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Total phenolics, flavonoids, tannins and antioxidant activity of lima beans conserved in a Brazilian Genebank

Fenólicos, flavonoides, taninos totais e atividade antioxidante de fava conservada em banco de germoplasma

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ABSTRACT

*The objective of this study was to characterize for the first time polyphenols and DPPH (2-diphenyl-1-picryhydrazyl radical) antioxidant activity in commonly cultivated accessions of **Phaseolus lunatus** from an ex situ germplasm collection maintained by Embrapa, in Brazil. Furthermore, the study aimed to detect changes in total polyphenols, total flavonoids and condensed tannin for the same accessions after regeneration in a greenhouse. The results showed the diversity of the lima bean collection for phenolic compounds, which were strongly correlated with antioxidant activity. Lima beans accessions with the highest polyphenols and antioxidant activity were those with colored seeds. Conservation through cold storage of **P. lunatus** seeds in a cold chamber in the germplasm collection did not necessarily affect phenolic compounds. Variations observed in values after regeneration seeds may be mainly results of biotic and abiotic factors, including not only cultivar, but also environmental conditions. This study suggests that polyphenols in the lima beans present antioxidant activity, with possible beneficial effects for human health. It was expected that the potential of this tasty legume can be also used as a functional food crop and/or as a new ingredient in gastronomy.*

Key words: ***Phaseolus lunatus**, genetic resources, germplasm collection, **Leguminosae**, polyphenols.*

RESUMO

*O objetivo deste estudo foi caracterizar, pela primeira vez, os polifenois totais e a atividade antioxidante por DPPH (2- difenil-1- picryhydrazyl radical) em acessos cultivados de **Phaseolus lunatus** L., provenientes de uma coleção de germoplasma mantida pela Embrapa, no Brasil, e detectar mudanças nos teores de polifenóis totais, flavonoides e taninos condensados, após a regeneração dos acessos em casa de vegetação. Os resultados mostraram a diversidade da coleção de fava para compostos fenólicos, que foram fortemente correlacionados com a atividade antioxidante. Os acessos de fava com os teores mais elevados de polifenóis e de atividade antioxidante foram aqueles com sementes coloridas. A conservação das sementes de **P. lunatus** na câmara fria da coleção de germoplasma não afetou, necessariamente, os polifenois, sendo que as variações após a regeneração das sementes parecem resultar principalmente de fatores bióticos e abióticos, incluindo não apenas cultivar, mas também condições ambientais. Este estudo sugeriu que os polifenóis da fava apresentam atividade antioxidante, com possíveis efeitos benéficos para a saúde humana. Esperamos que o potencial deste apetitoso legume também possa ser aplicado como alimento funcional e/ou como um novo ingrediente na gastronomia.*

Palavras-chave: ***Phaseolus lunatus**, recursos genéticos, coleção de germoplasma, **Leguminosae**, polifenóis.*

INTRODUCTION

Phaseolus lunatus L. has been domesticated in the Americas and presents two major gene pools: the Mesoamerican one, with small seeds and wild types distributed in Mexico, Central America and eastern part of the Andes, and the Andean pool, with large seeds and wild types distributed predominantly in the Western part of the Andes, in Ecuador and Northern Peru (GUTIÉRREZ SALGADO et al., 1995; SERRANO-SERRANO et al., 2010). In Brazil, the lima bean is considered

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the most important species of the genus, after the common bean (***P. vulgaris*** L.) and it is one of the most consumed legumes in the Northeast region (GUIMARÃES, 2007). Embrapa Genetic Resources and Biotechnology has an Active Genebank of ***P. lunatus*** L., with approximately 330 accessions collected predominantly in Brazil.

