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# Total flavonoids, phenolics, tannins and antioxidant activity in seeds of lentil and grass pea

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## Abstract

Lentil (*Lens culinaris* Medik.) and grass pea (*Lathyrus sativus* L.) are rich in polyphernols and antioxidants and in constant use as food and nutritional supplements in many traditional diets throughout the world. Both plants reportedly possess beneficial effects in jaundice, high fever, measles, diabetes, and cardiac problem. Despite immense potentiality, limited work was done regarding antioxidant potential in different genotypes of these two legumes, useful in phytomedicinal and pharmacological formulations.

The objective of the present study was to assess the antioxidant composition and activity of ethanolic extract (ethanol: water, 80:20 v/v) of seeds (edible grain) of three improved genotypes each in lentil and grass pea. Total phnolics and flavonoids were estimated by the Folin–Ciocalteu colorimetric method and aluminium chloride method, respectively. Condensed tannin (pro anthocyanidins) content was determined using leucocyanidin (LE) equivalent. Antioxidant activity was ascertained by different *in vitro* protocols such as DPPH, $\beta$-carotene bleaching assay, reducing power and free radical scavenging activity.

Among the genotypes, IPL 406 in lentil and BioL 212 in grass pea showed the best performance with highest phenolic and flavonoid content and superior antioxidant activity as evidenced in potential FRAP, bleaching assay and by the low $IC_{50}$ values in DPPH, hydroxyl and superoxide radical scavenging assay compared to WBL 58 and B 256 in lentil and B1 and BioR 231 in grass pea. The six genotypes differed significantly ($P < 0.05$) in antioxidant content and activity.

Both legumes are potential enough as phytomedicinal and functional foods with ample scope for genotype-wise phyto-nutrient profiling.

**Keywords:** Lens culinaris, Lathyrus sativus, polyphenols, antioxidant, β-carotene bleaching assay, free radical scavenging.

## Introduction

Both lentils (*Lens culinaris* Medik.) and grass pea (*Lathyrus sativus* L.) are cool-season food legumes with a cultivation period of more than 8000 years, and have been in constant use in many societies as traditional diets throughout the world [1, 2]. Usually, lentils are canned or dry-packaged, whole or split, for retail outlet or processed into flour. They are used in soups, stews, salads, snack foods, and vegetarian dishes because of high seed protein, fiber, minerals and antioxidant capacities [3]. Flour made from lentil seed is gluten free and may be added to cereal flour to make bread, cakes, candy, and baby foods [4]. Several ethnic traditional food and pharmacological preparations are now available with lentil in temperate and sub-tropical Indian Himalayas [2]. Beyond nutritional functions, lentils have several potential health-promoting effects, such as managing blood-sugar level, reducing cholesterol and the risk of cardiovascular diseases, cancer and erectile dysfunction [5,6]. Grass pea is also rich in protein content, essential amino acids, minerals, dietery fibers, flavonoids and other polyphenols [7]. Introduction of low seed neurotoxin containing genotypes, showing bold seed size with white/light seed coat color developed through mutagenesis and somaclonal techniques have resulted in extensive use of this hardy legume crop in food preparations [8, 9, 10]. Traditional uses of these two legumes have been reported in Indian Himalayan states in treating severe jaundice, mal-nutrition, measles, and in a type of diabetes for which stress markers have recently been identified [11].It has been widely accepted that significant health-promoting effects of plant foods are related to high contents of phenolic components [12]. Polyphenols and other natural bioactive plant products may act as antioxidants which can hinder the formation of free radicals and protect cellular protein, lipid and nucleic acid from oxidative damage [12]. Among cool season food legumes like lentils and grass pea, significant correlation may exist between potent antioxidant capacities and high contents of phenolic substances [1,3]. However, existence of genetic variations in lentil and grass pea genotypes as observed in natural and induced population [13, 14, 15] necessitates screening of different genotypes to develop a complete phytochemical profiles and bioactivities of both crops. Recent work on grass pea revealed significant changes in antioxidant activities due to change

