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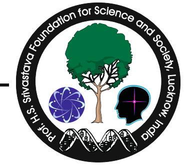

SHORT COMMUNICATION

Antioxidants from defatted Indian Mustard *(Brassica Juncea)* protect biomolecules against in vitro oxidation

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Abstract Indian mustard seeds were defatted by distillation with hexane and the residue extracted with methanol was analyzed for potential antioxidants; ascorbate, riboflavin, and polyphenols. Gallic acid (129.796 μg), caffeic acid (753.455 μg), quercetin (478.352 μg) and kaempferol (48.060 μg)/g dry seeds were identified by HPLC analysis of the extract. DPPH free radical scavenging activity and protection of lipids, proteins and DNA against metal induced oxidation was examined. Defatted mustard seed remnant had excellent free radical scavenging activity and protects biomolecules with IC_{50} value 2.0–2.25 mg dry seed weight. Significant content of polyphenols in methanol extract of defatted seeds accounts for high antioxidant potential. We are the first to report the detailed analysis of antioxidant composition and protection of biomolecules against oxidative damage by methanol extract of mustard seed remnant after oil extraction.

Keywords Brassica · Seedmeal · Polyphenols · Antioxidants

Introduction

Indian mustard (*Brassica juncea)* is an oilseed crop belonging to family Brassicaceae. It is a perennial herb grown as an annual or biennial throughout India. Although widely and extensively used as a vegetable, it is grown mainly for seeds, which yield essential oil and condiment. Mustard oil is the third-most important vegetable oil only after soya bean and oil palm (Anuradha et al. 2012). Mustard seeds are also used in the treatment of abdominal pain, anorexia, tumours and diabetes (Grover et al. 2002). Mustard leaf extracts are reported to have antioxidant potential and reduce lipid peroxidation under diabetic oxidative stress (Yokozawa et al. 2003). Brassica seed meal extract has been used in the ground meat to reduce lipid oxidation, extend shelf life and preserve quality (Lara-Lledo et al. 2012). An increased financial incentive to grow these crops can be achieved through the extraction of value added co-products from the seed meal remnant after oil extraction. Extract of *Brassica napus* seed meals has been reported to contain 1.5 % tannins and 1.0 % sinapine (Enami 2011). Manju et al. (2011) have reported free radical scavenging potential of gallic acid rich extracts of mustard seed meal. Dubie et al. (2013) have studied the effect of different extraction procedures on the amount of polyphenols in extract of *B. juncea.* The present study is undertaken with an objective to estimate the presence of various antioxidant compounds, evaluate the antioxidant activity and to check the possible protection of biomolecules by the oil free extract of mustard, to evaluate the possible use of mustard seed meal as source of antioxidants.

Materials and methods

The present investigation was carried out on Indian mustard (*B. juncea*) cultivar RH30. Seed material was procured from Oil Seed Section of Department of Genetics and Plant Breeding, CCS Haryana Agricultural University, Hisar, India. Crop was raised during rabi (2012–13) season following normal agronomic practices. Seed material was stored at

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room temperature till use. All chemicals used in present work were of analytical grade and were purchased from Sigma Aldrich, USA or Hi-media, India.

Mustard seeds were dried at 50 °C in hot air oven till constant weight was attained. Finely powdered seeds were defatted with n-hexane (1 g/40 ml) for 6 h in Soxhlet's apparatus. Hexane was removed and the residue was again extracted with fresh hexane for 2 h. The defatted seed remnant was extracted with 80 % methanol (1 g/10 ml) in a shaker at room temperature for 4 h. Residue was extracted with methanol again for 2 h. Collected methanol extract was filtered, centrifuged at 5,000 g for 5 min and concentrated in a vacuum evaporator and stored at −20 °C for further use. Antioxidants including ascorbate, riboflavin and polyphenols were estimated using the spectrophotometric methods as described in our earlier publications (Dua et al. 2013a,b). Polyphenols were identified and analyzed quantitatively by HPLC after acid hydrolysis (Ani et al. 2006) using gallic acid, caffeic acid, ellagic acid, ferulic acid, quercetin and kaempferol as standards.

Free radical 1, 1-diphenyl- 2-picryl hydrazine (DPPH) scavenging activity was monitored as described by Ani et al. (2006). Different dilutions of the extract were incubated with 1 ml of DPPH solution ($50x10^{-5}$M) in a final volume of 1.1 ml. The decrease in absorbance due to the scavenging of DPPH radicals by the extract was recorded at 517 nm after 5 min. The percent remaining DPPH was calculated and IC_{50}, the concentration at which 50 % of the initial DPPH could be scavenged was noted from the graph.