Phenolic compounds or polyphenols are widely distributed in nature, including simple molecules such as phenolic acids, compounds with intermediate molecular weight, such as flavonoids and long chain polymers with high molecular weight, such as condensed and hydrolyzed tannins. In plants, polyphenols are mainly associated with the attraction of insects and pollination, and plant defense and resistance (POCIECHA et al., 2009; ZUR et al., 2011). In humans, there is evidence that phenolic compounds are related to antioxidant capacity (WU et al., 2004; XU & CHANG, 2007; GRANITO et al., 2007). ***P. lunatus*** presents nutritional values that are very similar to the values of ***P. vulgaris***. However, the lima bean crop presents higher resistance to extreme climatic and agronomic conditions, if compared to the common bean (BETANCUR-ANCONA et al., 2004; GRANITO et al., 2007). In addition, an antagonism between the resistances to pathogens and to herbivores seems to take place in the species (BALLHORN, 2011).

Accessing the variability of bioactive polyphenols in a diverse lima bean sample can be important to improve understanding of the potential related to species adaptation and to resistance to biotic stresses and to improve the knowledge about the functional potential of ***P. lunatus***. This study aimed to characterize a sample of 50 commonly cultivated accessions of ***P. lunatus*** conserved in a Brazilian genebank for phenolic profiles and for antioxidant activity. In order to verify the stability of the results, the polyphenol profiles of a sample stored in a cold chamber of the Embrapa Base Collection was compared to a sample that had just been regenerated in a greenhouse. This study expected also to contribute to improve the use of this legume as a functional food crop and/or as a new ingredient in food diet.

MATERIAL AND METHODS

The first batch of beans originated from an *ex situ* germplasm collection of Embrapa that conserves seeds at -20°C for long-term purposes (the Base Collection), located at Embrapa Genetic Resources & Biotechnology (Cenargen), in Brasília, DF. The accessions conserved in this collection have been collected in different years and, most probably, under different conditions of cultivation. Fifty cultivated accessions of ***P. lunatus*** collected since the 60's and in different years were included in this batch. For each accession, 40 seeds were randomly chosen to compose the sample to be ground. The second batch of beans consisted of seeds collected from plants regenerated in a greenhouse during 2010, in Cenargen (15°46'47'' S; 47°55'47'' W). To compose this batch, 18 accessions of ***P. lunatus*** (Table 1) were selected from different groups that were formed according to a previous cluster analysis of phenolic compounds within the first batch. Forty dry seeds were ground to flour so that they passed through a 40-mesh sieve. All the chemical analyses were performed in both batches, except for the antioxidant activity and the HPLC fingerprinting of seed extracts, which were performed just on the second batch. Samples of ***Phaseolus vulgaris*** L. (black beans, "carioca" beans), ***Vigna angularis*** Willd. (adzuki beans), ***Vigna uinguiculata*** L. (cowpea) and ***Arachis hypogaea*** L. (peanut) were also analyzed for comparison.

Extraction efficiencies of different polarity solvent systems were tested using acetone-water (50:50 v/v), acetone-water-acetic acid (70:29.5:0.5 v/v), acetone-water-formic acid (70:29.5:0.5 v/v), ethanol-water (70:30 v/v) and ethanol-water (70:30 v/v pH 2.5 with formic acid). Acetone-water-acetic acid (70:29.5:0.5 v/v) presented the best efficiencies and was used as the extraction solvent. For total phenolics, total flavonoids, total condensed tannin and radical scavenging activity, 0.5g of ground dry seeds (in triplicate) was extracted with 10mL of extraction solvent, staying in an ultrasonic bath for 30min followed by 18h in the dark at room temperature. After centrifugation at 3500rpm at 10°C for 12min, the supernatant was retained for analysis and the residue was stirred in a vortex with 10mL of extraction solvent and then extracted again in an ultrasonic bath for 30min. Supernatant was added to the first extract. Acetone was vacuum evaporated at 40°C. The residue was transferred to 25mL flask and the volume was made up with ethanol. The reagent blank was made with extraction solvent prepared in the same way as the sample extracts.

The method for total phenolics was based on Folin-Ciocalteou's reagent (SINGLETON & ROSSI 1965), using a Lambda 25 Perkin Elmer® ultraviolet visible spectrophotometer. Total phenolic content was expressed as milligrams of gallic acid equivalent per g of dry bean (mg of GAE g^{-1}) through a calibration curve of gallic acid from 1-10µg mL^{-1} (r=0.999).

Table 1 - Characteristics of the ***P. lunatus*** accessions conserved in the genebank of Embrapa.