in nuclear ploidy level [16]. Thus, input of more and more information from different geographic regions is urgently required to get a comprehensive idea about quality and quantity of functional ingredients of these two extensively used legumes. Currently, very little information is available in the literature regarding antioxidant composition and activity of both lentils and grass pea [3, 9]. The cultivation and yield of these two crops are gradually increasing for diverse purposes, especially in the perspectives of food, medicinal and nutritional security of millions of people in Asia and Africa. With the current upsurge of interest about the efficiency and function of natural antioxidants in food and medicinal systems, the testing of antioxidant composition and activity of edible grain of legume crops has received huge attention [17]. The objective of the present study was, therefore, to determine and compare the antioxidant activity and content of flavonoids, phenolics and condensed tannins in seeds of lentil and grass pea which is being reported in this article.

## Material and Methods

### Plant material

Fresh, dry and healthy seeds of three improved lentil varieties (*Lens culinaris* cv. IPL 406, B 256, WBL 58) and three grass pea varieties (*Lathyrus sativus* L. cv. BioR 231, BioL 212, B1) were collected from local farmers from Kalyani agricultural farms, Kalyani, West Bengal, India and Pulses and Oilseed Research Station, Berhampore, West Bengal, India just after harvest during March, 2011. All the chemicals used were of analytical grade, purchased from Sigma-Aldrich, Bangalore, India, and Merck, Germany.

### Extract preparation

Seeds (edible grains) were ground and 5 g of samples were exhaustively extracted with 50 ml of chilled aqueous ethanol for 3 h at room temperature (ethanol: water, 80:20 v/v) after percolation with petroleum ether to remove fatty substances. Sample was then centrifuged at 3000 *g* for 25 min and the supernatant was removed. Extraction was repeated thrice and supernatants were pooled and subsequently, evaporated at 40 C. Phenolic compound was made to a final volume of 10 ml of distilled water and stored at -80 C until analyzed.

### Estimation of total phenolic content (TPC)

TPC was analysed by the Folin–Ciocalteau method using gallic acid as standard [18] with some modifications for lentil and grass pea seeds. Briefly, 1 ml of diluted samples of extract (1: 10) was oxidized with 1 ml of Folin-Ciocalteau reagent (Sigma-Aldrich, Bangalore, India), and left for 5 min incubation. The reaction was then neutralized with 2 ml of 70 g/l $Na_2CO_3$ solution (v/v). After 2 h incubation at 25 C, the resulting blue colour was measured at an absorbance of 750 nm. TPC was expressed as gallic acid equivalent (GAE) mg/g on dry weight basis (dwb).

### Determination of condensed tannins (CT)

For the extraction of tannins, 10 ml of aqueous acetone (70:30 v/v) was added to 1g of sample, and the mixture was subjected to ultrasonic treatment for 20 min at 25 C, then centrifuged for 10 min at 3000 *g* at 4 C and the supernatant was collected. After another round of extractions as described, the supernatants were pooled, evaporated at 30 C to remove solvent and stored at – 80 C until analysis. Condensed tannins (% dry matter) were estimated following earlier method [18] and were calculated as leucocyanidin (LE) equivalent by the formula: ($A_{550\ nm}$ 78.26 dilution factor) / (% dry matter). CT was expressed as mg LE/g [18].

### Estimation of total flavonoid content (TFC)

TFC was estimated spectrophotometrically using the earlier methods [18, 19] based on the formation of a flavonoid-aluminium complex with some modifications. An amount of 2% ethanolic $AlCl_3$ (aluminium chloride) solution (0.5 ml) was added to 0.5 ml of sample. After 45 min incubation at room temperature, the absorbance of the reaction mixture was measured at 420 nm. TFC was calculated by extrapolating the absorbance of reaction mixture on standard curve of catechin (CAE). The experiment was repeated four times, and the total flavonoid content was expressed as equivalent to CAE in mg/ g of the extracts.

