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In-vitro Antioxidant Activity and Total Phenolic content in Methanolic extracts of locally Grown Red Lentil (*lens culinaris* l.).

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ABSTRACT

Pulse crops, rich in flavonoids and polyphenolics possess not only free radical scavenging properties but also they are highly safe in the treatment of many diseases. Quantitative determination of phenols and flavonoids in seed coat, dehusked raw dal, dehusked cooked dal and whole seed of locally grown red lentil (*Lens culinaris* L.) was carried out using spectrophotometric methods. The amount of total phenols were analysed by using Folin-Ciocalteau assay and the amount of total flavonoids were analysed using aluminium chloride calorimetric assay. Gallic acid and catechin reagents were used as the standards For the calibration of phenols and flavonoids respectively. Hydrophillic and hydrophobic phenols were also estimated to confirm total phenolic composition. Orthodihydric phenols were analysed by Arnow's method. Free radical scavenging efficiencies and antioxidant activities were analysed by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay and ferric thiocyanate (FTC) assay respectively. Among the four treatments, seed coat extract showed highest scavenging activity followed by whole seed, dehusked raw dal and lowest found in dehusked cooked dal extracts in terms of their EC_{50} values. The results from analysis of variance (ANOVA) revealed that total phenolic content (TPC), total flavonoid content (TFC) and antioxidant properties varied among the different methanolic extracts significantly (p<0.05). Moreover, a highly significant correlation (p<0.05) between antioxidant activities/free radical scavenging efficiencies and phenolic contents as well as favonoids was observed. The results concluded that the lentil seed extracts may be valuable natural antioxidant sources and are potentially applicable in both medicine and healthy food industry.

Keywords: Lentil, antioxidant, flavonoids, phenolics, free radical scavenging activity.

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INTRODUCTION

Free radicals have been claimed to play an important role in affecting human health by causing chronic diseases such as cancer, cardiovascular diseases, immune system decline and Alzheimer's disease[1,2,3]. Free radicals are produced as by products of metabolic process in our body system. Level and effect of these free radicals are controlled by the antioxidants. Antioxidants are those necessary species which possess the capacity of protecting organisms from oxidative damage caused by free radical. Exogenous intake of antioxidants can help the body scavenge free radical effect effectively. Currently available synthetic antioxidants like butylated hydroxy anisole (BHA), butylated hydroxy toluene (BHT) and tertiary butylated hydroquinone and gallic acid esters have been suspected to cause negative health effects[4].

Figure 1: Lentil plant

Furthermore, these synthetic antioxidants also show low solubility and moderate antioxidant activity[5]. Hence, current research was directed towards finding naturally occurring antioxidant particularly of plant origin. Phenolic compounds are bioactive phytoconstituents having an important role in control and prevention of tissue damage by activated oxygen species. Flavonoids are a group of polyphenolic compounds with known properties like free radical scavenging, inhibition of hydrolytic and oxidative enzymes and anti-inflammatory actions. Till date, a number of plant species have been investigated in search of novel antioxidants but generally there was still a demand to find more information concerning the antioxidant potential of plant species. Legumes, the staple food in many regions of the world recently have been studied for their antioxidant properties. Lentils are one of the oldest crops cultivated by humans. The major lentil producing countries are India, Canada, Turkey, The United States, Australia, China and Iran. Beyond their nutritional functions, lentils have several potential health promoting effects. A variety of lentils exist with seed coat colours that range from yellow to red orange to green, brown and black,

whereas the cotyledon is yellow, red or green. On the other hand, phenolic compounds can be divided into many sub-classes such as flavanol-3-ols, flavonols, isoflavones, lignins, procyanidins and anthocyanins. This diversity of phenotype in lentil varieties indicates that complex phytochemical profiles may exists between the varieties. Among lentils, significant correlations have been found between potent antioxidant capacities and high contents of phenolic substances[6]. However, some information is available in the literature regarding phenolic compounds in lentils grown in Canada and Poland. But, there is little chemical information about lentil grown in India, a land of diversity. We do not know enough about the quality and quantity of health promoting components in Indian lentils. Hence, present study was undertaken to analyse polyphenolic components in local variety of lentil grown in northern India and effect of cooking and dehulling on its antioxidant potential.

