CHAPTER-XII

IN-VITRO FREE-RADICAL SCAVENGING POTENTIAL OF OLIGOPEPTIDES DERIVED FROM WHEAT AND MUNGBEAN

12.1 INTRODUCTION

Reactive oxygen species (ROS), like hydrogen peroxide, hydroxyl radical and superoxide anion are constantly produced in the form of free radicals in the living cell through oxidative metabolic pathways (Fridovich, 1978). These free radicals are responsible for causing diseases like cancer, multiple sclerosis, inflammation, coronary heart disease, cardiovascular disease, arthritis etc. Antioxidant components are capable of minimizing the effects of these free radicals and thus, help in prevention of these diseases (Halliwell and Gutteridge, 1999) and also used for preservation in many food industries (Andrea *et al.,* 2010). The inhibitory response of proteins towards oxidation reactions makes them a vital component of antioxidant based defence system. (Loganayakia *et al.,* 2011).

In recent years, numerous naturally occurring bioactive peptides have been isolated from different living organisms such as bacteria, fungi, plant and animals, which are found to be involved in regulation of physiological processes in plants like induction of α-amylase synthesis, regulation of stomatal opening in dark etc (Ghosh *et al.*, 2010). Recently, antioxidant potential of peptides derived from different plant sample has created a great attention among scientists. The peptides isolated from various plants like soybean (Moure *et al.,* 2006), maize (Zhu *et al.,* 2008), chickpea (Li *et al.,* 2008), pea seed (Pownall *et al.,* 2010), buckwheat (Tang *et al.,* 2009), alfalfa leaves (Xie *et al.,* 2008) have reported to possess potential antioxidant activity. The natural antioxidants based on peptides isolated from plant sample are effectively used for extending the storage period of food (Lagouri and Nisteropoulou 2009, Beermann *et al.,* 2009, Jacobsena *et al.,* 2008). The antioxidant activities are affected by molecular weight of peptides and also composition and sequences of amino acids present in that particular peptides (Saito *et al.,* 2003).

Several works have been done on high and low molecular weight peptides of various legumes and grains reporting their biochemical and physiological roles but the variation in their antioxidant potential are yet to be revealed. In this present study, an attempt was made to determine the antioxidant potential of peptides isolated from wheat and mungbean seedlings. The peptides of different molecular weight, ranging from 0.5 to

3 kDa and 3 to 10 kDa were worked out and a clear line of difference was established on the antioxidant activity of these isolated peptides.

12.2 MATERIAL AND METHODS

12.2.1 Plant material and culture conditions

Seeds of dicotyledonous plant material, Sonamung [*Vigna radiata* (L) Wilczek. cv. Sonali B1], collected from Central Pulses Research Institute (C.P.R.I.), Berhampur, West Bengal, India and seeds of wheat [*Triticum aestivum* L. (cv. Sonalika RR-21), Poaceae], collected from National Seeds Corporation Limited, was used in the present investigation. 500g of same-sized seeds were weighed out and surface-sterilized for 5 minutes in sodium hypochlorite (5% available chlorine) and rinsed 3 times in sterile deionized water. Seed germination was carried out in sterile petridishes containing two layers of filter papers and the culture conditions were maintained as specified in Chapter III Section 3.2.1.

12.2.2 Extraction and purification of low molecular weight peptides

Aqueous extraction of one week old whole wheat and mung bean seedlings (1kg) was performed in the same way as mentioned in Chapter III Section 3.2.2. Amphoteric components were separated through ion-exchange chromatography and ultrafiltered for isolating 3 KDa to 0.5 KDa pools of peptide molecules. Whole wheat and mung bean sample produced 176mg and 154mg of semipure peptides respectively which showed positive colour reaction with ninhydrin. The purified and lyophilized dry extract of wheat was semisolid and brownish. Different bioassays were performed with this semi-purified extract for determining the hormone like action of these peptides.

12.2.3 DPPH radical scavenging activity

DPPH is a relatively stable free radical which has been widely used to examine the free radical-scavenging activity of tested samples. The radical scavenging activity of the aqueous extracts was measured by DPPH method (Blois, 1958). The reaction mixture contained 1.8 ml of 0.1 mM DPPH and 0.2 ml of aqueous extracts. The reaction mixture was vortexed and kept in the dark at room temperature for 30 min. The absorbance was

measured at 517 nm. A reaction mixture without test sample was taken as control. The free radical scavenging activity of tested sample were expressed as percentage of inhibition and were calculated according to these equation:

$$\% \text{ inhibition of DPPH activity} = [(A_0-A_1)/A_0] \times 100\ \%$$

Where A_0 is the absorbance values of the blank sample i.e. control reaction and A_1 is the absorbance value of the tested sample. A curve of inhibition percent or percent scavenging rate against sample concentration was determined from where IC_{50} (concentration of the sample required to inhibit 50 % of free radicals) of tested sample were calculated.