### In vitro antioxidant activity

### DPPH radical scavenging activity

The free radical scavenging activity of seed extracts of lentils and grass pea was assayed using a stable free radical 1, 1 diphenyl-2-picryl hydrazyl (DPPH) following the protocol [20] with little modifications. A series of sample concentrations of 0.1 ml for each sample (10:90, 20:80, 30:70, 40:60 and 50:50) (extract: aqueous ethanol) was added to 2.9 ml of freshly prepared (DPPH radical dissolved in aqueous ethanol, ethanol: water, 80:20 v/v) DPPH radical solution. For the control, 0.1 ml aqueous methanol (methanol: water, 80:20 v/v) was added to 2.9 ml of DPPH radical solution. The mixture was shaken and allowed to stand in the dark at room temperature for 30 min. The absorbance of the mixture was then read at 517 nm. The radical scavenging activities of the samples were expressed in terms of $IC_{50}$ (concentration required for a 50% decrease in absorbance of DPPH radical) relative to the control (100%) and calculated as % inhibition of DPPH. DPPH radical scavenging activity (%): [$A_{517\ control}$- $A_{517sample}$] / $A_{517\ control}$ 100 where, $A_{517\ control}$ is the absorbance of the control (DPPH solution without test sample) and $A_{517}$ sample is the absorbance of the test sample (DPPH, solution plus antioxidant).

### Antioxidant activity (AOA)

β-carotene- linolate bleaching assay was carried out according to the method developed earlier [21]. Ten mg of β carotene (type I, synthetic, Sigma-Aldrich) was dissolved in 10 ml of chloroform and

its 3 ml was added to 20 µl of linoleic acid and 200 µl Tween ® 40. After removing chloroform under reduced pressure, 100 ml of oxygenated water was added slowly and mixed properly to obtain a stable emulsion. A portion (3 ml) of emulsion were added to mix with 40 µl of sample and incubated for 1 hr at 50 C. The absorbance was recorded at 0 and after 60 min of incubation at 470nm against a blank (emulsion without β-carotene). Butylated hydroxytoluene (BHT) was used as a synthetic reference.

## Ferric reducing antioxidant power (FRAP)

The FRAP assay for lentil and grass pea seed extract was done in four replications following earlier adopted methods [22]. Briefly, 100 ml of appropriately diluted samples of seed extracts was added to 3 ml of freshly prepared FRAP reagent, consisting of 300 mmol/l acetate buffer (pH 3.6), 10 mmol/l 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) in 40 mmol/l HCL and 20 mmol/l ferric chloride ($FeCl_3$. $6H_2O$). After 10 min incubation at 37 C, the absorbance was recorded at 593 nm. The absorbance changes in the test mixture were compared to those obtained from standard mixture of ferrous sulphate ($FeSO_4.7H_20$) (0.1 mmol/l - 1.0 mmol/l). The increasing concentration of $Fe^{3+}$ is expressed as mM of $Fe^{2+}$ /g.

## Hydroxyl radical scavenging activity

The deoxy ribose method was used for determining the scavenging effect on hydroxyl radicals as described earlier [23]. The reaction mixture contained ascorbic acid (50µM), $FeCl_3$ (20µM), EDTA (2µM), $H_2O_2$ (1.42mM), deoxy ribose (2.8mM), with different concentration of seed extracts in a final volume of 1ml in K-phosphate buffer (10mM, pH 7.4). It was incubated at 37 ºC for 1 h and then 1ml of 2.8 % TCA and 1ml of 1 % TBA were added. The mixture was heated in a boiling water bath for 15 min. It was cooled and absorbance was taken at 532 nm. $IC_{50}$ value (µg/ml) was calculated.

## Super oxide scavenging activity

Super oxide scavenging activity was measured by alkaline dimethyl sulfoxide (DMSO) method [24]. To the reaction mixture containing 0.1 ml of NBT (Nitroblue tetrazolium, 1 mg/ml solution in DMSO) and 0.3 ml of the extract and standard in DMSO, 1 ml of alkaline DMSO (1 ml DMSO containing, 5 mM NaOH in 0.1 ml water) was added to give a final volume of 1.4 ml and the absorbance was measured at 560 nm. $IC_{50}$ value (µg/ml) was calculated.

