).

Due to the nature of the assays and the variety of radicals applied, the relationship between the antioxidant activity and total flavonoids of legume extracts were complex and difficult to individually classify. Therefore, PCA was performed on the data sets of flavonoid contents, analyzing DPPH RSA and PF of Rancimat test of legume samples. The data sets were standardized by the re-scaling procedure automatically to form zero mean and unit standard deviation. The original variables were linearly combined by PCA, which is responsible for the overall antioxidant capacity of legume samples. The factor loading and variance information are shown in Table 2, and demonstrates that the percentage of total variance accounted for 96.7% by the two principal components (PC) extracted. The first PC (PC1) and the second PC (PC2) account for 74.8 and 21.9% of the total variance, respectively. From the estimated factor loadings, PC1 and PC2 were expressed by the equations as follows:
In general, antioxidants are divided into two groups according to their participation in the free radical chain reactions. These are “preventive” antioxidants, and "chain-breaking" antioxidants (Ou et al., 2001). According to the analysis above, it is suggested that PC1 account for the antioxidant ability of legume samples and is positively correlated with a large proportion of antiradical activity; PC2 might be positively connected to a large proportion of “preventive” radical-generating ability.

According to the factor scores in Figure 3, the legumes were clearly distinguished into four groups (A, B, C and D). Group A, containing kidney beans and rice beans, presents the highest capacity in both scavenging and “preventive” antioxidant action and is good for antioxidant sources. Group B, containing lentils and broad beans suggest that they are effective in breaking off radical chain reactions along with a weak inhibitory effect on lipid oxidation. Group C, containing black soybeans, mung
beans and peas presents moderate antioxidant ability. However, Group D, containing yellow soybeans, green soybeans and chickpeas showed a relatively low antioxidant activity compared to the other groups, which is in agreement with Xu et al. (2007).

Conclusion

The present study revealed the tested legume seeds to be a potential source of valuable nutrients and antioxidants. Besides, the data show that the flavonoids of legumes vary greatly in their antioxidant activities among the ethanolic extracts from the 10 experimental Chinese legume seeds evaluated. Kidney beans and rice beans were found to have high amount of total flavonoids and strong general antioxidant activity among four groups by PCA implementation. Because of increased consciousness of general nutrition, consumption of the legumes and related products is becoming popular worldwide. The findings in this study provide information for the selection of suitable legumes as health-promoting food sources.

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