Lipid peroxidation inhibition was monitored as the amount of malonaldehyde (MDA) produced by copper induced egg lecithin peroxidation (Dua et al. 2013a). Different dilutions of the extract were added to 1 ml of reaction mixture containing 2.5 mM lecithin, 250 mM $CuCl_2$ in 50 mM Tris–HCl buffer (pH 7.4). After incubation at 37 °C for 15 min, 2 ml of reagent containing 0.37 % Thiobarbituric acid (TBA), 15 % TCA, 0.04 % Butylated hydroxytoluene (BHT) and 2 % ethanol was added. Mixture was heated at 100 °C for 15 min and centrifuged at 3,000 rpm for 10 min. Absorbance of supernatant at 535 nm is an index of malonaldehyde concentration. IC_{50}, the concentration inhibiting 50 % peroxidation was noted from the graph.

Oxidative modification in albumin was induced by copper in presence and absence of different dilutions of the extract (Dua et al. 2013a). The reaction mixture containing albumin (10 mg/ml), 100 mM $CuCl_2$ in 50 mM Tris–HCl buffer (pH 7.4), in a total volume of 0.3 ml was incubated at 37 °C for 2 h with or without extract. After incubation, 1.6 ml of 0.125 M phosphate buffer (pH 8.0) containing 12.5 mM EDTA plus 10.0 M urea and 0.1 ml of 50 mM phosphate buffer (pH 7.0) containing 10 mM 5,5-Dithio-bis(2-nitrobenzoic acid) (DTNB) were added. The absorbance was recorded at 412 nm, as an index of cysteine-SH residue. Percent inhibitory ratio was calculated as follows: % inhibition=[(Abs $_{control}$ – Abs $_{sample}$)/Abs $_{control}$] x 100

Hydroxyl radicals generated by Fenton's reaction were used to induce oxidative damage to DNA (Ani et al. 2006). The reaction mixture (9 μl) contained 3 μg of calf thymus DNA in 20.0 mM phosphate buffer saline (pH 7.4) and different concentrations of the extract were preincubated for 15 min at ambient temperature. The oxidation was induced by incubating DNA with 1.0 mM $FeSO_4$ and 10.0 mM ascorbic acid for 1 h at 37 °C. The reaction was terminated by the addition of loading buffer (xylene cyanol, 0.25 %; bromophenol blue, 0.25 % and glycerol, 30 %). The mixture was subjected to gel electrophoresis in 1.5 % agarose/TAE buffer run at 60 V. DNA was visualized and photographed by chemidoc (Biorad) to assess the damage and protection.

The statistical analysis was performed with the statistical software SPSS/Windows (SPSS 10.0. LNK) followed by Tukey test. The results were expressed as the means ± SEM to show variations in a group. Differences were considered significant at $p \leq 0.05$.

Results and discussion

Reactive oxygen species (ROS) generated in biological systems in various vital processes harm the structural and functional machinery of the cells by causing oxidation of biomolecules (Grover et al. 2002; Yokozawa et al. 2003). ROS play an important role in the degenerative or pathological processes such as aging, cancer, atherosclerosis, diabetes and Alzheimer (Aruoma 1994). There are many epidemiological surveys revealing an inverse association between diet rich in antioxidants and the risk of cardiovascular diseases and certain forms of cancer (Kannappan and Anuradha 2009). Plants have ascorbate, riboflavin, tocopherol and polyphenols with redox potential high enough to scavenge or terminate ROS (Schafer et al. 2003). Polyphenols can further contribute as metal ion chelators due to the presence of various hydroxyl groups (Shan et al. 2005). In the present study the defatted mustard seed extract was analyzed for the presence of ascorbate, riboflavin and polyphenols known to have antioxidant activity. Defatted seed extract had 858.88 μg total ascorbate, and 8.81 μg riboflavin/g of dry seeds. Reduced ascorbate is about 50 % of the total ascorbate (Table 1). The methanolic extract had considerably high amount of polyphenols (15.726± 0.15 mg Gallic acid equivalent/g dry weight of seeds). Dubie et al. 2013 have reported comparatively lesser polyphenols when extracted with water (13.79 mg Sinapic acid equivalent/g dry seeds) or ethanol (8.53 mg SAE/g dry seeds). Amount of polyphenols in the extracts increased with increase in time of extraction from 24 h to Seven days (Dubie et al. 2013), but the same was not reflected in antioxidant activity. Higher amount of polyphenols in methanol extracts is reported as compared to

Table 1 Antioxidant profiles of defatted mustard seed extract

Compound	Amount (µg/g dry wt.)
Oxidized ascorbate	858.88±5.15
Reduced ascorbate	406.91±3.36
Total ascorbate	451.97±4.82
Riboflavin	8.81±053
Total Polyphenols	15.726±0.15*
Gallic acid	129.796
Caffeic acid	753.455
Quercetin	478.352
Kaempferol	48.060

*(mg GAE/g dry wt.)

other solvents like ethanol, diethyl ether and hexane from other plant sources also (Saxena et al. 2011). High amount of polyphenols could be extracted from defatted mustard seeds using 80 % methanol in the present study.