ID	Seed color	100 seed weight (g)	Collection date	Municipality/Province of collection
8394	White	69.4	29/03/1983	Floriano/Piauí
8385	Brown	72.5	25/07/1968	Viçosa/Minas Gerais
8543	Light Brown	29.5	10/06/1967	Ipanema/Minas Gerais
8594	White	147.0	15/08/1975	Bocaiuva/Minas Gerais
8637	Red	25.2	17/06/1980	Taiobeiras/Minas Gerais
8650	Brown	27.9	17/06/1980	R. Pardo de Minas/Minas Gerais
31294	Pink	31.3	-	-
31298	Brown	50.5	-	-
31299	Brown	44.7	-	-
38102	Black	64.9	23/09/1966	Salvador/Bahia
38175	Brown	32.3	20/07/1967	Rio Casca/Minas Gerais
38206	White	28.7	15/08/1975	Porteirinha/Minas Gerais
38216	White	33.3	15/08/1975	Porteirinha/Minas Gerais
38217	Brown	46.1	15/08/1975	Mortugaba/Bahia
38231	White	28.2	15/08/1975	Arcos/Minas Gerais
38243	White	41.2	15/08/1975	Mortugaba/Bahia
38244	Brown	28.1	15/08/1975	Porteirinha/Minas Gerais
38282	Brown	32.0	03/1984	Nova Venécia/Espírito Santo

ID: identification of accessions in the Base Collection.

Total flavonoids were quantified according to ZHISHEN et al. (1999). The results were expressed as milligrams of rutin equivalent per g of dry bean (mg of RUE g^{-1}) through a calibration curve of rutin from 1-100µg mL^{-1} (r=0.9999).

The analysis of total condensed tannins (procyanidins) was determined following BROADHURST & JONES' (1979). The results were expressed as milligrams of catechin equivalent per g of dry bean (mg of CAE g^{-1}) through a calibration curve of catechin between 2.5 and 30µg mL^{-1} (r=0.999).

For antioxidant activity, the free-radical scavenging capacity was evaluated with DPPH assay (MILIAUSKAS et al., 2004) slightly modified. For each 3mL of daily prepared methanolic solution of DPPH ($9x10^{-5}$M), 200µL of the bean extract was added and the mixture was allowed to stand in the dark. After 60min, the absorption was measured at 515nm. Radical scavenging activity (%) was calculated by the formula, according MILIAUSKAS et al., 2004.

For polyphenols fingerprint, defatted extracts were evaporated to dryness under vacuum and then re-dissolved in ethanol-water 80:20 (v/v) adjusted to pH 2.5 with formic acid to a final volume of 2mL. HPLC profile was carried out on a ProStar Varian® system (Mulgrave, VIC, Australia) equipped with ternary pump, autosampler and photodiode array detector, according to ROMANI et al. (2003) with modifications. The column was Phenomenex® (USA) Luna C18 (2) 3µm 150x4.6mm, using mobile phase of acetonitrile - water adjusted to pH 3.0 with formic acid (gradient starting from 5% acetonitrile to 100% acetonitrile in 27min; flow 0.8mL min^{-1}). The detection was performed between 190 and 450 nm and chromatograms were acquired at 275 and 315nm.

For morphological characteristics, seed color and seed weight had been previously evaluated in the whole lima bean genebank at Embrapa, following IPGRI (2001). For seed weight, the 100 seeds weight descriptor was applied.

All chemical analyses were performed in four replicates. Statistical analysis was performed using R 2.14.1 for Windows. First, the Manhattan matrices of generalized distance were used to measure the dissimilarity of variables in the cluster analysis of the 50 bean accessions by the method of average linkage between groups. The optimal number of groups was determined based on analysis of variance (ANOVA) at 0.05 of significance level to test the hypothesis of the differences between average vectors of each group. Correlation between different methods (multivariate analysis) was performed by principal component analysis (PCA).