MATERIAL AND METHOD

Plant material

A bulk of healthy seeds of lentil var. Sapna (LH-84-8) having reddish brown seed coat and red cotyledon was procured from Pulses section, Department of Genetics & Plant Breeding, CCS Haryana Agricultural University, Hisar (India). These seeds were sorted manually, to remove stones, damaged and immature seeds. After sorting, major portion of these were dehusked to obtain seed coat and dehusked seed/dal. Dehusked dal was divided into two parts. The first part was kept for extraction and second part was cooked in distilled water in ratio of 1:10 (w/v) at 100°C, dried at 55°C. Thus, we obtained four samples of lentil, 80 gm each for further analysis.

Chemicals

The commercially available chemicals from Sigma-Aldrich, Qualigens, Merk and Ranbaxy, of high purity were used for various experimental procedures.

Preparation of extracts

All the four samples were ground in an electric grinder and converted into fine powdered form. The powdered samples were then extracted separately with methanol by refluxing for six hours. Extracts were filtered and filterates were concentrated under reduced pressure on rotatory evaporator at 40°C. These concentrated filterates were stored at 5°C for further analysis.

Determination of total phenolic content

The total phenolics were determined by the Folin-Ciocalteu reagent method using gallic acid as standard[7]. Appropriately diluted extracts (1ml) were added to a 50 ml volumetric flask filled with 1.0 ml of Folin–ciocalteau reagent (diluted to 1:2 ratio) and 2.0 ml of Na_2CO_3 (20% w/v) were

mixed and the volume was made to 50 ml. The mixture was allowed to stand for 50 min. and centrifuged at 6000 rpm for 10 minutes. The absorbance of supernatant solution was measured at 730 nm using Shimadzu UV-Vis spectrophotometer (UV-2600) and against a blank prepared similarly with the same solvent but omitting the extract. The results were expressed as equivalent to milligrams of gallic acid per gram of extract (mg GAE/g).

Determination of content of hydrophilic and hydrophobic phenolics

The 50 ml of crude extracts of each of four samples were fractionated into its hydrophilic and hydrophobic components by mixing with 100 ml of deionized water and 100 ml of n-butanol in separating funnel by using Wettasinghe & Shahidi method[8]. The mixture was allowed to stand till layers got separated. The separated layers were concentrated using rotavapor set at 40°C. Weight of each fraction was recorded and the content of phenolics in each fraction was determined as per Shahidi and Naczk method[7].

Determination of Ortho-dihydric phenols

The estimation of ortho - dihydric phenols in methanol extracts was done by Arnow's method using catechol as standard[9]. To 0.4 ml of each test sample 1 ml 0.5N HCl, 1 ml Arnow's reagent and 2ml 1N NaOH was added and final volume was made to 10 ml using distilled water. Arnow's reagent was made by dissolving 10g $NaNO_2$ and 10g $NaMoO_2$ in 100 ml of distilled water. Prepared a blank sample without adding the extract. The absorbance for pink colour was measured at 515 nm. The final results were expressed as equivalent to milligrams of catechol per gram of extract (mg COE/g).

Determination of flavonoids

The aluminium chloride colorimetric assay, as described by Zhishen et al. was used[10]. Briefly, 1 ml of diluted extracts was added to test tubes containing 4 ml of double distilled water. After five minutes, 0.3 ml 5% $NaNO_2$ was added to the test tubes, followed by 0.3 ml 10% $AlCl_3$. Immediately, 2 ml 1M NaOH was added and the total volume was made upto 10ml with double distilled water. The solution was mixed thoroughly and the absorbance was measured at 510 nm. Total flavonoids in testing samples were expressed as mg catechin equivalent per gram of the extract (mg CAE/g).

