Chemical Science Review and Letters ISSN 2278-6783

Research Article

Alterations in antioxidant activity and free radical scavenging capacity in seeds of lentil (Lens culinaris Medik.) genotypes under arsenate exposure

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Abstract

Arsenic (As) is a wide-spread carcinogenic and toxic metalloid. Seeds of lentil (*Lens culinaris* Medik.) genotypes are rich in proteins, antioxidants (flavonoids, phenolics) and free radical-scavenging capacity. Despite grown in As-contaminated soil in vast areas of south and south-east Asia and sensitivity of lentil genotypes to As-induced oxidative stress, effects of As exposure to seed nutritional composition in this important food legume is still unknown. Present study was endeavored to investigate effects of arsenate (As V) on antioxidant and free radical scavenging activity on seeds of three lentil genotypes in laboratory conditions. Plants were grown in pot soil spiked with 100 mg As kg^{-1}, added as Sodium arsenate, in a completely randomized block design till maturity. As exposure significantly reduced antioxidant activity and free radical scavenging capacity of IPL406 and WBL58 seeds but alteration was not significant in case of B256 genotype. Total phenolics and flavonoid contents, potential FRAP test, DPPH free radical scavenging capacity, and hydroxyl and superoxide scavenging capacity was decreased in As-treated IPL406 and WBL58 seeds compared to control. Total seed protein content, however, did not show any significant alterations in As-treated three genotypes in relation to control. The results suggested genotypic differences of lentil varieties in seed phytochemical constituents under As exposure and seeds of B256 was found superior in antioxidant and free radical scavenging capacity. Obviously, a focused germplasm strategy is required to identify genotype/s of lentil which is relatively safe to consume in As-contaminated regions.

Keywords: *Arsenic, antioxidant activity, free radical scavenging, lentil seeds*.

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Introduction

Lentil (*Lens culinaris* Medik.), being one of the first crops to be domesticated and cultivated by man, have been and continue to be an important food source for over 8000 years [1]. The high protein content of lentil has made them a nutritious substitute for meat. In fact, 100 grams of lentil has as much protein as 130 grams of meat in addition to beneficial dietary fiber [2]. The low phytate content of lentil makes micronutrients like iron and zinc easily available to human nutrition. Lentil and lentil-based food items are excellent to lower rates of cardiovascular disease, angina and type-II diabetes and hypertension. The balance of about ¼ protein and ¾ carbohydrate and fiber is ideal for regulating blood sugar–a low glycemic index [3, 4]. Thus, lentil has become more important to the diets of people in the Middle East, West Asia, parts of Europe and South Asia where they are placed on the table in some forms for nearly every meal [3-9].

Arsenic (As) is a wide-spread toxic metalloid. Groundwater contamination by As and its entry into crops through water-soil-plant system has caused great environmental concern. The bioaccumulation of As in crop plants has huge negative impact for public health issues [10], and this is of great environmental concern because As is known to be a carcinogen and a powerful co-mutagen [11, 12]. Alarmingly, as is gradually entering into pulse food system through its accumulation in major legumes like chickpea, pea, lentil, beans and grasspea [13-18]. Negative impact of as on nodulation and nitrogen-fixation capacity has been reported in Indian genotypes of black gram (*Vigna mungo*) [19]. As can induce severe oxidative stress in legume plants through alteration in cellular and metabolic system, severely jeopardizing plant growth and inflicting damage to yield and nutritional quality of grain, and may also render the

crops vulnerable to other biotic and abiotic stresses [20-26]. In India, groundwater contamination by As and toxicity of As-laced vegetables, cereals and pulses have been reported in Gangetic West Bengal and some parts of neighboring state of Bihar. New As-contaminated zones have been reported in central-north Indian plain [27]. Lentil has been grown extensively as a dependable cropper in these regions, either as monocrop, relay cropping with rice or mixed with other winter crops. More than 85% of the annual global production of lentils occurs in four specific regions in which eastern half of the Indo-Gangetic plain of south Asia including India, Nepal, and Bangladesh occupies the major (32%) share [28]. Among the lentil growing areas in South Asia, lower Indo-Gangetic plains comprising Gangetic South Bengal (India) and Bangladesh occupies major share in production and consumption of the crop, where vast areas of cultivated lands are irrigated, and water as well as soil are heavily contaminated with As [23, 29]. Lentil in this region is grown in the dry season; therefore irrigation is needed for successful cultivation [10]. The people of eastern part of India and Bangladesh irrigate their crops with groundwater, the major source of soil contamination by As [30, 31]. In the irrigated areas of these regions, soil As concentrations often can reach up to 83 mg As kg^{-1} [32]. Recent report revealed that lentil is sensitive to As and that higher concentrations of As in irrigation water produced As contents of this crop higher than the recommended values for forage [32]. As concentration in lentils has been found to be higher than in beans, most vegetables and some rice samples[33]. Transport and bioaccumulation of As in lentil genotypes grown in As-contaminated soil of Gangetic west Bengal, India, has recently been reported and thus danger of severe As-toxicity in this densely-populated region through daily meal of lentil grain is looming large [23, 34]. Use of As-contaminated lentil-based food items may, thus, pose great threat to huge human population inhabiting in contaminated areas. Despite immense importance of lentil crop as food and fodder in As-affected areas, phytonutritional quality of lentil seeds under As exposure has not been studied. This paucity of knowledge may greatly aggravate the food and nutritional security of millions of people and escalate mal-nutrition in the form of 'hidden hunger' in As-contaminated regions. A detail investigation is thus required to decipher the alterations in antioxidant activity and free radical scavenging capacity in seeds of lentil genotypes under arsenate exposure.