RESULTS AND DISCUSSION

Wide variability was observed for polyphenols in the lima bean stored in the Embrapa genebank. The values of total phenolics evaluated in the seeds of the 50 ***P. lunatus*** accessions conserved in the cold chamber of the Embrapa Base Collection ranged from 0.11±0.02 to 9.72±0.4mg GAE g^{-1}. Seven statistically different groups were formed based on cluster analysis. Total flavonoid content in this same sample ranged from 0.2±0.1 to 17.3±3.0mg RUE g^{-1}, forming five different groups. Condensed tannin ranged from no tannin detected to 8.85mg CAE g^{-1} and formed seven significantly different groups.

For the other legumes evaluated (black beans, "carioca" beans, adzuki beans, cowpea and peanut) the values ranged from 1.47±0.04 to 8.18±0.12mg GAE g^{-1} for total phenolics, from 1.90 to 15.56mg RUE g^{-1} for flavonoids and from traces to 9.50mg of CAE g^{-1} for condensed tannin.

Genetic characteristics associated with secondary metabolite are developed and selected on the basis of biotic and abiotic factors expressed throughout plant evolution. Phenolic compounds often play important roles in plant defense and in resistance against insects and pathogen attacks (POCIECHA et al., 2009; ZUR et al., 2011), and many flavonoids protect plants from radiation damage (POCIECHA et al., 2009; NEUGART et al., 2012). Taxonomic position of plants and their specificity to biotic and abiotic environment, such as microbial (and other) symbionts are among the factors that affect the variability of polyphenols in plants (GOTTLIEB et al., 2000).

A weak negative correlation between seed size and total phenolics (-0.51) was identified. However, considering that the studied sample was not well represented with accessions of the Andean gene pool (large seeds), the present study cannot reach substantial conclusions about the relationship between polyphenols and gene pool origin.

As observed for lima beans stored in the Embrapa genebank, regenerated lima beans also showed a large diversity of phenolic compounds. Total phenolic analyzed in fresh seeds of 18 ***P. lunatus*** accessions that have just been regenerated in a greenhouse ranged from 0.06 ±0.00 and 7.61±0.09mg of GAE g^{-1} (Table 2) and formed five

Table 2 - Polyphenols and antioxidant activity in seeds of ***P. lunatus*** accessions (ID) conserved in the genebank cold chamber (GB seed) and regenerated in a greenhouse (fresh seed).

ID	Total Phenolics ----------(mg of GAE g-1) ---------		Condensed Tannins --------- (mg of CAE g^{-1}) ---------		Flavonoids ----------(mg of RUE g^{-1}) ---------		Antioxidant activity (%)
	GB seed	Fresh seed	GB seed	Fresh seed	GB seed	Fresh seed	Fresh seed
8394	0.7±0.1a	0.1±0.0b	0.6±0.0a	0.0±0.0b	0.6±0.1a	0.9±0.1b	5.6±1.1
8385	5.5±0.2a	5.1±0.2a	5.6±0.3a	5.8 ±0.3a	9.4 ±1.3a	8.81±0.7a	83.0±3.0
8543	7.4±1.2a	7.6±0.1a	9.9±1.9a	9.0±0.4a	17.3 ±3.0a	10.3 ±0.9a	94.4±1.2
8594	0.1±0.0a	0.9±0.1b	0.0±0.0a	0.0±0.0a	1.5±0.3a	0.8±0.1b	2.6±0.6
8637	8.0±0.1a	7.5±0.3b	10.3±0.4a	6.6±0.4b	19.5 ±2.5a	14.1±0.9b	92.2±2.9
8650	3.1±0.2a	6.3±0.3b	3.0±0.2a	7.7±0.4b	4.5 ±0.8a	10.5±0.6b	92.2±1.9
31294	4.1±0.1a	5.2±0.5b	4.3±0.2a	5.2±0.4b	5.2±0.2a	8.3±0.7b	84.8±2.5
31298	6.1±0.3a	5.2±0.1b	8.1±0.7a	6.0 ±0.2b	11.9 ±1.7a	8.7±0.4b	88.2±4.5
31299	5.3±0.2a	6.7±0.2b	5.6±0.4a	6.5±0.5b	9.7 ±0.6a	6.6±0.4b	81.6 ±4.9
38102	5.0±0.3a	5.1±0.3a	4.4±0.4a	4.5 ±0.3a	7.8±0.5a	8.8±0.7a	63.2±5.1
38175	6.5±0.1a	6.9±0.2b	7.0±0.8a	7.3 ±0.4a	12.9 ±0.5a	13.0±0.6a	85.7±2.9
38206	2.1±0.2a	3.6±0.3b	1.2±0.1a	2.7±0.2b	4.0±0.2a	4.5±0.3a	61.6±2.7
38216	1.3±0.1a	3.8±0.2b	0.8±0.1a	3.0±0.1b	3.1±0.4a	3.9±0.2b	61.0 ±6.5
38217	4.4±0.1a	6.5±0.2b	3.7±0.1a	6.1 ±0.3b	8.8±0.4a	10.9±0.6b	85.0±6.1
38231	0.7±0.1a	0.7±0.0a	0.0±0.0a	0.1±0.1b	1.5±0.4a	0.9±0.0b	3.8±0.6
38243	1.4±0.1a	4.3±0.2b	1.2±0.1a	3.4±0.1b	4.7±0.4a	5.7±0.5b	74.1±5.1
38244	6.1±0.2a	5.4±0.3 b	5.9±0.2a	5.4±0.4a	12.1 ±0.8a	10.7 ±0.3b	79.9±2.0
38282	9.8±0.7a	5.8±0.2b	7.0±0.7a	7.1±0.3a	11.4 ±2.1a	9.8 ±0.6a	91.9±2.3
General	4.3±2.8a	4.8±2.2a	4.1±3.1a	4.8±2.7a	7.6 ±5.0a	7.8±3.9a	68.4±30.9