## Statistical analysis

Each antioxidant activity assay was done four times from the same extract in order to determine their reproducibility. Analysis of variance was used to test any difference in antioxidant composition and activities resulting from these methods. Multiple means were separated by Duncan's multiple range tests using SPSS v.10 (SPS Inc., USA) software. Correlations among data obtained were calculated using Pearson's correlation coefficient (*r*) by 'Microsoft Excel data analysis (v. 2007) tool pack' software. A level of $P < 0.05$ was considered significant.

# Results and Discussions

Natural phenolics exert their beneficial health effects mainly through their antioxidant activity. Typical phenolics that possess antioxidant activity are known to be mainly phenolic acid and flavonoids [13]. TPC contribute to the overall antioxidant activities of plant foods. Proanthocyanidins (condensed tannins) are a class of phenolic compounds widely distributed in the plant kingdom including legume seeds [18]. In the present study, genotypes differed significantly for TPC, TFC and CT contents in seeds of both pulse crops. Results in table 1 indicated that IPL 406 contained highest level of TPC, TFC and CT, followed by WBL 58 and B 256 in lentil. The results agreed well with the comparative analyses of phenolic composition, and antioxidant capacity of lentil varieties grown in the cool-temperate regions of USA where considerable genotypic differences were found in their individual phenolic compounds, as well as chemical and cellular antioxidant activities [1]. Moderate to high level of TPC and TFC was also found in methanolic extracts from leaves and pods of lentil grown in Pakistan [25]. Similar results were obtained in Iranian germplasm of lentil seeds in different solvent (hexane, methanol and acetone) extractions [26]. In the present study, ethanolic extract of seeds were used. The three lentil lines used here have been developed through inter-crossing and subsequent selection for high grain yield in India and released for cultivation in different parts of the country. Despite extensively used in daily meal, different cuisines and ethno-medicinal purposes, no reports are available regarding antioxidant composition of lentils grown in India. The present revelation, thus, assumes significance in this regard.

For grass pea, highest content of TPC and TFC was estimated in the variety BioL 212, followed by B1 and BioR 231 (Table 1). However, CT content was much higher in B1 than that in Bio L 212 and BioR231 (Table 1). The results indicated significant differences ($P < 0.05$) in TPC, TFC and CT content in Indian grass pea genotypes, exhibiting much higher TPC content than genotypes grown in Mediterranean Europe with TPC average value of 175.0±8.39 mg GAE/100 g in Italy [27]. Even the lowest average TPC value (8.34 ± 1.4 mg GAE/g) measured in the present variety BioR231 was about 2-fold higher than genotypes grown in south Spain (430 mg/100 g) [7], strongly confirming genotypic differences in TPC composition of Indian grass pea genotypes from the Mediterranean region. Variation in flavonoid content has also been explored in grass pea, and a strong connection between flavonoid composition and nitrosylation of thiol antioxidant glutathione has recently been established [19]. The variation in CT content may be manifested in diverse seed coat colour in grass pea genotypes, as also explained in lentil also [1, 28]. Presence of polyphenols was also found in seeds of Spanish grass pea genotypes [7].

The medicinal capabilities of a particular plant and plant parts are absolutely dependent on their free radical scavenging capacities [29]. All the biological molecules present in our body are at risk of being attacked by free radicals. Such damaged molecules can