Experimental

Materials and Reagents

Plant material

Fresh, dry and healthy seeds of three improved lentil varieties (*Lens culinaris* cv. IPL 406, B 256, WBL 58) 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 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 flavonoid content (TFC) & total phenolic content (TPC)

TFC was estimated spectrophotometrically using the earlier methods [35] based on the formation of a flavonoid-aluminium complex with some modifications. 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. TPC was analysed by the Folin-Ciocalteau method using gallic acid as standard with some modifications for lentil [35]. In brief, 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)[35]. Soluble seed protein content was measured following earlier methods [35]

In vitro antioxidant activity

DPPH radical scavenging activity

The free radical scavenging activity of seed extracts of lentils was assayed using a stable free radical 1, 1 diphenyl-2-picryl hydrazyl (DPPH). 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).

Hydroxyl radical scavenging activity

The deoxy ribose method was used for determining the scavenging effect on hydroxyl radicals as described earlier [36]. The reaction mixture contained $FeCl_3$ (20μM), EDTA (2μM), ascorbic acid (50μM), 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 (10 mM, pH 7.4). It was incubated at 37 °C 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 [37]. 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.

Ferric reducing antioxidant power (FRAP)

The FRAP assay for lentil seed extract was done in four replications [38]. 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 (FeSO4.7H20) (0.1 mmol/l - 1.0 mmol/l). The increasing concentration of Fe^{3+} is expressed as mM of Fe^{2+} /g [38].

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

The three lentil genotypes differed significantly ($P < 0.05$) in their seed antioxidant and free radical scavenging capacity under untreated control and 100 mg As Kg^{-1}. Total phenolic content (TPC) and total flavonoid content (TFC) were significantly higher in IPL406 than those in WBL58 and B256 (**Table 1**). The result is consistent with earlier reports on phytonutrient compositions of seeds of these three genotypes [35] and signifies the true breeding nature of

the genotypes for seed antoxidant compositions. However, seeds obtained from the genotypes grown in soil spiked with sodium arsenate exhibited altered trend. Both TPC and TFC values reduced significantly in IPL 406 and WBL 58 but did not change significantly ($P > 0.05$) in case of B 256 (Table 1). It is noteworthy here that both phenolic acids and flavonoids constitute natural phenolics which greatly contribute to antioxidant values of plant based foods [39, 40]. The genotypic differences which are observed in the present case agreed well with reports regarding antioxidant activity and phenolic compounds of lentils grown in USA, Pakistan and Iran [35, 41-43].

Table 1 Alterations in total phenolics (TPC, mg GAE/g, dwb), flavonoids (TFC, mg CAE/g, dwb), antioxidant activity, FRAP, and free radical (DPPH, hydroxyl and superoxide) scavenging capacity in seeds of three lentil genotypes under untreated control (0 mg As Kg^{-1}) and treated (100 mg As Kg^{-1}) conditions in pot experiment. Data are means ± SE of at least three replicates. Different lowercase letters indicates significant differences ($P < 0.05$) among genotypes for seed nutritional traits studied.

Traits studied	Untreated genotypes			Treated genotypes		
	IPL406	WBL58	B256	IPL406	WBL58	B256
TPC	25.71±1.2a	16.70 ± 1.1b	8.53 ± 0.98c	12.56 ± 1.0b	10.17 ± 0.9b	8.48 ± 0.79c
TFC	1.95 ± 0.59a	1.41 ± 0.40b	0.94 ± 0.20c	1.03 ± 0.28c	0.97 ± 0.29c	0.90 ± 0.18c
Total seed protein %	24.45 ± 2.8a	23.89 ± 2.7a	22.88 ± 2.5a	24.12 ± 2.6a	23.39 ± 2.5a	22.23 ± 2.3a
DPPH (IC_{50}, µg/ml)	175.59 ± 2.4d	189.88 ± 2.7c	219.76 ± 3.9a	202.45 ± 2.6b	211.63 ± 3.3a	220.51± 4.0a
FRAP (mM Fe^{2+}/g)	105.3± 1.9a	99.76 ± 1.7a	67.91 ± 0.7c	77.66 ± 0.6b	56.89 ± 0.5d	65.82 ± 0.7 c
Hydroxyl radical scavenging assay (IC_{50}, µg/ml)	113.31± 4.1e	175.65 ± 5.8d	290.76± 7.5a	189.72± 6.2c	212.67± 6.8b	292.41±7.6a
Superoxide radical scavenging assay (IC_{50}, µg/ml)	135.55 ± 3.3e	195.34 ± 4.6c	366.71 ± 8.8a	187.45± 4.1d	229.87± 5.9b	369.56 ± 8.5a