For each analysis, the same letter in the line indicates no significant differences between values of the sample from the cold-storage collection and samples from fresh seeds.

groups based on the cluster analysis. The groups that presented the lowest levels of total phenolics were formed just by white seeds (groups 1 and 5). Group 2, with intermediary levels of phenolic contents (5.07-5.75mg of GAE g^{-1}), was formed by six accessions with different seed colors (brown, black, and pink). Group 4 with high total phenolic content (6.31-6.85mg of GAE g^{-1}) was formed by four accessions of brown seeds. The group with the highest level of total phenolics (7.54-7.61mg of GAE g^{-1}) was formed by two accessions (light brown and red). Like the red lima bean from the last group, red adzuki beans presented the highest total phenolics (8.18±0.12mg GAE g^{-1}) among the other legumes evaluated in our laboratory. The level of total phenolics obtained for the black lima bean accession (5.07±0.28mg GAE g^{-1}) was lower than results of XU & CHANG (2007) for black common bean (6.89±0.15mg GAE g^{-1}) and for black soybean (6.18±0.11mg GAE g^{-1}), suggesting variability between these species.

Total flavonoids analyzed in seeds from regenerated accessions ranged between 0.81±0.09 and 14.1±0.99mg of RUE g^{-1} (Table 2) and formed six groups according to the cluster analysis. Condensed tannins ranged between 0.0±0.0 and 8.97±0.39mg of CAE g^{-1} and formed three groups. As happened for total phenolics, the two groups with the smallest amounts of flavonoids (0.81-4.47mg of RUE g^{-1}) were of white seed colors and group 1 with the smallest amounts of condensed tannin (0.0-0.1mg of CAE g^{-1}) was only formed by white seeds. XU & CHANG (2007) found 1.53 to 7.53mg GAE g^{-1} for total phenolics and 1.40 to 8.78mg CAE g^{-1} for condensed tannin in 8 commonly consumed legumes. HEIMLER et al (2005) found 1.17 to 4.40mg of GAE g^{-1} for total phenolics in four landraces of common beans. These comparisons show wide variability for polyphenols (total phenolics, total flavonoids and condensed tannins) in the commonly cultivated accessions of ***P. lunatus*** conserved in the Brazilian genebank.