impair cell functions and even lead to cell death eventually resulting in diseased states. Dietary antioxidants may have promising therapeutic potential in delaying the onset as well as in preventing the various life style and age-related diseases and related complications [29]. Measurement of antioxidant activities is one of the primary steps towards development of a phytomedicinal profiling of a particular plant. In the present study, both lentil and grass pea genotypes exhibited high antioxidant activities, although significant ($P < 0.05$) variations existed (Table 1). The DPPH $IC_{50}$ (µg/ml) value was minimum in IPL 406 (177.43 ± 1.1) of lentil and in BioL 212 (191.8 ± 1.5) of grass pea. Highest value was recorded in lentil B 256 (212.7 ± 1.3) and grass pea cv. BioR 231 (236.78 ± 1.7). Lentil cv. WBL 58 and grass pea cv. B1showed intermediate values (Table 1). DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. Antioxidant on interaction with DPPH, transfer electron or hydrogen atom to DPPH and thus neutralizing its free radical character and the degree of discoloration indicates the scavenging activity of the drug [30]. In antioxidant activity estimation, β-carotene undergoes rapid decolouration in the absence of an antioxidant because of the coupled oxidation of β-carotene and linoleic acid, while during reducing power assay the presence of reductant in extracts would result in reducing ferric to ferrous. In both β-carotene-linolate and FRAP assay, IPL 406 in lentil and BioL212 in grass pea manifested highest antioxidant activity, followed by WBL 58 and B 256 in lentil and B1 and Bio R 231 in grass pea, showing significant differences among themselves (Table 1).

**Table 1: Total phenolics (TPC, mg GAE/g, dwb), flavonoids (TFC, mg CAE/g, dwb), condensed tannins (CT, mgLE/g, dwb) and antioxidant activity, reducing power and free radical (DPPH, hydroxyl and superoxide) scavenging capacity in seeds of lentil and grass pea genotypes**

| | Lentil | | | Grass pea | | |
|---|---|---|---|---|---|---|
| **Parameters** | **IPL 406** | **B 256** | **WBL 58** | **BioL 212** | **B1** | **BioR231** |
| TPC | $25.67^{a}$ ±1.1 | $8.56^{c}$ ± 0.87 | $16.67^{b}$± 1.0 | $22.69^{a}$ ± 1.7 | $19.31^{a}$ ± 0.98 | $8.34^{c}$ ± 1.4 |
| TFC | $1.93^{a}$ ± 0.65 | $0.96^{c}$ ± 0.19 | $1.37^{b}$ ± 0.19 | $1.82^{a}$ ± 0.21 | $1.43^{b}$ ± 0.27 | $0.94^{c}$ ± 0.34 |
| CT | $3.31^{a}$ ± 1.8 | $1.49^{c}$ ± 0.97 | $2.98^{b}$ ± 1.7 | $1.67^{c}$ ± 0.10 | $3.67^{a}$ ± 1.6 | $2.45^{b}$ ± 1.1 |
| DPPH ($IC_{50}$, µg/ml) | $177.43^{c}$ ± 1.1 | $212.71^{a}$ ± 1.3 | $191.41^{b}$± 2.1 | $191.83^{b}$ ± 1.5 | $203.62^{a}$ ± 2.0 | $236.78^{a}$ ± 1.7 |
| β-carotene-linolate bleaching assay (AOA, %), BHT-56.34 ± 1.2 | $80.34^{a}$ ± 1.3 | $56.67^{c}$ ± 1.1 | $71.56^{b}$ ± 1.5 | $87.35^{a}$ ± 1.4 | $76.87^{b}$ ± 2.3 | $66.78^{c}$ ± 1.9 |
| FRAP (mM Fe2+/g) | $103.6^{a}$ ± 1.4 | $67.89^{c}$ ± 1.2 | $97.67^{b}$ ± 1.5 | $110.23^{a}$ ± 1.6 | $93.56^{b}$ ± 1.1 | $67.31^{c}$ ± 1.3 |
| Hydroxyl radical scavenging assay ($IC_{50}$, µg/ml) | $112.23^{a}$ ± 3.9 | $285.71^{c}$ ± 3.8 | $198.34^{b}$ ± 4.0 | $160.62^{a}$ ± 2.8 | $191.83^{b}$ ± 2.8 | $278.23^{d}$ ± 3.1 |
| Superoxide radical scavenging assay ($IC_{50}$, µg/ml) | $137.67^{a}$ ± 2.6 | $365.38^{c}$ ± 3.1 | $197.54^{b}$ ± 2.9 | $122.45^{a}$ ± 3.1 | $190.32^{b}$ ± 1.8 | $234.33^{c}$ ± 2.2 |