Measurement of antioxidant activity and free radical scavenging capacity are two important criteria to assess therapeutic values of edible parts of plants. In the present case, The DPPH IC_{50} (µg/ml) value was minimum in IPL 406, which was followed by WBL58 and B256 (Table 1). Similarly, IC_{50} (µg/ml) values for superoxide radical scavenging assay and hydroxyl radical scavenging assay were significantly lower in seeds of IPL406 than those in WBL58 and B256 (Table 1). On the other hand, FRAP value (mM Fe^{2+}/g) was markedly higher in seeds of IPL406 and WBL58 than that in B256. The results suggested high antioxidant and free radical scavenging activity in seeds of IPL406, followed by WBL58 and B256 under un-treated conditions which agreed well with earlier findings on these three genotypes [35]. However, considerable deviations were observed in seeds of plants grown in arsenate-spiked pot soil. The DPPH IC_{50} (µg/ml) and IC_{50} (µg/ml) values for superoxide radical scavenging assay and hydroxyl radical scavenging assay were significantly decreased in As-treated IPL406 and WBL58 but no significant change for the above traits was observed in seeds of B256 (Table 1). The FRAP value was also decreased in As-treated former two genotypes but did not change significantly for B256 (Table 1). DPPH As a stable free radical, DPPH accepts an electron or hydrogen radical to become a stable diamagnetic molecule. When interacts with DPPH, antioxidant molecule/s transfer electron or hydrogen atom to DPPH and thus neutralizing its free radical capacity [44]. Similarly, superoxides and hydroxyl are the major free radicals in aerobic cells, and the latter is the product of biological system undergoing the Haber-Weiss and Fenton type reactions. The antioxidants receive transition metal ions through chelation, resulting in the suppression of hydroxyl generation and inhibition of peroxidation processes of biological molecules. Low IC_{50} values indicate strong antioxidant and free radical scavenging capacity of both IPL406 and WBL58 under un-treated condition but As-exposure significantly deteriorated this capacity in these two genotypes, as was evident from rising IC_{50} values. Furthermore, As-exposure significantly reduced FRAP values in these two genotypes, suggesting decreasing capacity of metal ion scavenging capacity of IPL406 and WBL58. This decreasing

trend of antioxidant activity and free radical scavenging capacity was accompanied by reduced TPC and TFC level in seeds of IPL406 and WBL58. Surprisingly, no significant change was observed in case of B256. A strong negative correlation between TPC/TFC values and IC_{50} values for DPPH, superoxide and hydroxyl radical scavenging assay (r = -815*, -797*, -808*, $P < 0.05$, n = 10) suggested As exposure significantly affected TPC and TFC values which in turn negatively interfered with antioxidant activity and free radical scavenging capacity of lentil seeds. A strong positive correlation between TPC and FRAP (r = 811*, $P < 0.05$, n = 10) and between TFC and FRAP under As exposure suggested significant contribution of both TPC and TFC towards metal ion scavenging capacity which was significantly reduced in plants grown in As-spiked soil. As-exposure reportedly compromised nodulation and nitrogenase activity in roots of black gram (*Vigna mungo*) [19] and reduced mineral nutritional compositions as well as essential amino acids in rice seeds [45]. In As-treated mung bean genotypes of Bangladesh, phosphorus (P), potassium (K), magnesium (Mg) and sulfur (S) concentrations decreased with increasing As levels [46]. Uptake of copper, manganese, iron and P was seriously affected in As-exposed *Pistia stratiotes* plants, also [47]. In the present study, total seed protein level, however, did not change significantly in any of the three As-treated lentil genotypes. Whether or not protein quality was compromised, further study is needed to decipher it.

Conclusions

Present study for the first time revealed that As-exposure can significantly reduce seed antioxidant activity and free radical scavenging capacity. However, the genotypes differed sharply in their responses to As-treatment. While IPL406 and WBL58 exhibited reduced capacity, no change was observed for B256 under As exposure. The results also pointed out that a detail germplasm strategy is required in lentil crop for identification of genotypes suitable for stress breeding and further improvement for better phytonutrients.

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Publication History

Received	23rd Jun 2016
Accepted	18th Jul 2016
Online	30th Sep 2016