2, 2'-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay

The antioxidant activity of the extracts was determined by DPPH free radical scavenging method. The antioxidant effect of extracts on DPPH free radical was estimated as per method of Hatano *et al*[11]. Solutions of different concentrations 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0 mg of each extract, were added to methanolic solution of DPPH (0.025 g L^{-1}, 2 ml). The mixture was shaken

and the absorbance of the resulting solution was measured by using Shimadzu UV- Vis spectrophotometer (UV-2600) at 1,5,10 min. interval at 515 nm until the reaction reached at its plateau gradually. For each sample separate determinations were carried out. Similarly, a control sample was also prepared. The antioxidant activity was expressed as the percentage of decline of the absorbance after 70 min, relative to the control, corresponding to the percentage of DPPH scavenged. The percentage of DPPH, which was scavenged (% $DPPH_{sc}$) was calculated using:

$$\% DPPH_{sc} = \{(A_{cont} - A_{samp})/A_{cont}\} \times 100$$

Where A_{cont} is the absorbance of control and A_{samp} is the absorbance of sample.

Ferric thiocyanate (FTC) method

The FTC method of Kikuzaki and Nakatani was used to evaluate the antioxidant activity of the extract[12]. Reagents prepared for this method were 2.51% (w/v) linoleic acid in ethanol, 30% (w/v) ammonium thiocyanate, 0.02 mol/L ferrous chloride in 3.5% (v/v) hydrochloric acid, 75% ethanol and 0.2 M phosphate buffer, pH 7.0 (39.0 ml of 0.2 M solution of monobasic sodium phosphate and 61.0 ml 0.2 M solution of dibasic sodium phosphate were mixed, diluted to a total of 200 ml). A linoleic acid emulsion was prepared by mixing linoleic acid (0.28 g), Tween 20 (0.28 g) and phosphate buffer (50 ml, 0.2 M, pH 7.0). Different test samples of conc. 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0 mg of each extract, were mixed with 5 ml of linoleic acid emulsion and final volume made to 10 ml with phosphate buffer (0.2 M, pH 7.0) and incubated at 37°C for 96 hours (4 days). The mixture prepared as above without the test sample served as control. Aliquots (0.1 ml) were drawn from the incubation mixture at intervals of 24 hour and mixed with 0.1 ml of 30% ammonium thiocyanate, 0.1 ml of 20 mM ferrous chloride in 3.5% HCl and final volume made to 10 ml with 75% ethanol and allowed to stand at room temperature for 3 minutes. The colour developed was measured at 500 nm in a spectrophotometer. This method depends on peroxide formation in the aqueous emulsion of linoleic acid. In this method, the higher the absorbance increase is, the higher the concentration of peroxide formed and hence, the lower the antioxidant activity of the sample tested. Antioxidant activity was expressed as:

Antioxidant activity (%) = {1- (increase in abs. of sample/ increase in abs. of control)} x 100

Statistical analysis

Four replicates of each sample were used for statistical analysis and resulting values are expressed as mean± S.D. One way analysis of variance (ANOVA) and F-test were carried out to asses for any significant differences between the means ($p<0.05$). Correlation analyses of antioxidant activity, flavonoids and total phenolic content were carried out using Pearson correlation programme in Online Statistical Analysis (OPSTAT www.hau.ernet.in).

RESULTS AND DISCUSSION

Figure 2, 3 and 4 present the calibration plot for determination of phenols, flavonoids and orthodihydric phenols respectively. Phenolic compounds are class of antioxidant agents which act as free radical terminators and their bio-activities are related to their abilities to chelate metals, inhibit lypoxygenase and scavenge free radicals[13].