12.2.4 $ABTS^{\bullet+}$ scavenging activity

The spectrophotometric analysis of $ABTS^+$ radical cation(s) scavenging activity was determined according to Re *et al.* (1999) method with some modification. This method is based on the ability of antioxidants to quench the $ABTS^+$ radical cation, a blue/green chromophore with characteristic absorption at 734 nm, in comparison to that of BHT. The $ABTS^+$ was obtained by reacting 7 mM $ABTS^+$ radical cation(s) in H_2O with 2.45 mM potassium persulphate ($K_2S_2O_8$), stored in the dark at room temperature for 6 hrs. Before usage, the $ABTS^+$ solution was diluted to get an absorbance of 0.750 ± 0.025 at 734 nm with sodium phosphate buffer (0.1 M, pH 7.4). Then, 2 ml of $ABTS^+$ solution was added to 1 ml of the aqueous extract. After 30 min, absorbance value was recorded at 734 nm, relative to a blank absorbance. The percentage inhibition of the samples was calculated as:

$$\text{Inhibition } \% = (1 - A/A_0) \times 100$$

Where A_0 is the absorbance at 734 nm of the control, A is the absorbance at 734 nm of the sample mixture.

12.2.5 Reducing power

The assay was performed according to the method of Oyaizu (1986) with some modifications. Extracts were diluted at different concentrations of 2.5 ml of the 0.2 M phosphate buffer (pH: 7.0) and 2.5 ml of 1% potassium ferricyanide solution were added

with tested sample and vortexed. The mixtures were incubated at 50° C for 20 min in a water bath. The tubes were cooled at room temperature and 2.5 ml of 10% trichloroacetic acid was added and centrifuged at 3000 rpm for 10 min. 2.5 ml upper layer was mixed with 2.5 ml of distilled water and 250 µl of 0.1% aqueous ferric chloride. Fluorescent green colour was appeared and absorbance of the final solution was recorded at 700 nm.

12.2.6 Metal chelating activity

Determination of chelation of iron (II) ions by extracts was carried out as described by Dinis *et al.*, (1994). To a mixture of 400 µL of extracts and 1.6 ml of methanol, 40 µL of 2 mM $FeCl_2$ solution and 80 µL of 5mM ferrozine were added. After 10 min of incubation, absorbance of the solution was recorded at 562 nm.

12.2.7 Nitric oxide Scavenging assay

Nitric oxide was generated from sodium nitroprusside and measured by the Greiss reaction (Marcocci *et al.*, 1994). 320 µL extract, 360 µL (5 mM) sodium nitroprusside-PBS solution, 216 µL Greiss reagent (1% sulfanilamide, 2% H_3PO_4 and 0.1% napthylethylenediamine dihydrochloride) was mixed and incubated at 25^0C for one hour. Finally 2 ml water was added and absorbance was taken at 546 nm.

Radical scavenging activity was expressed as percentage inhibition from the given formula:

$$\% \text{ inhibition of } NO\cdot \text{ radical} = [(A_0 - A_1)/A_0] \times 100;$$

Where A_0 is absorbance of control and A_1 is the absorbance of sample.

12.2.8 Superoxide anion radical scavenging activity

The superoxide radical scavenging activity was measured by the method of Nishikimi *et al.*, (1972) with slight modification. All solutions were prepared in 0.05 M phosphate buffer (pH 7.8). The photo-induced reactions were performed using fluorescent lamps (20W). The reaction mixture contained 1ml of NBT solution (312 µM prepared in phosphate buffer, pH- 7.4), 1ml of NADH solution (936 µM prepared in phosphate buffer, pH-7.4), and 1ml of methanolic extract of different concentrations. After 5 min incubation, 100 µl of PMS (120 µM prepared in phosphate buffer, pH-7.4) was added to