Defatted mustard seed extract was subjected to HPLC analysis after hydrolyzing glycosidic bonds with HCl and different standards were run simultaneously to analyze the phenolics. Gallic acid (129.796 µg), caffeic acid (753.455 µg), quercetin (478.352 µg) and kaempferol (48.06 µg) are identified in the extract. Glycosides of caffeic acid, quercetin and kaempferol are also reported in *B. oleracea* (Olsen et al. 2009) and other plants of family Brassicaeace (Heimler et al. 2006). Presence of high amount of polyphenols especially caffeic acid and quercetin indicates high efficacy of defatted mustard seed remnant as an antioxidant source.

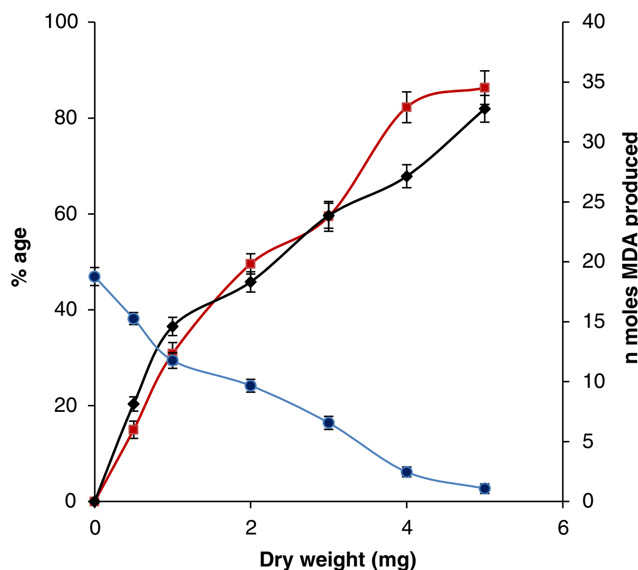

Fig. 1 Antioxidant activity of defatted mustard seed extract. DPPH scavenging (■) and protein oxidation inhibition (●) represented as percent of control; lipid peroxidation (●) shown as MDA produced in presence of extract equivalent to different amounts of defatted mustard seeds

Table 2 IC_{50} for different assays

Assay	IC_{50} (mg)
DPPH Scavenging	2.00
Lipid peroxidation	2.10
Protein oxidation	2.25

The mustard seed meal extract exhibited a concentration dependent elimination of DPPH free radicals (Fig. 1). Methanol extract equivalent to 4 mg or more of dry seed weight caused 85 % eradication of free radicals in the reaction mixture. Differences in the means were found to be significant by Tukey's test. IC_{50} of the mustard seeds is 2 mg for DPPH free radical scavenging activity (Table 2). The results indicate that antioxidants in defatted mustard seed extracts are effective electron or hydrogen donors and this activity contributes to the antioxidant capacity of mustard seeds. Polyphenols are reported to contribute reducing properties and antioxidant characteristics in different herbs and spices (Naveen et al. 2011). Methanol and aqueous extracts of various plant sources are reported to have better free radical scavenging activity than dichloromethane or ethyl acetate extracts, indicating the polar nature of the antioxidant biomolecules (Saxena et al. 2011). A positive correlation between the DPPH radical scavenging activity and polyphenol content of the extracts of various spices has been reported (Shan et al. 2005; Ani and Naidu