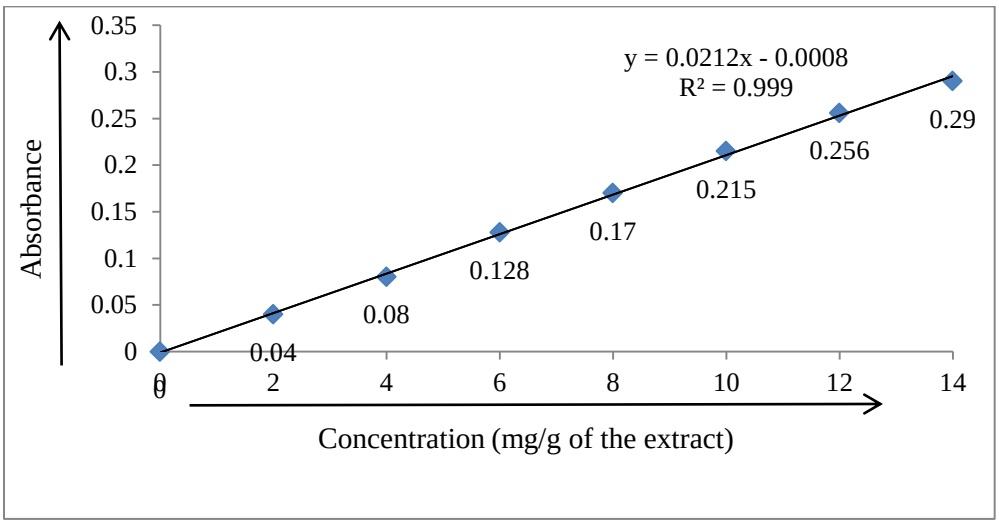

Figure 2: Gallic acid Calibration plot for total phenols

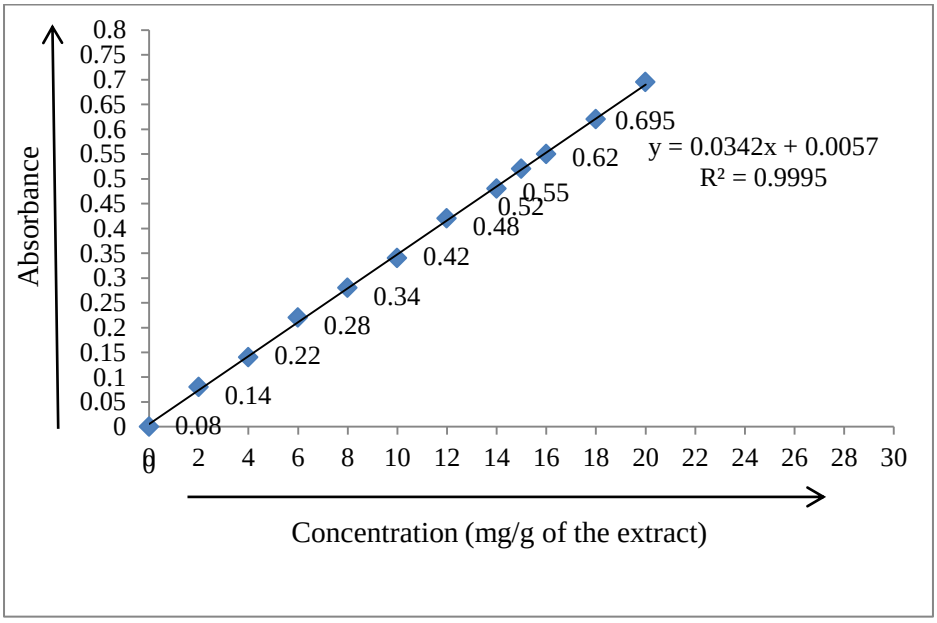

Figure 3: Catechin calibration plot for flavonoids

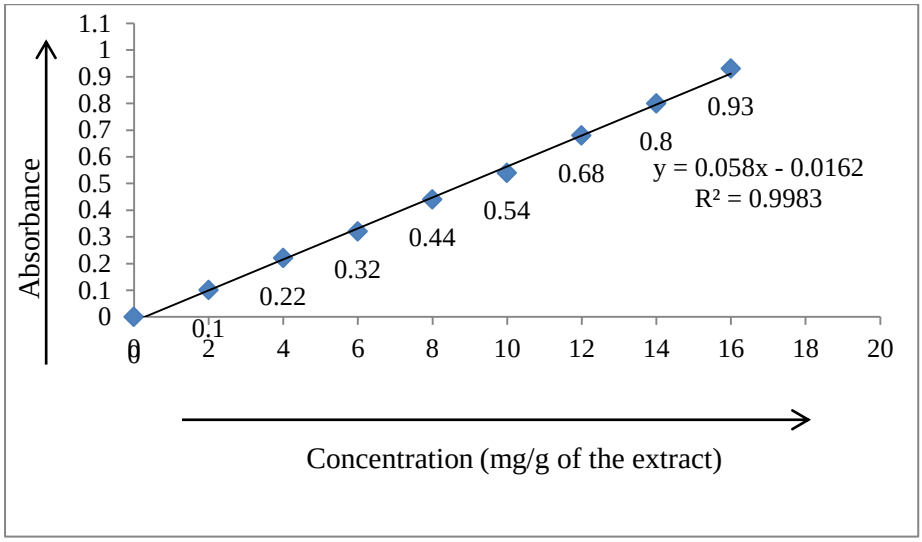

Figure 4: Catechol calibration plot for ortho-dihydric phenols

In present study total phenols were expressed as mg of gallic acid equivalents/gm of extract using the standard curve equation: y= 0.0212x-0.0008, R^2 =0.999 where y is absorbance at 760 nm and x is total phenolic content in mg/gm of the test sample. Table 1 summarises total phenolic contents and flavonoid contents of seed coat, dehusked seed, cooked dehusked seed and whole seed extracts of lentil. All values are obtained by average of four replicates ± standard deviation. The present study revealed the phenolic content's variation between 95.75 to 12.02 mg gallic acid equivalent (GAE)/g of the extract. Phenolics analysed in different extracts of lentil exhibited the following order: seed coat > whole seed > dehusked seed > cooked dehusked seed. Methanolic extract of seed coat contain 95.75 ± 4.5 mg GAE/g extract while whole seed contained 32.65 ± 0.62 mg GAE /g extract. According to Amarowicz *et al*, whole seed of lentil extracted in 80% acetone possessed 58 ± 2mg/g of the extract[14]. Here substantial variability is influenced by many complex factors which includes