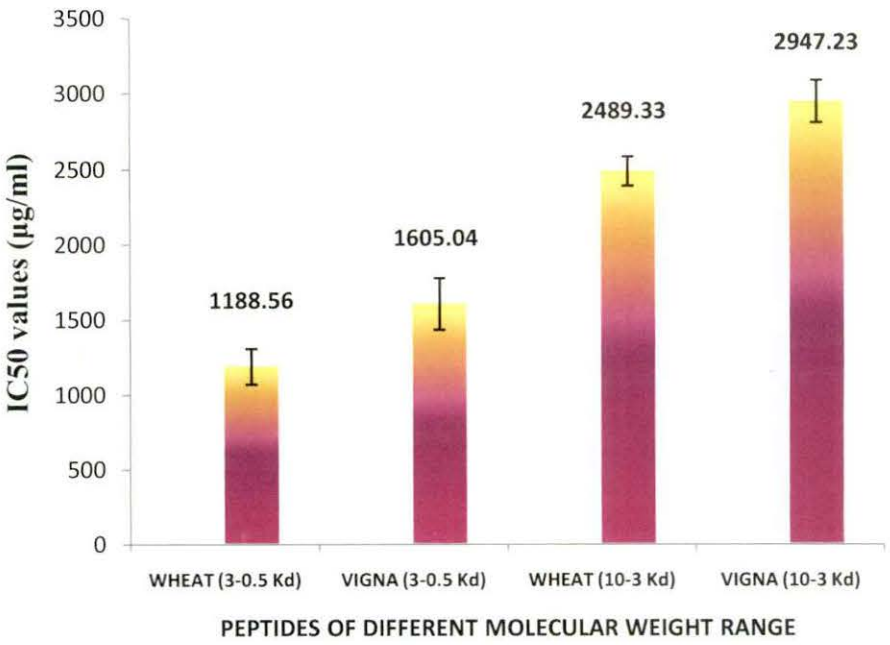

Figure 12.1 DPPH radical scavenging activity (IC_{50} values) of LMW and HMW peptides isolated from *T. aestivum* and *V. radiata*

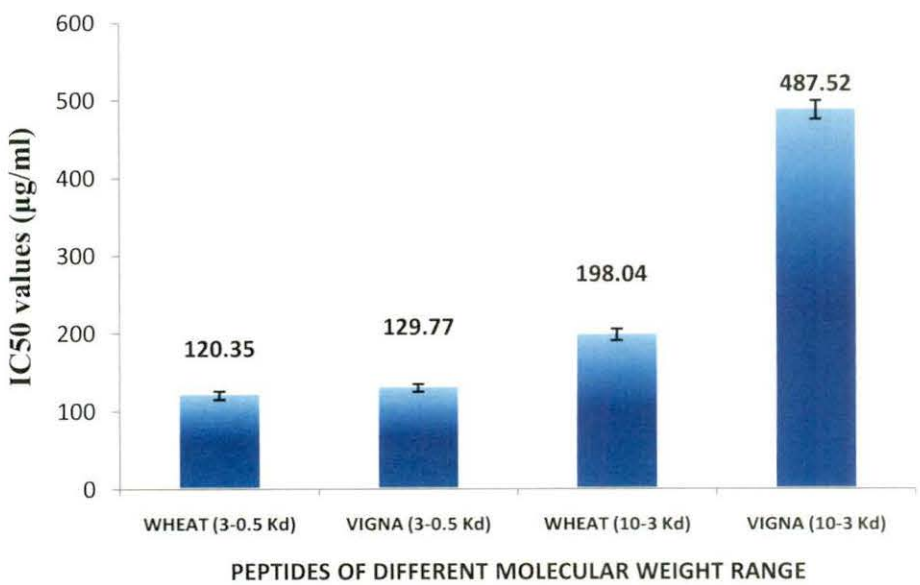

Figure 12.2 ABTS+ radical scavenging activity (IC_{50} values) of LMW and HMW peptides isolated from *T. aestivum* and *V. radiata*

the reaction mixture. The reactant was illuminated at 25°C for 30 min and the absorbance was measured at 560 nm against methanol as control. The inhibition percentage of superoxide anion generation was calculated by using the following formula:

Superoxide radical scavenging effect (%) = [Abs. of control – Abs. of sample / Abs. of control] x100.