Changes in polyphenol contents of samples stored in the genebank were compared with samples from recent regeneration (Table 2). From the 18 accessions that were analyzed again for total phenolics after regeneration (batch 2), 22% presented no significant change, when compared to the seeds of the same accessions conserved in the genebank cold chamber (batch 1). For total flavonoids and condensed tannins, 33 and 39% of the tested accessions, respectively, presented no significant change in their contents. These results indicated that the conservation in the genebank cold chamber at -20°C does not always affect total phenolic content in ***P. lunatus*** seeds.

However, the majority of the accessions tested presented significant variation in their polyphenols between the two batches. The predominance of significant changes in the amounts of polyphenols, when comparing seeds that have been recently regenerated in the same period vs. cold stored seeds, are in accordance with the literature. POCIECHA et al. (2009), ZUR et al. (2011) and NEUGART et al. (2012) affirm that both biotic and abiotic factors present variable effects on plant polyphenols, including little or no change, significant losses or enhancement. The cultivar, the year, the harvest time and other environmental conditions are among the factors that affect the phenolic compound profile in plants.

Considering the multivariate analysis for all polyphenols evaluated (total phenolics, tannins and flavonoids), accessions that had been just regenerated showed only white accessions grouped in two clusters and all accessions of colored seeds grouped in a third cluster. The first group with white seeds presented the lowest content of polyphenols; a second group included three remaining accessions of white seeds, with medium content of phenolic compounds. All brown, pink, red and black accessions of ***P. lunatus*** were grouped in a third cluster with the highest phenolic content. These results suggest that accessions of lima beans with white seeds may be clearly discriminated by polyphenol contents, indicating that white seeds tend to have lower polyphenol contents than the colored seeds.

Fingerprint of regenerated accessions showed variations in HPLC profile of extracts, indicating polyphenol diversity of the ***P. lunatus*** collection. The maximum absorptions of phenolic compounds were smaller than 315nm, indicating the presence of phenolic acids and/or isoflavonoid derivatives, predominantly. To the best of our knowledge, no other studies report the polyphenol composition in ***P. lunatus***. However, ferulic acid was the most abundant phenolic acid present in beans of 15 varieties of ***P. vulgaris***, whereas intermediate levels of p-coumaric acid and sinapic acid were extracted from all the bean samples (LUTHRIA & PASTOR-CORRALES, 2006). Isoflavonoid derivatives have been identified mainly in soybean seeds (ROMANI et al., 2003).

Polyphenols of lima beans were strongly correlated with antioxidant activity. Radical scavenging activity (% RSA) analyzed in the seeds of the 18 regenerated accessions of ***P. lunatus*** ranged

from 2.62±0.62 to 94.4±1.24%. Cluster analysis detected two heterogeneous groups. One of these clusters had just three accessions of white seeds; these accessions showed low antioxidant activity, ranging from 2.6 to 5.6%. The other cluster had fifteen accessions of white, black, red, pink and brown seeds; this group presented high antioxidant activity, ranging from 61 to 94.4% for scavenging DPPH free radical. Natural phenolics exert their beneficial effects for health predominantly through their antioxidant activity.

Multivariate analysis of the different polyphenol contents and antioxidant activity of the regenerated seeds showed strong and positive correlation (0.96) between these variables. The first principal component (PC1) was responsible for 93.5% of the total variation and was strongly correlated to total phenolics (0.99), condensed tannins (0.98), flavonoids (0.95) and antioxidant activity (0.96). High positive correlation between polyphenols and antioxidant activity indicate the capacity that these phenolic compounds have to deactivate the free radicals or to turn them into stable compounds, and supports the beneficial health effects that these polyphenols may present. This study expected to contribute to improve the use of this legume as a functional food crop and/or as a new ingredient in food diet.

CONCLUSION

The seeds of cultivated accessions of ***P. lunatus*** conserved at Embrapa genebank presented wide diversity in polyphenols (total phenolics, flavonoids and condensed tannins). Conservation through cold storage of ***P. lunatus*** seeds for a long period of time in the cold chamber of the Base Collection did not always affect polyphenol contents of the accessions. Accessions of white seeds of lima beans were clearly discriminated by polyphenol contents. Polyphenols in the lima beans were highly and positively correlated with antioxidant activity, with possible beneficial effects for human health.

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