Data are means ± SE of at least four independent experiments; means followed by same lowercase superscript letters are not significantly different at $P < 0.05$ level by DMRT test

Hydroxyl and superoxides are predominant free radicals, continuously generated as the fall out of aerobic life cycle. The presence of transition metal ions in a biological system could catalyse
the Haber-Weiss and Fenton type reactions, leading to the generation of hydroxyl radicals. However, antioxidants could form chelates with the transition metal ions, resulting in the suppression of hydroxyl generation and inhibition of peroxidation processes of biological molecules. Among the six genotypes, lowest $IC_{50}$ value for hydroxyl radical (112.23) was recorded in lentil cv. IPL 406, and was closely followed by grass pea cv. BioL 212 (191.83) and lentil cv. WBL 58 (198.34)(Table 1). Highest value (285.71) was measured in lentil cv. B256. The results indicated high metal ion scavenging activity of the ethanolic leaf extract in the present study which is probably mediated through formation of sigma bonds by chelating agents, with the metal and was effective as secondary antioxidants [31]. Superoxides are produced from molecular oxygen due to oxidative enzymes of body as well as via non-enzymatic reaction. In the present assay protocol, super oxide free radical was formed by alkaline DMSO which reacts with NBT to produce coloured diformazan. The results (Table 1) showed that both lentil and grass pea genotypes are efficient scavenger of both

hydroxyl and superoxide radicals, indicating the potency of legume seed extract against free radical scavenging activity in the lentil and grass pea system. Lentil cv IPL 406 and grass pea cv. BioL 212 were the most efficient in radical scavenging activity, followed by other genotypes as was evidenced by $IC_{50}$ values. The results confirmed the efficacy of legume seed extracts in antioxidant activity for the first time in lentil and grass pea, although antioxidant composition and free radical scavenging activity of leaves and pod extracts of chickpea, pea, beans and lentils have been reported [25]. The apparent mechanism of scavenging the superoxide anions may be due to the occurrence of phenolic compounds that are present in the extracts and their uptake of generated superoxide in *in vitro* reaction mixture [25]. The differences between genotypes and also between two legumes as observed in the present study might be due to variation in TPC and TFC content and concomitant change in antioxidant capacities, although involvement of other small molecular weight antioxidants such as ascorbate and glutathione and enzymatic defense compounds cannot be ruled out [32, 33].

The results of the different antioxidant assays used in the present study were compared and correlated ($n$= 10) with TPC and also with each other. TPC showed significant ($P$ < 0.05) correlation with most of the antioxidant assays, such as FRAP (r = 0.818), β-carotene-linoleic acid test (r = 0.695), hydroxyl radical scavenging assay (r = 0.792) and superoxide radical scavenging activity (r = 0.784), although correlation between TPC and DPPH assay was less strong (r= 0.289). Good linear correlations between antioxidant activity tests and TPC were also reported in other preparations [1, 34], whereas weak correlation between TPC and DPPH was found in American lentil genotypes [1] and in beans [18]. There was also good relation among different antioxidant assays. DPPH radical scavenging assay showed close relation with superoxide anion scavenging activity (r = 0.951). This may be due to the reason that many other compounds such as carotenoids, thiol components, tocopherol and vitamin C other than total phenols and total flavonoids also contribute to antioxidant activity [32, 34]. Good correlation (r = 0.885) was also found between FRAP and β-carotene-linolate test in both legumes in the present study.

## Conclusion

The present investigation revealed that seed extracts of both lentil and grass pea genotypes have high phenolic, flavonoid and condensed tannin (proanthocyanidin) content with comsiderable level of antioxidant and free radical scavenging activity. However, in both legumes, genotypes differed significantly in antioxidant composition and activity, for which a comparative profiling for the first time is revealed in the present study. This variability in phenolic and flavonoid content among different genotypes could be useful for breeders and farmers to select high-phenolic cultivars to plant. The food industry may prefer lentils and grass pea with high antioxidant composition as essential source of phytonutrients which can be used as ingredients for manufacturing functional foods or nutraceuticals for promoting consumer health.

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