12.3 RESULTS AND DISCUSSION

12.3.1 DPPH and $ABTS^{\cdot+}$ scavenging activity

The results of DPPH and $ABTS^{\cdot+}$ free radical-scavenging activity of purified peptides are shown in Figure 12.1 and Figure 12.2 respectively. The both free radical-scavenging effects of peptide samples isolated from wheat and *V. radiata*, increased in a concentration-dependent manner. The result indicated that the DPPH and $ABTS^{\cdot+}$ radical scavenging activities of the peptides are molecular size dependent. The LMW peptides exhibited better DPPH radical scavenging activities than that of HMW. Higher antioxidant activity *i.e.* lower IC_{50} values were exhibited by LMW wheat peptides (1188.56 µg/ml) in comparison to HMW peptides which showed higher IC_{50} values (2489 µg/ml). *V. radiata* peptides showed 50% scavenging potential at concentrations of 1605.04 µg/ml (LMW) and 2947.24 µg/ml (HMW). This result is in agreement with the previous findings reported by Aluko and Monu (2003) in quinoa seed, and Girgih *et al.* (2011) in hemp seed, which showed that LMW peptide fractions had higher DPPH scavenging activities than HMW peptides. It was also observed that wheat peptides have comparatively higher scavenging activity than mung bean peptides in both molecular weight ranges.

12.3.2 Reducing power Assay

In reducing power assay, the reducing capacity of a biological compound plays a significant indicator of its potential antioxidant activity (Kallithraka *et al.*, 2001). As shown in Figure 12.3, the low molecular peptides exhibited effective reducing ability which increased with an increase in sample concentration. The reducing ability of the peptides was determined with ascorbic acid equivalent. Lower ascorbic acid equivalent value indicates lower reducing ability of samples. Wheat indicated higher ascorbic acid

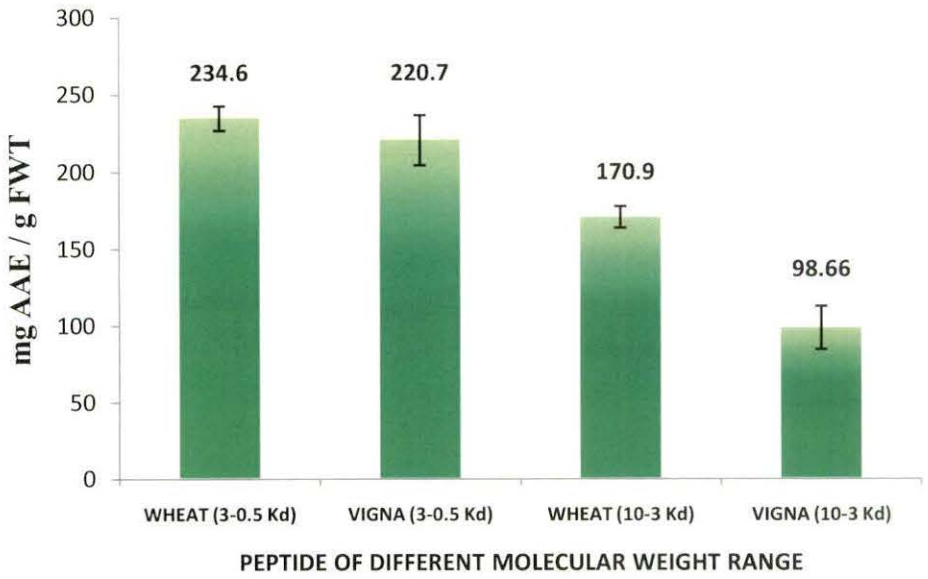

Figure 12.3 Reducing power activity (mg AAE / g FWT) of LMW and HMW peptides isolated from *T. aestivum* and *V. radiata*