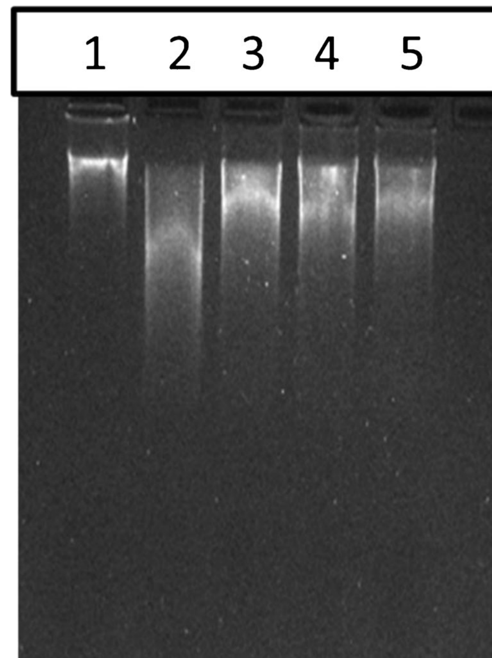

Fig. 2 Protection of DNA against H2O2 induced damage by defatted mustard seed extract. Lane1-control DNA (1.5 µg); Lane2-DNA + Fenton's reagent with ascorbic acid; Lane3- mustard (0.5 µg) + DNA + Fenton's reagent with ascorbic acid; Lane 4- mustard (1.0 µg) + DNA + Fenton's reagent with ascorbic acid; Lane5- mustard (1.5 µg) + DNA + Fenton's reagent with ascorbic acid

2011). DPPH scavenging activity of *B. juncea* seed extracts in ethanol (Dubie et al. 2013) and methanol (Manju et al. 2011) are also correlated to the polyphenolic content of the extracts. In the present study, high polyphenol content, with caffeic acid and quercetin as major phenolics, seems to be the major free radical scavenger.

ROS in the cells attack polyunsaturated lipids, cause damage to the membranes and affect various processes such as apoptosis, autogenesis and carcinogenesis (Schafer et al. 2003). Lipid oxidation leads to the spoilage of fats and fatty acids of foods (Lara-Lledo et al. 2012). MDA produced by copper induced oxidation of egg lecithin in presence and absence of different dilutions of mustard seed extract was determined (Fig. 1). In controls, 18.769±0.32 nmoles of MDA was produced and the production of MDA was reduced to 1.07±0.39 nmoles in presence of the extract equivalent to 5 mg of the mustard seeds. Differences in the means were found to be significant by Tukey's test. IC_{50} calculated from the curve is 2.1 mg (Table 2). The results indicate that antioxidants from *Brassica* are efficiently preventing the lipid oxidation either by metal chelation or by inhibiting the propagation reactions. Metal ion chelation plays a significant role in antioxidant mechanism since it reduces the concentration of the transition metal catalyzing lipid peroxidation. IC_{50} for lipid oxidation inhibition is similar to DPPH scavenging indicating that reducing property of polyphenols is contributing to the antioxidant activity in protecting lipids against oxidation. Inhibition of lipid peroxidation by the polyphenols from other plant sources has also been reported (Ani and Naidu 2011; Dua et al. 2013a,b) .

Sulfhydryl groups of the proteins are susceptible to oxidation in presence of ROS. Deleterious impact of oxidative stress in biological systems is related to the damage of proteins, enzymes and various transcriptional factors (Kannappan and Anuradha 2009). Oxidative modifications were induced in BSA by incubation with copper ions in presence and absence of mustard seed extract (Fig. 1). The process was inhibited by 82 % in presence of extract equivalent to 5 mg of seeds. Presence of extract equivalent to 2.25 mg dry seeds is required to inhibit the metal induced protein oxidation upto 50 % (Table 2). IC_{50} for protection of protein oxidation is slightly higher than that for lipid oxidation, indicating that electron transfer to lipids is more efficient than to proteins.

Oxidation of nitrogenous bases of nucleic acids may cause mutations. H_2O_2 generated by Fenton's reaction in vitro can cause breaks in calf thymus DNA and it moves to a greater extent in the gel (Fig. 2). Presence of extract equivalent to 0.5, 1.0 and 1.5 μg mustard seeds in the incubation mixture could prevent the damage. This protective impact of the extract indicates that antioxidant principles from mustard seeds can efficiently quench hydroxyl radicals from the reaction mixture. Inhibition of DNA damage by polyphenol rich cumin (Ani et al. 2006; Dua et al. 2012), fennel (Dua et al. 2013a) and fenugreek (Dua et al. 2013b) seed extracts has also been reported.

The results of the present study indicate that oil free methanol extract of Indian mustard seeds has polyphenols including gallic acid, caffeic acid, quercetin and kaempferol responsible for the high free radical scavenging and metal chelating activity of mustard seeds. Natural antioxidants present in mustard seeds even after oil extraction, protect important biomolecules like lipids, proteins and DNA against oxidative stress. Antioxidants present in mustard seed meal extracts may be explored as a component of composite food formulations in order to stabilize or enrich food with antioxidant properties. Use of Brassica seed remnant as pharmaceutical and neutraceutical agent should be further explored.

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