phenotype, crop location, weather conditions and environmental stress as well as post harvest environments. Further, total hydrophilic phenols and hydrophobic phenols were also calculated to confirm the results analysed for total phenolics. The ratio of hydrophilic: hydrophobic phenol for seed coat, dehusked seed, cooked dehusked seed and whole seed is 1.1, 1.09, 0.71, 1.6 respectively. In lentil, as o-dihydric phenolic composition was considered, the variation in all the four extracts followed same pattern as in total polyphenolic composition. In present study, o-dihydric phenols were analysed by Arnow's method using a standard calibration plot of catechol (y= 0.058x-0.0162, R^2 = 0.9983) in terms of catechol equivalent (COE)/g of the extract. Flavonoids as one of the most diverse and wide spread group of natural compounds are probably the most important natural phenols. Using the standard plot of catechin (y=0.0342+0.0057, R^2 = 0.9995), the flavonoid contents of these extracts are found varying from

31.9 to 6.04 mg catechin equivalent (CAE)/g of the extract (p<0.05). Highest flavonoid contents were possessed by seed coat replicates i.e. 31.94 ± 2.1 mg CAE/g extract. As steps like dehulling and cooking were being carried out the flavonoid's concentration went on decreasing. Hence, least concentration was contained in cooked dehusked seed (6.04 ± 0.6 mg CAE/g).