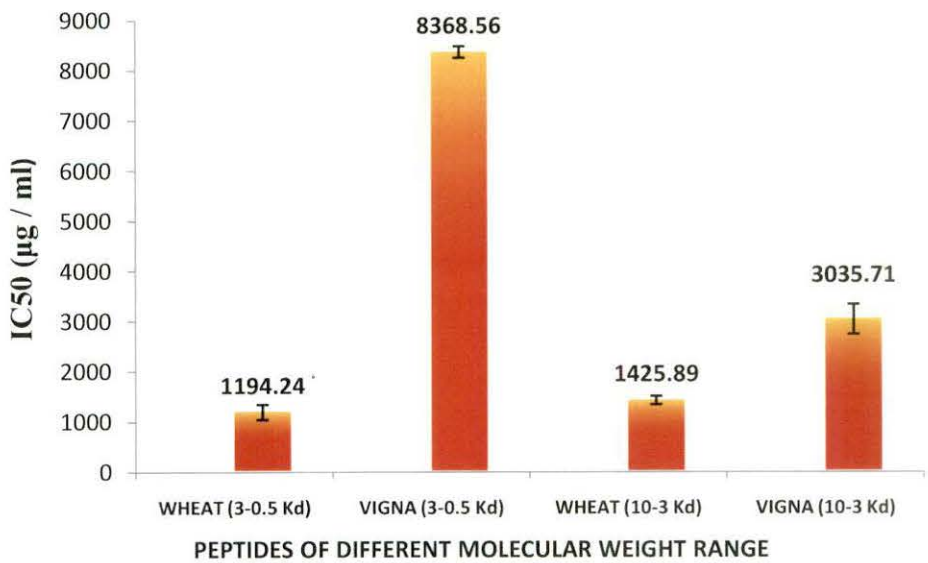

Figure 12.4 Metal chelating activity (IC_{50} values) of LMW and HMW peptides isolated from *T. aestivum* and *V. radiata*

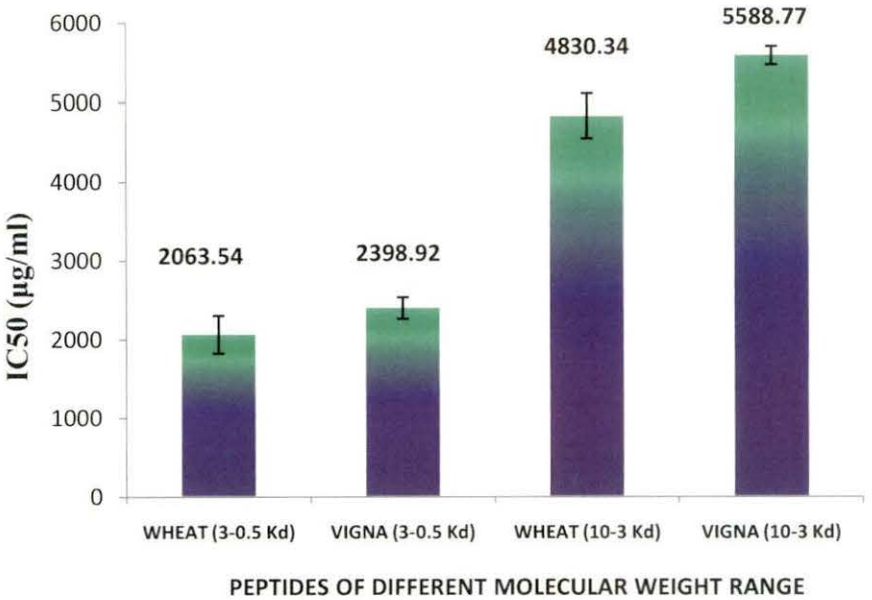

Figure 12.5 Nitric oxide scavenging activity (IC_{50} values) of LMW and HMW peptides isolated from *T. aestivum* and *V. radiata*

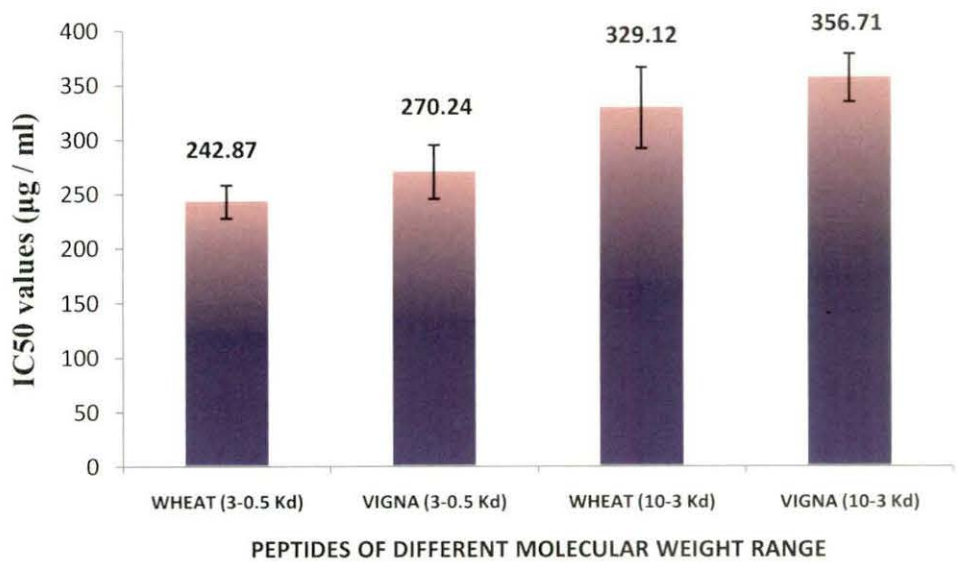

Figure 12.6 Superoxide scavenging activity (IC_{50} values) of LMW and HMW peptides isolated from *T. aestivum* and *V. radiata*

equivalent value [234.0 mg/g fresh weight tissue (FWT)] and *V. radiata* has relatively lower ascorbic acid equivalent value (220.7 mg/g FWT) in presence of 1000 mg/ml peptides (LMW) solution. The LMW peptides had significantly higher reducing power when compared to those of HMW peptide(s). HMW peptide of wheat and *V. radiata* had 170.9 mg/g FWT and 98.66 mg/g FWT ascorbic acid equivalent value respectively. These results indicate that smaller size peptides exhibited better reducing power than high molecular weight fractions. In contrast, Girgih *et al.* (2011) reported that the reducing power activity was enhanced with the increase in molecular weight of peptides in hemp seed protein.

12.3.3 Metal chelating activity

The chelation of transition metal ions Fe^{2+} and Cu^{2+} by antioxidative peptides could prevent the oxidation reaction (Dinis *et al.*, 1994). In Figure 12.4, it is apparent that the metal chelating activity of LMW peptides was markedly reduced in *V. radiata* (IC_{50} value is 8368 µg/ml) in comparison to wheat (IC_{50} values 1194.24 mg/ml). Carboxyl and amino group in the side chains of the acidic (Glx and Asx) and basic (Lys, His and Arg) amino acids play an important role in chelating metal ions (Saiga *et al.*, 2003). These residues have been reported to contribute to the metal chelating effect of protein hydrolysates, which is commonly related to its imidazole ring (Nam *et al.*, 2008).

12.3.4 Nitric oxide Scavenging assay

The nitric oxide generated from sodium nitroprusside reacts with oxygen and form nitrite. The extract inhibits nitrite formation by contending with oxygen to react with nitric oxide directly and also inhibits its synthesis (Nikkhah *et al.*, 2009). In these experiment peptides from wheat showed higher nitric oxide scavenging activity than peptides from *V. radiata*. The IC_{50} values of wheat and *V. radiata* LMW peptides were 2063.54 µg/ml and 2398.9 µg/ml respectively (Figure 12.5). In case of HMW peptides, IC_{50} values of wheat and *V. radiata* were much higher than their LMW peptide counterparts, i.e. 4830 µg/ml and 5588.78 µg/ml respectively (Figure 12.5). Like previous observations, nitric oxide scavenging activities of these peptides are molecular size dependent.

12.3.5 Superoxide scavenging assay

Superoxide is considered as an initial free radical which is formed from mitochondrial electron transport systems, to create other cell-damaging free radicals, such as hydrogen peroxide, hydroxyl radical, or singlet oxygen (Bloknina *et al.*, 2003). Peptides can protect the cell against toxic effect of some superoxide free radicals (Ajibola *et al.*, 2011).The result shown in Figure 12.6 clearly indicates that peptides isolated from wheat have more potential superoxide scavenging activity than the *V. radiata* peptide because wheat possess low IC_{50} values *i.e.*, highly effective antioxidant activity. It was also observed that in both the samples low molecular weight peptides exhibited stronger superoxide activity than large size peptides. Similarly, Li *et al.*, 2008 reported that low molecular weight peptides from chickpea protein hydrolysates exhibited strong superoxide radical scavenging activity and was found to contain higher concentrations of Phe, Ile, Leu and Val in comparison to other fractions. Therefore it can be suggested that the superoxide scavenging activity of peptides might be related with the concentration of these hydrophobic amino acids in peptide sequence.

12.4 CONCLUSIONS

Results of this study demonstrated that peptides derived from seedlings of wheat and *V. radiata* possess antioxidant properties. Small sized peptides (0.5 to 3 kDa) seem to be more effective free radical scavenger such as $ABTS^{\cdot+}$, DPPH, nitric oxide and superoxide. The peptides isolated from wheat seedlings had better antioxidant properties than peptides of *V. radiata*. Hence, these peptides might be used for formulation of functional foods and nutraceautical. Also these seedlings might be explored as natural source of antioxidants and preservatives in the food industry for storage of food products and to maintain freshness during production.

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