Many evidences suggest that biological actions of phenolic compounds are related to their antioxidant activity. Antioxidants are basically radical scavengers. Here, free radical scavenging efficiencies of lentil extracts were determined by using 2, 2'-diphenyl-1-picrylhydrazyl (DPPH) radical which is one of the stable and commercially available nitrogen radical. When an electron donating antioxidant is added to DPPH, decolouration of DPPH solution takes place whose absorbance is measured at 517 nm. More is the decolouration efficiency, more is the antioxidant activity of that test sample. Antioxidant activities of lentil extracts in methanol are shown in table 2 in terms of their EC_{50} values. Analysis of variance of means (p<0.05) showed that there was significant statistical difference between all the extracts. Lowest EC_{50} values were reported in seed coat extract i.e. 0.16 ± 0.02 mg/ml of the extract. Hence, order for free radical scavenging efficiency of different extract follows: seed coat > whole seed > dehusked seed > cooked dehusked seed. The same order of antioxidant property was further confirmed by ferric thiocynate assay which measured the amount of peroxide produced during initital stages of lipid oxidation.

Table 1: Phenolic composition of lentil seed extracts

Parameters	Seed Coat	Dehusked Dal	Cooked Dal	Whole Seed
Yield (%) of methanolic extracts	5.6±0.14	5.33±0.22	2.34±0.08	4.9±0.15
Total phenolics (mgGAE/g)	95.75± 4.5	23.61±2.9	12.02±0.71	32.65±0.62
Hydrophilic phenols (mgGAE/g)	50.94±1.3	12.15±0.26	5.3±0.56	20.54±1.19
Hydrophobic phenols (mgGAE/g)	44.47±0.68	11.17±0.29	7.4±0.57	12.28±0.55
Ortho-dihyric phenols (mgCOE/g)	41.63±1.9	8.59±0.19	5.8±0.32	14.45±1.09
Flavonoids (mgCAE/g)	31.94±2.1	12.04±1.5	6.04±0.67	17.62±0.77

Values are mean of four replicates ± standard deviation and means are different from each other by F-test (p<0.05)

Table 2: Antioxidant activity (EC_{50} values) in Lentil seed extracts

Test sample	Anti-oxidant activity by DPPH (mg/ml of the extract)	Antioxidant activity by FTC (mg/ml of the extract)
Seed Coat	0.16±0.22	0.43±0.03
Dehusked Dal	0.28±0.03	0.56±0.03
Dehusked Cooked Dal	0.45±0.02	0.65±0.03
Whole Seed	0.37±0.017	0.48±0.02
Butylated hydroxytoulene (BHT)	0.41±0.01	0.63±0.02
Butylated hydroxyanisole (BHA)	0.60±0.03	0.70±0.01

Values are mean of four replicates ± standard deviation and means are different from each other by F-test ($p<0.05$)

More the peroxide formation takes place, higher is the absorbance measured at 500 nm and consequently lowers the antioxidant activity of the test sample. In both the experimental techniques for measuring EC_{50} values BHT and BHA were used as standards.

The antioxidant properties of polyphenols and flavonoids are due to several different mechanisms such as scavenging of free radicals, chelation of metal ions such as iron and copper. Depending on the structure, flavonoids are able to scavenge practically all known reactive oxygen species (ROS) including superoxide anions, hydrogen peroxide, hydroxyl radicals, singlet oxygen, alkoxy, aroxy and peroxyl radicals as well as alkyl, aryl and nitrogen derived radicals[15]. Antioxidant activity may also come from the presence of other antioxidants secondary metabolite but correlation matrices of total phenol, flavonoids and their antioxidant activities were highly significant ($p < 0.05$). Therefore, it may be suggested that natural mixtures of phenolic compounds in lentil extracts are contributing significantly to their antioxidant properties.

CONCLUSION

The methanolic extracts of lentil (raw as well as processed), showed high amount of total phenolic and flavonoid contents. As per result of this study,whole seed of red lentil grown in Haryana exhibit high antioxidant property as compared to cooked dehusked dal. Hence, it is advisable to consume dal with seed coat for better antioxidant defense system.These phenolic compounds are known to antioxidants which are reported to have potent protective role in many human ailments including organ protecting properties. Hence, it can be concluded that Indian lentils may be potential source of antioxidant principles and can be used in nutraceutical industries.

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